The biological basis and clinical correlates of the association between EBV-positive Hodgkin lymphoma and HLA class I

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Abstract

Classical Hodgkin lymphoma (cHL) is one of the commonest lymphomas in the developed world, frequently affecting young adults. The majority of patients will be cured of their disease, but the toxicities of the therapy required to achieve this can lead to long-term morbidity in survivors. In addition, whilst most patients are cured, there remains approximately 15% -20% of patients who will not respond to primary therapy and may ultimately die of their disease.

Approximately one-third of cases of cHL in the developed world are associated with the Epstein-Barr virus (EBV), where this association is believed to be causal. A ubiquitous herpesvirus, persistently infecting more than 95% of the world's population, precisely how this virus causes malignant disease in a minority of immune competent hosts remains ill-understood.

Epidemiological evidence points to inherited genetic factors. Long-recognised to have an association with the class I human leukocyte antigen (HLA) system, recent studies have confirmed that risk of EBV-associated cHL is related to an individual's HLA-A* allotype, with HLA-A*01:01 being associated with increased risk of disease and HLA-A*02:01 being protective. Heterozygotes are observed to have an intermediate risk. HLA plays a central role in the recognition and cell killing of virally-infected or malignant cells by the cytotoxic T lymphocytes (CTLs) of the cell-mediated immune system. The exact mechanism whereby HLA-A* exerts its effect on risk of cHL unknown, but CTL responses to EBV in this context are hypothesised to be crucial.

The CTL response to EBV is well-studied. Immunodominant epitopes restricted through common class I alleles have been described, many directed towards

peptides derived from proteins expressed in the lytic cycle of viral infection. In spite of intensive study, no confirmed HLA-A*01:01-restricted EBV-specific CTL responses have been described, raising the possibility that absent or weak CTL responses specifically to EBV might lead to elevated risk of disease. However, particularly given the intermediate risk of disease seen in HLA-A*01:01 heterozygotes, it remains a possibility the HLA-A*01:01-associated risk might be due to qualitative or inhibitory changes to the EBV-specific immune response.

The work of this thesis set out to address a number of specific questions regarding the role of HLA class I in the aetiology and clinical outcome of cHL. Firstly, whether an HLA-A*01:01 allele could modify the magnitude of the CTL response to HLA-A*02:01-restricted epitopes was examined. In a study of healthy adults examining CTL responses using interferon-γ ELISPOT, overall HLA-A phenotype did not significantly affect the EBV-specific CTL response restricted through HLA-A*02:01. However, exploratory analysis of cytokine levels in response to stimulation with EBV peptides demonstrated significantly higher secretion of IL-10 (with a nearly 10-fold difference), IL-17 and IL-5 in response to stimulation with EBV peptides in HLA-A*02:01/A*01:01 heterozygotes, compared to other HLA-A*02:01 phenotype groups. This suggests a possible effect of HLA-A*01:01 in HLA-A*02:01/A*01:01 heterozygotes which might begin to explain some of the HLA-associated differences in risk of developing EBV+ve cHL.

Secondly, again in a study of healthy EBV-seropositive adults, and using sensitive methodologies, HLA-A*01:01-restricted EBV-specific CTL responses were sought, and, in an exploratory analysis, cytokine responses were examined. No HLA-A*01:01-restricted CTL responses to EBV were detected in this study, however, exploratory analysis demonstrated statistically significant differences in cytokine

levels following simulation with EBV, with HLA-A*01:01 homozygotes generating much higher levels of IL-6.

Lastly, given the importance of class I HLA in determining risk of developing EBV+ve cHL, a study of 424 patients with cHL was performed to determine if HLA-A*01:01 and A*02:01 alleles are a factor in determining clinical outcome. In this study, HLA-A*02:01 was associated with inferior overall survival (OS) and disease-specific survival (DSS) in EBV+ve cHL, and was independently prognostic in an adjusted analysis. Given the extremely poor outcomes seen in this study in HLA-A*02:01 carriers with EBV+ve disease (61.7% 10-year OS), it is possible that this group of patients is not currently being well-served by standard first-line therapy and may benefit from novel therapies.

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This thesis is dedicated to my father,

Thomas Farrell (1946-1990).

Author Declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature _____

Printed name _____

Date _____

Symbols and Abbreviations.

Symbols

α	alpha
ß	beta
γ	gamma
Δ	delta
κ	kappa
°C	degrees centigrade
µ	micro
%	per cent

Abbreviations (also defined at time of first use in the text).

AA	amino acid
ABCC1	ATP-binding cassette, sub-family C
ABVD	doxorubicin, bleomycin, vinblastine, dacarbazine
AIDS	acquired immune deficiency syndrome
Akt	also known as Protein Kinase B (PKB), a serine/threonine-specific protein kinase
*Bam*H1	Bacillus amyloli restriction endonuclease
BARTS	*Bam*HI A rightward transcripts
BCIP	5-bromo-4-chloro-3'indolylphosphate p-toluidine salt
bcl-2	B cell lymphoma 2
BCR	B cell receptor
BD-PBMC	B cell depleted PBMC
BEACOPP	bleomycin , etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisolone
BOB1	B cell Oct binding protein 1
CCL	chemokine (C-C motif) ligand
CCM	complete culture medium
CD	cluster of differentiation
CEF	CMV, EBV, 'flu
CGH	comparative genomic hybridisation
cHL	classical Hodgkin lymphoma
CI	confidence interval

CIITA	class II, major histocompatibility complex, transactivator
CM	culture medium
CMV	cytomegalovirus
CO_2	carbon dioxide
COPP	cyclophosphamide, vincristine, procarbazine, prednisolone
COSHH	control of substances hazardous to health
CR	complete response
CRF	clinical research facility
CSF	colony stimulating factor
CSF1R	colony stimulating factor 1 receptor
CT	computed tomography
CTL	cytotoxic T lymphocyte
CVID	chronic variable immune deficiency
DLBCL	diffuse large B cell lymphoma
DMSO	dimethyl sulfoxide
DNA	deoxy-ribonucleic acid
DSS	disease-specific survival
EA	early antigen
E2A	E2A immunoglobulin enhancer binding factors E12/E47
EBER	EBV virus encoded small RNA
EBF	early B cell factor
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EBV+ve cHL	EBV-associated classical Hodgkin lymphoma
EBV-ve cHL	EBV-negative classical Hodgkin lymphoma
ECD	electron coupled dye, also known as PE-Texas red
EDTA	ethylenediamine tetra-acetic acid
EFS	event-free survival
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
ERK	extracellular signal-regulated kinases
ESR	erythrocyte sedimentation rate
FBS	foetal bovine serum
FCR	immunoglobulin constant region
FDG	fluorodeoxyglucose
FFTF	freedom from treatment failure
FITC	fluorescein isothiocyanate

FOXP3	forkhead box P3
Fyn	FYN oncogene related to SRC, FGR, YES
g	x gravity
GC	germinal centre
GEP	gene expression profiling
GHSG	German Hodgkin Study Group
GM-CSF	granulocyte-macrophage colony-stimulating factor
GWAS	genome-wide association study
Gy	Gray
HBSS	Hank's Balanced Salt Solution
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HL	Hodgkin lymphoma
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
HRS	Hodgkin and Reed-Sternberg
IARC	International Agency for Research on Cancer
IE	Immediate early
IFN	interferon
Ig	immunoglobulin
IgD	immunoglobulin D
IgG	immunoglobulin G
IgM	immunoglobulin M
IgV	immunoglobulin variable
IHC	immunohistochemistry
IκB	IκB kinase
IL	interleukin
IM	infectious mononucleosis
IPS	international prognostic score
IRAS	integrated research application service
JAK	Janus kinase
LCL	lymphoblastoid cell line
LD	linkage disequilibrium
LDHL	lymphocyte deplete Hodgkin lymphoma
LMP	latent membrane protein
LRF	Leukaemia Research Fund
LRHL	lymphocyte rich Hodgkin lymphoma

M	molar
MALDI	matrix-assisted laser desorption and isolation
MAPK	mitogen-activated protein kinase
MCHL	mixed cellularity Hodgkin lymphoma
MHC	major histocompatibility complex
MIG	macrophage-induced gene, also known as CXCL9
min	minute
miRNA	micro ribonucleic acid
ml	millilitre
MMB	minimacs buffer
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MUM1	melanoma associated antigen (mutated) 1
NaOH	sodium hydroxide
NBT	nitro blue tetrazolium chloride
NF-κB	nuclear factor κ B
NHL	non-Hodgkin lymphoma
NK	natural killer
NLPHL	nodular lymphocyte predominant Hodgkin lymphoma
NPC	nasopharyngeal carcinoma
NSHL	nodular sclerosis Hodgkin lymphoma
Oct2	organic cation transporter 2
OR	odds ratio
ORF	open reading frame
OS	overall survival
PAX5	paired box 5
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	progressive disease
PD-1L	programmed death 1 ligand
PE	phycoerythrin
PEL	primary effusion lymphoma
PET	positron emission tomography
PFS	progression-free survival
PHA	phytohaemagglutinin

PI3K	phosphoinositide-3-kinase
PML	promyelocytic leukaemia gene
PR	partial response
PTLD	post-transplant lymphoproliferative disease
PU1	transcription factor binding to the PU-box
QC	quality control
RANK	receptor activator of nuclear factor-kappa B, also known as tumour necrosis factor receptor superfamily member 11A
RANK-L	RANK-ligand
RANTES	regulated on activation, normal T cell expressed and secreted
RAS	rat sarcoma
Rel	v-rel reticuloendotheliosis viral oncogene homolog
RNA	ribonucleic acid
RT	room temperature
s	seconds
SAP	SLAM-associated protein
SD	standard deviation
SEM	standard error of the mean
SFC	spot-forming cells
SHARE	Study of Healthy Adult Responses to EBV
SIR	standardised incidence ratio
SLAM	signalling lymphocytic activation molecule
SNBTS	Scottish National Blood Transfusion Service
SNEHD	the Scotland and Newcastle epidemiological study of Hodgkin's disease
SNP	single-nucleotide polymorphism
SOCS1	suppressor of cytokine signalling 1
SP	side population
SSO	sequence specific oligonucleotide
STAT	signal transducers and activators of transcription
Syk	spleen tyrosine kinase
TAP	transporter associated with antigen processing
TARC	Thymus and activation regulated chemokine, CCL17
TCR	T cell receptor
TE	tris-edta
Th	T helper
TIA-1	TIA1 cytotoxic granule-associated RNA binding protein
TIMP	tissue inhibitor of metalloproteinases
TNF	tumour necrosis factor

TNFAIP3	tumour necrosis factor alpha induced protein 3
TNFR	tumour necrosis factor receptor
T_{reg}	T regulatory cell
Ts	T suppressor cell
UK	United Kingdom
VCA	viral capsid antigen
VZV	varicella zoster virus
WAS	Wiskott-Aldrich syndrome
WHO	World Health Organisation
x	times
XLP	x-linked lymphoproliferative syndrome

Chapter 1. Introduction

1.1 Hodgkin Lymphoma - Introduction

Hodgkin lymphoma (HL) is a B lymphocyte malignancy, characterised by the presence of the mononuclear Hodgkin and multinucleate Reed-Sternberg cells, collectively known as Hodgkin and Reed-Sternberg (HRS cells). First described in 1832 by Thomas Hodgkin (Hodgkin, 1832) and subsequently called Hodgkin's disease, it has now been re-named Hodgkin lymphoma following its recognition as a clonal lymphoid disease (Stein *et al*, 2008). HL is unique in a number of regards, which presents special challenges to elucidating its complex biology and translating this into tangible clinical progress.

HL is one of the commonest lymphomas in the developed world, occurring with an incidence of approximately 3 per 100,000 person-years (Information Services Division, 2010) and accounting for approximately 10-30% of new lymphomas diagnosed worldwide (Stein *et al*, 2008). In contrast to non-Hodgkin lymphomas (NHLs), HL commonly affects young adults and is the second commonest cancer in teenagers and young adults in Scotland (Information Services Division, 2010). Approximately 170 individuals in Scotland will be diagnosed with HL per year, and the incidence is increasing (Information Services Division, 2010). Moreover, HL has served as the paradigm for treatment of malignant disease with successful outcomes now reported for the majority of patients presenting with the disease. A sizeable minority, however, will relapse or have resistant disease, and ultimately succumb to their disease. Identifying and treating this group is becoming an increasing priority of the international HL research community.

HL is one of a small number of cancers recognised by IARC (International Agency for Research on Cancer) (IARC Working Group on the Evaluation of Carcinogenic Risk to Humans, 2009) to have a viral origin in a proportion of cases. In

approximately one third of cases of HL, Epstein-Barr virus (EBV) is present within the malignant cell, where its presence is thought to be causal (Jarrett, 2002). EBV is itself oncogenic, and the virus plays a central role in HRS cell survival and proliferation. Important differences are seen in the epidemiology and biology of EBV-associated (EBV+ve) cHL, as compared with EBV-negative (EBV-ve) cHL. The interaction of the immune system, particularly cytotoxic T lymphocytes (CTLs), with the virus in EBV+ve cHL is being recognised as increasingly important. This is reflected in the class I human leukocyte antigen (HLA) associations with risk of developing EBV+ve cHL, and is the subject of this thesis.

1.2 Hodgkin Lymphoma - Pathology

The World Health Organisation (WHO) in its "Classification of Tumours of Haematopoietic and Lymphoid Tumours" defines HL as comprising two disease entities, classical Hodgkin lymphoma (cHL) and nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), Table 1.1.

Table 1-1 Classification of Hodgkin Lymphoma

Sub-type of Hodgkin Lymphoma	Abbreviation	Proportion of all cases	Malignant cell	Incidence	Association with EBV
classical Hodgkin lymphoma	cHL	95%	Hodgkin and Reed-Sternberg (HRS) cell	Bimodal age distribution (peak at 15-35 and > 50 years)	Approximately 1/3 of cases associated with EBV
Nodular lymphocyte predominant Hodgkin lymphoma	NLPHL	5%	Lymphocyte predominant (LP) cell, also known as "popcorn cell"	Unimodal age distribution (median age 35)	Not associated with EBV

There is increasing evidence that the cell of origin, pathogenesis and clinical course of NLPHL are distinct from cHL. Accounting for 5% of all HL diagnoses, NLPHL is increasingly recognised as a separate disease entity. It is not associated

with EBV. The other 95% of HL diagnoses are cHL. Discussion in the remainder of this thesis will be restricted to cHL, unless stated otherwise.

One striking feature of cHL is the rarity of the malignant cell in the tumour with the malignant HRS cell typically accounting for around 1% of the tumour mass (range 0.1-10%) (Kuppers, 2009b). Usually diagnosed on excision biopsy or core biopsy of a lymph node, a diagnosis of cHL requires the presence of the classical Reed-Sternberg cells which are large, with abundant cytoplasm and binucleate, or their mononuclear counterpart, the Hodgkin cell, collectively referred to as HRS cells. The remainder of the tumour comprises a mixed inflammatory cellular infiltrate, thought to be crucial in the survival of the malignant cells. This infiltrate includes small lymphocytes, often rosetting the HRS cell, eosinophils, neutrophils, plasma cells, and fibroblasts (Figure 1.1). The nature and importance of this inflammatory background is discussed in Section 1.3.7.

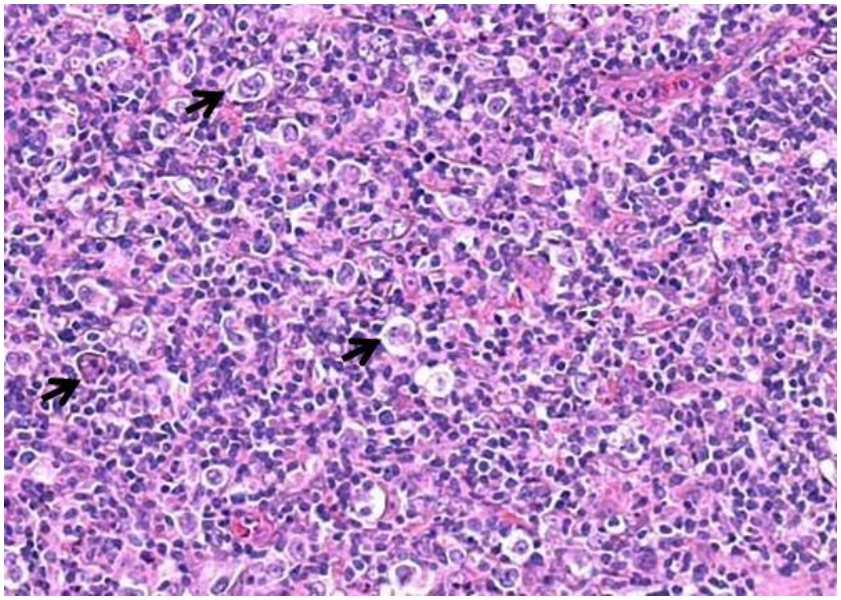

Figure 1-1 Lymph node biopsy, classical Hodgkin lymphoma

Haematoxylin and eosin staining (x400 original magnification) showing classical appearance of nodular sclerosis Hodgkin lymphoma, with large mononuclear and binucleate Hodgkin and Reed-Sternberg cells (black arrows) in a polymorphous background of cells which includes small lymphocytes and eosinophils. With thanks to Dr. G. Bryson, Department of Pathology, Southern General Hospital, Glasgow.

The modern classification of HL is based on the morphological appearances of the disease, particularly the composition of the background cellular infiltrate (Lukes & Butler, 1966). Within cHL there is further sub-classification into four histological subtypes (Table 1.2).

Table 1-2 Sub-types of classical Hodgkin Lymphoma

Subtype of cHL	Abbreviation	Proportion of cHL	Association with EBV	Outcome
Nodular sclerosis Hodgkin lymphoma	NSHL	70%	Approximately 10-30% of cases associated with EBV (depending on local patterns)	No strong association with outcome
Mixed cellularity Hodgkin lymphoma	MCHL	20-25%	More strongly associated with EBV (approximately 75% of cases)	No strong association with outcome
Lymphocyte rich Hodgkin lymphoma	LRHL	Approximately 5%	Less commonly associated with EBV	Associated with better outcome
Lymphocyte depleted Hodgkin lymphoma	LDHL	< 1%	Strong association with EBV	Associated with poor outcome

The four subtypes of cHL continue to be reported to clinicians, and recorded by cancer registries. Although they share the same immunophenotype and malignant cell, these subtypes vary in their epidemiology, clinical features, sites of involvement, degree of fibrosis, cellular microenvironment and frequency of association with EBV. There is some evidence that histological subtype is associated with outcome; it is recognised that lymphocyte depleted HL (LDHL), although rare, has an extremely poor outcome compared with other subtypes (Klimm *et al*, 2011). Lymphocyte rich (LR) histology probably has the best outcome, with nodular sclerosis (NS) and mixed cellularity (MC) disease falling somewhere in the middle. Histological subtype is also associated with age, with

NSHL seen more commonly in young adults, and MCHL seen more commonly with increasing age. The increased incidence of MCHL in older adults may, in part, reflect the proportion of cases due to EBV. At present, treatment of cHL does not vary between histological subtypes.

Immunohistochemistry (IHC) is crucial to the diagnosis of cHL. The HRS cell has a downregulated B cell programme (Section 1.3.3). Although HRS cells express B cell transcription factors, such as melanoma associated antigen (mutated) 1 (MUM1) and paired box 5 (PAX5), they are typically negative for pan B cell markers, such as cluster of differentiation (CD) 19 or CD20, and also the pan-leucocyte marker CD45 (Hertel *et al*, 2002; Schwering *et al*, 2003). Instead, the tumour necrosis factor receptor (TNFR) family member CD30 (Figure 1.2) and the aberrantly expressed myeloid marker CD15 are present in most cases, and provide the basis of diagnosis.

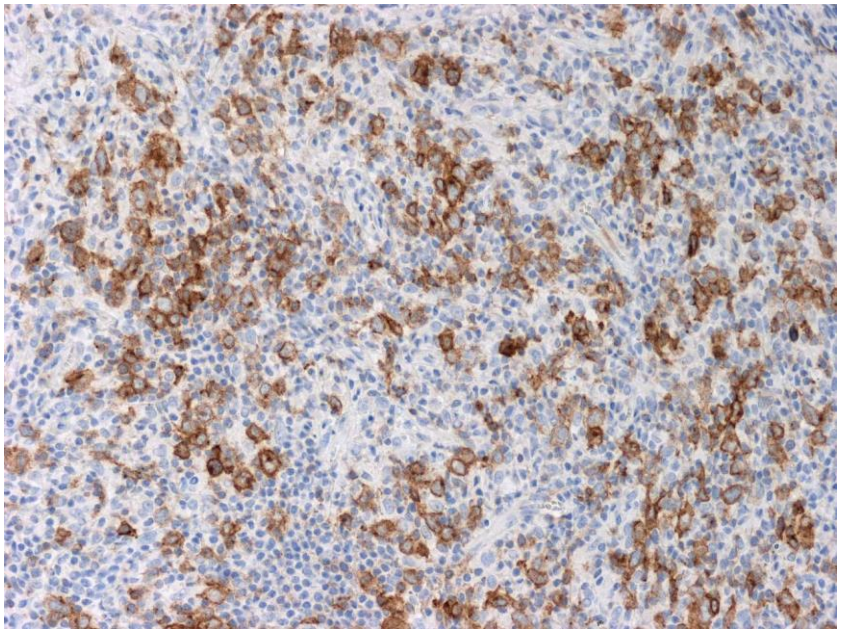

Figure 1-2 Lymph Node Biopsy, classical Hodgkin lymphoma, stained for CD30

Same case as in Figure 1.1, x200 original magnification, immunohistochemical staining for CD30. The tumour necrosis factor receptor family member CD30 is expressed by the malignant Hodgkin and Reed-Sternberg cells. With thanks to Dr. G. Bryson, Department of Pathology, Southern General Hospital, Glasgow.

In approximately 40% of cases (Stein *et al*, 2008), the B cell marker CD20 is expressed, usually on only a minority of cells, and at low surface intensity. There is some evidence that CD20 expression may be associated with outcome (discussed in Section 1.4.4). In addition, the putative "stem-cell" which has been described in cHL (discussed in Section 1.3.2) is also CD20 positive. Although the clonal expansion of the HRS cells occurs after the germinal centre (GC) (Section 1.3.1), the HRS cells are negative for the plasma cell markers CD138 and CD38. Diagnostic difficulty can arise in cases of B cell NHL with anaplastic morphology and CD30 positivity, or with anaplastic T-cell NHL. True biological overlap conditions can arise in the "grey-zone" lymphomas with pathological features and phenotype intermediate between cHL and diffuse large B cell lymphoma (DLBCL) where outcome is generally poor. Determination of the EBV status of the cHL is by in-situ hybridisation for EBV virus encoded small RNA (EBER) or IHC for EBV latent membrane protein (LMP) 1, (Gulley *et al*, 2002).

1.3 Biology of cHL

1.3.1 Cellular origin of cHL

The cell of origin of cHL remained elusive for many years, the rarity of the malignant cell contributing to the difficulty in characterising these cells. However, in the 1990s, microdissection, coupled with immunoglobulin (Ig) gene re-arrangement analysis of single cells revealed that the malignant cell in cHL is usually a clonal B cell. HRS cells from nearly all cases have re-arrangements of Ig heavy and/or light chain genes, confirming a B cell origin (Kuppers *et al*, 1994; Kuppers *et al*, 1996). The finding of identical mutations within each case confirmed the clonal nature of the disease (Kanzler *et al*, 1996; Marafioti *et al*, 2000). In addition, the variable regions of the immunoglobulin gene (IgV)

demonstrate somatic hypermutation, suggesting a GC or post-GC derivation (Kuppers, 2002). In the HRS cell, these somatic hypermutations are also clonal, suggesting that the HRS cell arises from a relatively late stage of B cell differentiation (Brauninger *et al*, 2006).

In normal post-GC B lymphocytes, the somatic hypermutations in the IgV genes result in cells which recognise antigen with high affinity, and are thus selected to proliferate as B-memory lymphocytes and to undergo further differentiation to plasma cells (Figure 1.3). B lymphocytes which do not express a functional Ig as part of a B cell receptor (BCR) complex, would normally undergo apoptosis (Kuppers & Hansmann, 2005). In HRS cells non-functional or "crippling" mutations of IgV, e.g. through acquisition of a stop codon, are detected in approximately 25% of cHL cases (Kuppers *et al*, 1994; Kanzler *et al*, 1996; Brauninger *et al*, 2006; Kuppers *et al*, 2001). The ability of the HRS cell to survive in the face of apoptotic signalling is considered central in disease pathogenesis. It was later shown that nearly all cases with such crippling mutations are EBV+ve supporting an essential role for EBV in the development of HRS cells from germinal centre B cells with these types of unfavourable mutations (Brauninger *et al*, 2006).

In rare cases (< 2%), HRS cells have been demonstrated to harbour T-cell receptor gene rearrangements rather than Ig gene rearrangements. These cases appear to represent true/ genuine cases of cHL, and so a T-cell origin in a minority of cases remains a possibility (Muschen *et al*, 2000; Muschen *et al*, 2001; Seitz *et al*, 2000).

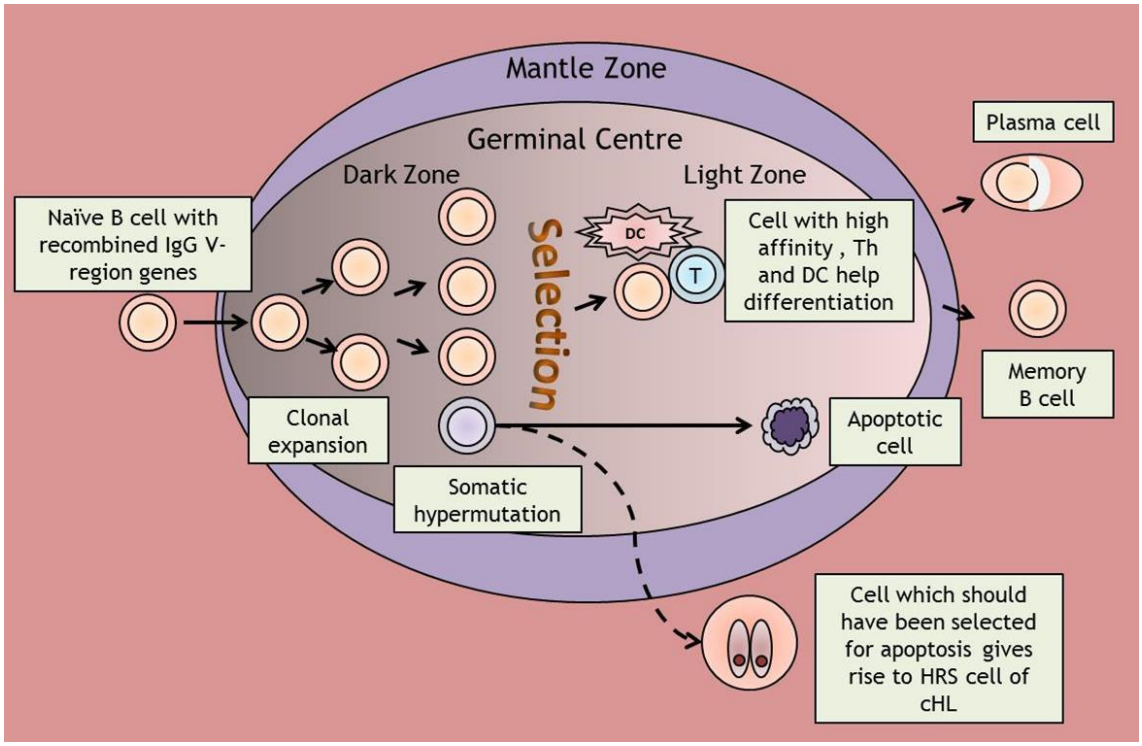

Figure 1-3 The germinal centre origin of the Hodgkin and Reed-Sternberg cell

Mature but antigen-naïve B lymphocytes, with re-arranged immunoglobulin (Ig) variable genes (V-genes), following encounter with an antigen, are driven into follicles within the lymph nodes and spleen. There the cells undergo clonal expansion and somatic hypermutation producing an Ig molecule with high affinity for the antigen. Those high-affinity cells are selected for differentiation, with the help of T-helper (Th) cells and dendritic cells (DC) differentiate into antibody-producing plasma cells, or long-lived memory B cells. Germinal centre, somatically hypermutated B cells which do not produce a functional Ig normally undergo apoptosis. This is the population thought to give rise to the Hodgkin and Reed-Sternberg cells of classical Hodgkin lymphoma.

1.3.2 A Hodgkin lymphoma stem cell?

True stem cells, as in acute leukaemia, are recognised by a capacity for unlimited self-renewal and generally a facility to produce progeny of multiple lineages. However, malignant lymphomas, including cHL, arise from mature lymphocytes. Mononuclear Hodgkin cells are capable of self-replication and generation of the multinucleate Reed-Sternberg cells through endomitosis (nuclear division without cellular division). The Reed-Sternberg cells are generally arrested cells unable to undergo further division (Ikeda *et al*, 2010).

The existence of a "lymphoma stem cell" or, to be more precise, a "lymphoma originating cell" has yet to be proved and remains controversial.

A recent study used flow cytometry for the stem cell marker aldehyde dehydrogenase to detect rare populations of small cells within cHL derived cell lines (Jones *et al*, 2009). These small cells were CD20 positive and shown to be capable of generating cultures of large HRS cells. They were also demonstrated to be present in the peripheral blood of patients with the disease by flow cytometry. The presence of rare (0.5%) side population (SP) cells in both cHL derived cell lines and primary tissue specimens has also been documented (Shafer *et al*, 2010). SP cells are defined by the capability of the ABC transporter to expel chemotherapeutic drugs from the cell; they share features with cancer stem cells and may cause disease relapse if not eradicated.

Much of this work is controversial and criticisms based on technical concerns have been raised (Kuppers, 2009a; Kuppers, 2010). No investigator has yet proven that these cells are clonogenic. However, one of the strongest pieces of circumstantial evidence validating the concept of the CD20 positive "cHL-originating cell" was the finding in a subsequent clinical study that persistence of detectable circulating clonotypic B cells was associated with a greater relapse frequency ($p < 0.05$) (Kasamon *et al*, 2012).

1.3.3 Cellular re-programming

Despite its B cell origin, the HRS cell does not express a typical B lymphocyte phenotype. Typical B lymphocyte lineage surface markers, such as CD20, CD19, CD79 and surface Ig are not present, and the B lymphocyte transcription factors, such as organic cation transporter 2 (OCT2), B cell Oct binding protein 1 (BOB1)

and transcription factor binding to the PU-box (PU1) are downregulated (Hertel *et al*, 2002; Schwering *et al*, 2003). The B lymphocyte-specific transcription factor PAX5 usually continues to be expressed, although often at low levels, but when present can be useful to the pathologist in distinguishing cHL from T cell NHLs (Foss *et al*, 1999). As has been discussed, HRS cells classically express CD30, normally expressed on activated B and T cells, and the myeloid/monocyte lineage marker CD15. HRS cells are observed to aberrantly express other lineage markers, such as T lymphocyte markers CD3, CD4 and granzyme B (Drexler, 1992), and dendritic cell markers, fascin and thymus and activation regulated chemokine (TARC) also known as chemokine (CC-motif) ligand (CCL) 17) (Peh *et al*, 2001). This loss of B cell signature may be one of the mechanisms adopted by the HRS cell to survive without the tonic BCR signalling normally required to prevent apoptosis.

Epigenetic modification plays an important role in the transcriptional downregulation of genes associated with the B cell phenotype. Groups of genes are silenced by promoter methylation, suggesting the involvement of a master transcriptional regulator(s) (Ushmorov *et al*, 2004). Activation of Notch1 signalling may be important as it promotes transcription of genes associated with a T lymphocyte phenotype, and suppresses B cell differentiation through interference with B cell transcription factors. Notch1 promotes the degradation of E2A immunoglobulin enhancer binding factors E12/E47 (E2A) (Nie *et al*, 2003) and blocks the ability of early B cell factor (EBF) to bind to deoxy-ribonucleic acid (DNA) (Smith *et al*, 2005), and has been observed to inhibit PAX5 at a transcriptional and post-transcriptional level (Jundt *et al*, 2008).

1.3.4 Survival and proliferation signals

Cellular re-programming is only one of many survival strategies the HRS cell employs. Numerous transcription factors, the most crucial of which is nuclear factor-κB (NF-κB), are altered to promote survival and proliferation. NF-κB is a collection of transcription factors which comprises RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) which are involved in inflammatory responses and cell fate decisions (Ghosh *et al*, 1998). NF-κB is constitutively active in HRS cells (Bargou *et al*, 1997; Bargou *et al*, 1996), where it is required for proliferation and survival.

NF-κB can be activated in a number of ways in cHL. First, many of the tumour necrosis factor (TNF)-family receptors upstream of NF-κB, including CD30, CD40 and CD95, are upregulated (Fiumara *et al*, 2001; Carbone *et al*, 1995). The cellular microenvironment in which the HRS cell resides (see below) provides ligands of these receptors, resulting in unregulated paracrine stimulation. Second, EBV can directly activate NF-κB via LMP1 which mimics CD40 signalling (see below). Third, mutations in the genes for inhibitors of NF-κB, IκB (IκB kinase) have been described in 10-20% of cases of cHL (Emmerich *et al*, 1999; Jungnickel *et al*, 2000; Lake *et al*, 2009; Cabannes *et al*, 1999), and genomic gains of c-Rel have been described in nearly 50% of cases (Barth *et al*, 2003). Lastly, inactivating mutations of the TNF-α induced protein 3 (TNFAIP3) gene, which encodes the NF-κB inhibitor A20, have been described in approximately 40% of cases of cHL (Kato *et al*, 2009; Schmitz *et al*, 2009a), particularly EBV-ve cases.

Also implicated in the proliferation and apoptosis resistance of HRS cells is the Janus kinase – signal transducers and activators of transcription (JAK-STAT)

pathway, one of the central mechanisms of cytokine signal transduction. The cytokines which initiate JAK-STAT signalling, including interleukin (IL)-6, IL-9, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF), are produced in the cHL tumour and activate the downstream STAT3, STAT5 and STAT6 (Hinz *et al*, 2002; Skinnider *et al*, 2002a; Kube *et al*, 2001). Genomic lesions affecting the JAK-STAT pathway have also been described, including frequent genomic gains of JAK2 (Joos *et al*, 2003) and inactivating mutations of suppressor of cytokine signalling 1 (SOCS1), a negative regulator of JAK-STAT signalling (Weniger *et al*, 2006).

Additional pro-proliferation signalling pathways constitutively activated in cHL include Notch1 (Jundt *et al*, 2008; Jundt *et al*, 2002), the phosphoinositide-3-kinase (PI3K/Akt) pathway (Dutton *et al*, 2005) and the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinases (ERK) pathway (Zheng *et al*, 2003). The myeloid cell receptor colony-stimulating factor 1 receptor (CSF1R) functions as an oncogene in cHL and is activated by de-repression of an endogenous long terminal repeat upstream of the gene (Lamprecht *et al*, 2010).

Micro ribonucleic acid (miRNAs), small non-coding RNAs that bind to and influence translation of messenger RNA (mRNA), have over recent years been shown to be important in the development of cancer. A number of miRNAs have been shown to be dysregulated in cHL as compared to normal B cells (Kluiver *et al*, 2005; Van Vlierberghe *et al*, 2009), and many of these could putatively be associated with targets linked to proliferation, although functional studies have to date not been performed.

Thus, multiple signalling pathways and transcriptional regulators are disrupted in the HRS cell, where they are thought to cooperate to induce proliferation, suppress apoptosis and promote a favourable cellular microenvironment (Section 1.3.7).

1.3.5 Chromosomal abnormalities

In contrast to a number of B cell NHLs, which are characterised by a single recurrent chromosomal abnormality, there are no recurrent cytogenetic abnormalities which characterise cHL. However, most cHL malignant cells have complex cytogenetics. Conventional and array comparative genomic hybridisation (CGH) studies (Joos *et al*, 2003; Kluiver *et al*, 2007; Chui *et al*, 2003; Steidl *et al*, 2010b) have demonstrated a number of recurrent imbalances including gains of 2p (which includes the REL oncogene), 9p (on which is located JAK2), 12p, 16p, 17p, 17q, 19p, 19q, 20q, 21q and 22q, and losses of 1p, 6q, 7q, 8p, 11q and 13q. Minimal regions were defined which harboured genes of interest, including REL, IκB, CD40, MAP3K14, TNFAIP3, JAK2, programmed death 1 ligand (PD-1L) and class II, major histocompatibility complex, transactivator (CIITA) (Steidl *et al*, 2011; Steidl *et al*, 2010b).

1.3.6 Genome-wide association studies

Over recent years a number of genome-wide association studies (GWAS) have been performed to look for germ-line variations associated with development of cHL (Enciso-Mora *et al*, 2010; Cozen *et al*, 2011; Urayama *et al*, 2012). These have shown strong associations with the HLA region (discussed in Section 1.7.2) and also identified a number of non-HLA loci associated with risk of disease. Susceptibility loci have been described (Enciso-Mora *et al*, 2010) at 2p16.1 (rs1432295, REL, odds ratio (OR) = 1.22, $p = 1.91 \times 10^{-8}$), 8q24.21 (rs2019960,

PVT1, OR = 1.33, p = 1.26×10^{-13}) and 10p14 (rs501764, GATA3, OR = 1.25, p = 7.05×10^{-8}). That the three loci identified are of genes described as functionally important in the HRS cell is striking.

1.3.7 Microenvironment

Nearly all of the cells present in a cHL tumour are those of a reactive infiltrate, which includes T cells, B cells, macrophages, eosinophils, fibroblasts, mast cells and plasma cells (Schmitz *et al*, 2009b). It is likely that this infiltrate in part reflects the immune response to the tumour; however, this response is ineffective. There is evidence that the HRS cell itself recruits these infiltrating cells to the tumour tissue, where the paracrine signals they provide generate a favourable environment. This in turn supports cell survival, proliferation and immune escape. The symbiotic relationship between the HRS cell and the cellular microenvironment therefore appears central to the pathogenesis of cHL.

Most of the infiltrating cells are CD4+ve T cells, characteristically with a T helper (Th) 2 (Th2) type phenotype. These cells secrete a cytokine profile which supports the humoral immune response and B cell proliferation. In contrast, Th1 cells which assist the cellular immune response, CD8+ve CTLs and natural killer (NK) cells are notable by their paucity. The Th2 cells are thought to be drawn in to the node by chemokines produced directly by the HRS cell: TARC (van den Berg *et al*, 1999), CCL5 and CCL22 (Skinnider & Mak, 2002). Th2 cells also express CD40 ligand, which may engage with and stimulate the CD40 molecules on the surface of the HRS cells, triggering the activation of NF-κB as mentioned above.

Abundant among the infiltrating CD4+ve T cells are CD4+ve, CD25+ve, forkhead box P3 (FoxP3)+ve cells T regulatory lymphocytes (T_{regs}) (Marshall *et al*, 2004). T_{regs} normal function is in immune homeostasis where they are important in inducing peripheral tolerance to autologous antigens and regulating effector immune responses. They are increasingly recognised as important in suppressing tumour antigen specific cellular immunity, and may therefore be important in protecting the HRS cell from attack, particularly in EBV-associated disease where viral antigens may be expected to trigger a CTL response (Li *et al*, 2009a; Marshall *et al*, 2007). The HRS cell is able to attract T_{regs} via secretion of cytokines including galectin 1 (Juszczynski *et al*, 2007; Gandhi *et al*, 2007), IL-10 and transforming growth factor-beta (TGF-β) (Marshall *et al*, 2004) and chemokines TARC, CCL5, CCL20 and CCL22 (Skinnider & Mak, 2002; Aldinucci *et al*, 2008). CD8+ve regulatory T cells, also known as T suppressor (Ts) cells, have also been described. Acting via secretion of IL-10 or via direct cell to cell contact, several types of Ts cell have been described; the majority originate from CD8+ve CTLs and acquire a suppressor phenotype after antigenic stimulation. The particular inducers of this phenotype in CD8+ve CTLs are not known, but it is postulated that different peptide repertoires may induce a suppressor phenotype (Filaci *et al*, 2011).

Eosinophils are often present in nodal tissue, bone marrow and peripheral blood of patients. The HRS cells and the fibroblasts in the tumour microenvironment secrete eotaxin, IL-5, IL-9, CCL5, CCL11 and CCL28, all of which are implicated in eosinophil proliferation and recruitment (Teruya-Feldstein *et al*, 2000; Teruya-Feldstein *et al*, 1999).

Further cytokines central in the pathogenesis of cHL include IL-13, which is secreted by HRS cells and, as HRS cells also express the IL-13 ligand, may act as an autocrine growth factor (Kapp *et al*, 1999; Skinnider *et al*, 2002b), IL-6 and IL-10. IL-6 has both pro-inflammatory and anti-inflammatory functions, and is thought to exert potent anti-viral effects. Elevated levels are noted in patients with HL (Gause *et al*, 1992), and in addition, the HRS cell expresses both IL-6 and IL-6 receptor, such that the cytokine may function as an autocrine growth factor (Jucker *et al*, 1991). In EBV-associated classical Hodgkin lymphoma (EBV+ve cHL), the EBV protein LMP1 leads to higher levels of IL-6 expression which may contribute to the proliferation and survival of the malignant cell in this setting (Herbst *et al*, 1997). IL-10 is an anti-inflammatory cytokine secreted by Th2 cells and a phenotypically distinct subset of Ts cells (Noble *et al*, 2006). In addition to the immune suppressive role, there is also some evidence that IL-10 can enhance B cell proliferation (Guedez *et al*, 2001) and IL-10 has been particularly implicated in the pathogenesis of EBV+ve cHL (Ohshima *et al*, 1995; Baiocchi, 2008).

Systemically, the cellular and cytokine responses can result in a degree of immune suppression observed in patients with cHL. cHL patients demonstrate anergic responses to skin tests for antigens including tuberculin (Schier, 1954) and typhoid (Rottino & Hoffmann, 1950). It is also recognised that patients with cHL, even before or after treatment, are at high risk of the almost uniformly fatal transfusion-associated graft-versus-host-disease (Spitzer *et al*, 1990), which normally occurs only in the setting of severe cell-mediated immune compromise e.g. following allogeneic bone marrow transplant or treatment with purine analogues. Such systemic immune suppression most likely contributes to the

impairment of an effective EBV-specific immune response at the time of oncogenesis.

An overview of the HRS cell and its interactions in the cHL microenvironment is given in Figure 1.4. A summary of the many cytokines, chemokines, receptors and ligands (Mani & Jaffe, 2009; Roullet & Bagg, 2007) implicated in the cross-talk between the HRS cell and the reactive infiltrate is shown in Table 1.3.

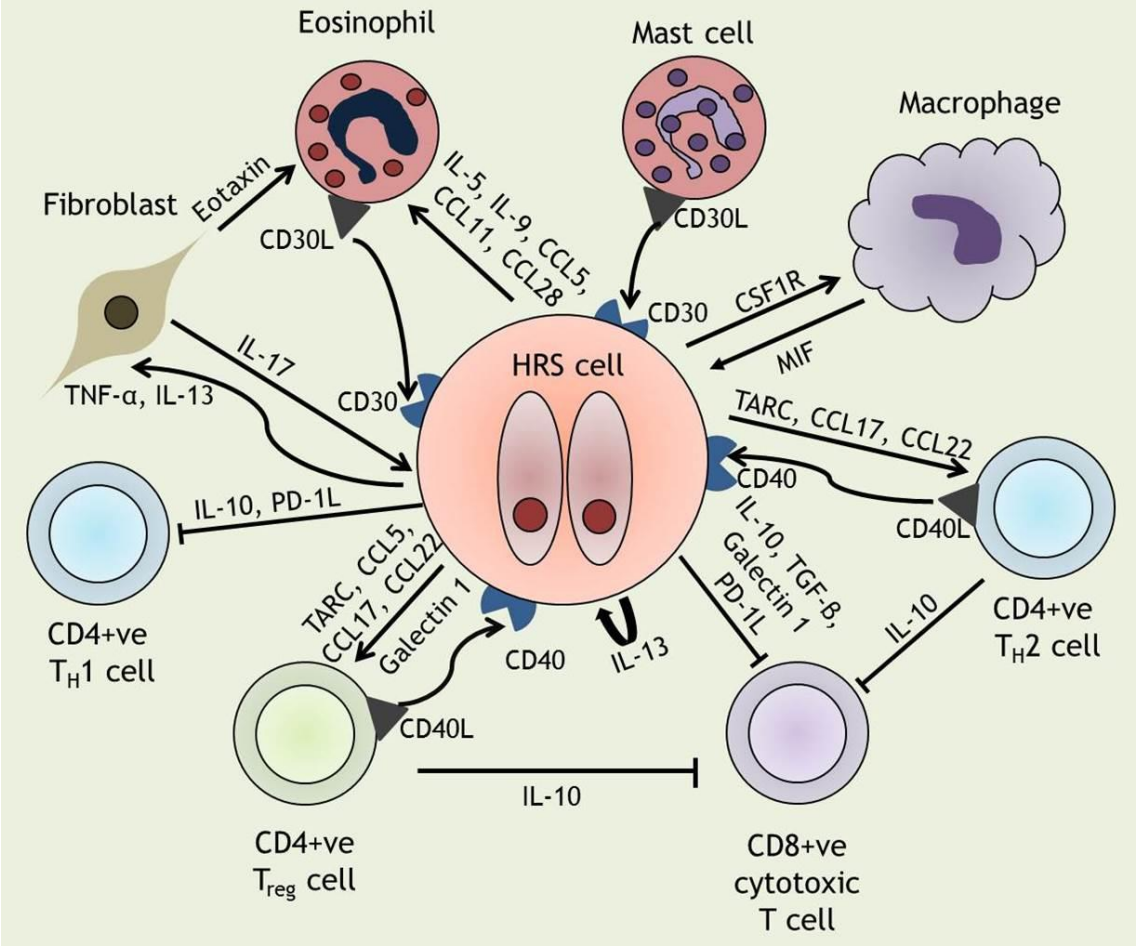

Figure 1-4 The HRS cell and its interactions with the cellular microenvironment

A simplified schematic of the HRS cell and its interactions with the reactive infiltrate. The HRS cell secretes cytokines and chemokines to recruit cells which result in immune escape or provide survival/proliferative signals. The effective CD4+ve Th1 and CD8+ve cytotoxic T lymphocyte (CTL) immune response is inhibited directly by the HRS cell and via IL-10 from T_{reg} and Th2 cells. Other surrounding cells of physiological importance in providing a favourable cellular environment are fibroblasts, eosinophils, mast cells and macrophages. Further detail is given in the text. CCL, CC chemokine ligand; CD, cluster of differentiation; CSF1R, colony stimulating factor 1 receptor; HRS, Hodgkin and Reed-Sternberg; IL, interleukin; PD-1L, programmed death 1 ligand; TGF, transforming growth factor; Th, T helper cell; TNF, tumour necrosis factor; T_{reg}, T regulatory cell; TARC, thymus and activation-regulated chemokine; MIF, macrophage inhibitory factor.

Table 1-3 Cytokines, chemokines and receptors altered in classical Hodgkin lymphoma.

Function	Cytokine/ Chemokine/ Receptor/ Ligand	Reference
Promote Th2 Response	TARC/CCL17	(van den Berg *et al*, 1999)
	MDC/CCL22	(Teruya-Feldstein *et al*, 1999; Hedvat *et al*, 2001)
	CCL-20	(Baumforth *et al*, 2008)
	MIG	(Teruya-Feldstein *et al*, 1999; Buri *et al*, 2001)
	IP-10	(Teruya-Feldstein *et al*, 1999)
	IL-13	(Skinnider *et al*, 2002b)
	GATA-3	(Kuppers *et al*, 2003; Atayar *et al*, 2005)
	CCR-4	(Ohshima *et al*, 2003)
Suppress Th1 Response	IL-10	(Aldinucci *et al*, 2010)
	TGF-β	(Kadin *et al*, 1993; Newcom & Gu, 1995)
	PD-1L	(Yamamoto *et al*, 2008)
	Galectin-1	(Juszczynski *et al*, 2007; Gandhi *et al*, 2007)
Promote influx of eosinophils	IL-5	(Samoszuk & Nansen, 1990)
	IL-9	(Merz *et al*, 1991)
	Eotaxin (produced by fibroblasts)	(Jundt *et al*, 1999)
	CCL28	(Hanamoto *et al*, 2004)
Promote influx of mast cells	IL-9	(Merz *et al*, 1991)
	RANTES/CCL5 (also monocytes, T cells & eosinophils)	(Aldinucci *et al*, 2008)
Promote influx of plasma cells	IL-6	(Jucker *et al*, 1991)
Promote influx of neutrophils	IL-8	(Foss *et al*, 1996)
	GM-CSF	(Byrne *et al*, 1986)
Promote influx of fibroblasts	IL-13	(Skinnider *et al*, 2002b)
	TGF-β	(Kadin *et al*, 1993; Newcom & Gu, 1995)
	TNF-α	(Kretschmer *et al*, 1990; Sappino *et al*, 1990)
	MMP	(Thorns *et al*, 2003)
	TIMP1 & 2	(Pennanen *et al*, 2004)
Autocrine growth factor	IL-13/IL-13-R (via STAT6 activation)	(Skinnider *et al*, 2002b)
	TIMP-1	(Pennanen *et al*, 2004)
	IL-6	(Jucker *et al*, 1991)
Activates NF-κB	CD40/CD40-R	(O'Grady *et al*, 1994)
	CD30/CD30-R	(Pinto *et al*, 1996; Horie *et al*, 2002)
	TNF-α	(Kretschmer *et al*, 1990; Sappino *et al*, 1990)
	RANK/RANK-L	(Fiumara *et al*, 2001)

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Abbreviations given at first use in the text or on pages 17-22.

1.4 Hodgkin lymphoma – Clinical Aspects

1.4.1 Clinical Staging

The vast majority of patients present with painless lymphadenopathy, although the disease may be asymptomatic and picked up as an incidental finding e.g. on a chest X-ray. Clinical outcome in HL depends partially on clinical stage of disease as assessed using the internationally accepted clinical staging system, the modified Ann Arbor staging system (Lister *et al*, 1989) (Table 1.4).

The B symptoms of HL (night sweats, weight loss of at least 10% of body weight or unexplained fever) occur in approximately 40% of patients at presentation and are associated with poorer outcome. These are thought largely to be the systemic result of cytokines released from the malignant HRS cells. Other systemic manifestations of disease such as pruritus, fatigue and alcohol-induced pain, are not prognostic and are not included as B symptoms for this reason.

All clinical stage IA and IIA disease is classified as "early stage" disease. All clinical stage III and IV disease, and any patient with stage I or II disease with B symptoms or disease bulk (nodal mass ≥ 10 cm or a mediastinal mass ≥ 0.33 of the intra-thoracic diameter) is classified as "advanced stage disease". Treatment decisions are based on this classification.

Further prognostic refinement of early stage disease based on the presence of "risk factors" can be used to define "early-stage unfavourable disease" which may require consideration for more intensive treatment at baseline. Such factors vary worldwide depending on the criteria of the national research group but in the United Kingdom (UK) include: erythrocyte sedimentation rate (ESR) > 50 (units); > 3 nodal areas; age > 50 years; and bulk ≥ 5 cm but < 10 cm.

Table 1-4 Ann Arbor staging system for Hodgkin lymphoma (Cotswold modification)

Stage I	Involvement of single lymph node region or localised extranodal site*
Stage II	Involvement of two or more lymph node regions or localised extranodal sites*, or both, on the same side of the diaphragm
Stage III	Involvement of lymph node regions or localised extranodal sites*, or both, on both sides of the diaphragm
Stage IV	Diffuse or disseminated involvement of one or more extra-lymphatic organ(s), with or without associated lymph node involvement. Involvement of liver or bone marrow is considered stage IV

* designated by the suffix E, e.g. stage IE, IIE etc.

† Subsets A and B are designated by the absence (A) or presence (B) of systemic symptoms, namely night sweats, weight loss of at least 10% of body weight or unexplained fever.

(Lister et al, 1989)

The most common presentation of cHL is with cervical lymphadenopathy (60-70% of patients); other common sites of disease include the mediastinum, supra-clavicular, axillary and para-aortic nodes (Sasse & Engert, 2007). Approximately half of patients have a mediastinal mass at presentation. In contrast to NHL, disease of the inguinal and pelvic nodes is less common. It is not known if this distribution of disease reflects potential antigen exposure e.g. oral exposures to antigen, or potentially EBV reactivation in the oropharyngeal mucosa. Primary extranodal involvement by cHL is rare and, where it occurs, usually arises as a local invasion of nodal disease. Bone marrow involvement is also rare (< 5% of cases).

Clinical staging is performed at diagnosis using computed tomography (CT) scanning; a three-dimensional scanning technique which uses x-rays to provide detailed cross-sectional imaging at high resolution. Involved anatomical areas can be defined and sized, and biopsies targeted to involved areas if necessary.

Functional imaging, using positron emission tomography (PET) scanning, which is based on the increased uptake of the radiolabelled glucose analogue ^{18}F-fluorodeoxyglucose (FDG) in metabolically active cells as compared with surrounding tissue, is increasingly being used at baseline for accurate staging. It is particularly useful in those patients thought to have early stage disease, and in whom the inability to correctly detect stage III or IV disease, particularly bony disease, could mean potentially undertreating the patient (Richardson *et al*, 2012; El-Galaly *et al*, 2012). In a study of ^{18}F-FDG PET scanning in staging (Cerci *et al*, 2011), up to 24% of patients thought to have early stage disease were up-staged to a higher clinical stage and in 15% clinical management was changed as a result. Staging ^{18}F-FDG PET is also useful in providing a baseline against which subsequent (interim or end-of-treatment) scans can be compared.

1.4.2 Therapy

Treatment of cHL is tailored to the patient's clinical stage and is generally based on combinations of different chemotherapeutic agents, with or without the addition of radiotherapy. Neither histological subtype or EBV status influence treatment decisions. HL was one of the first malignancies treated successfully with radiotherapy in the 1940s and chemotherapy in the 1960s (Kaplan, 1968; Kaplan & Rosenberg, 1968). Prior to this, the disease was almost universally fatal. Successive improvements in treatment protocols over subsequent decades mean that the majority of patients presenting today are cured of their disease. Factors involved in the decision regarding which therapeutic approach to take include: efficacy in controlling disease; options and prognosis for second-line therapy should it be required; and the toxicity, late effects and quality of life following treatment. The majority of patients with cHL achieve a lasting CR;

hence toxicity & quality of life have increased in importance when choosing treatment.

Most patients with early-stage disease are cured, and modern treatment protocols for this group emphasise cure with the lowest toxicity possible (Connors, 2005). Successive refinements of chemotherapy and radiotherapy have sought to define the *minimal* level of treatment which will result in an acceptable level of disease control. Abbreviated chemotherapy, based on doxorubicin, bleomycin, vinblastine and dacarbazine (ABVD) (Bonadonna *et al*, 1975), followed by involved-field radiotherapy (the combination referred to as combined-modality treatment) is the standard of care in this group. The GHSG (Engert *et al*, 2010) have most recently definitively shown that the combination of two cycles of ABVD chemotherapy and 20 Gray (Gy) of involved-field radiotherapy is sufficient in early-stage favourable disease, and results in an event-free survival (EFS) of 91% and OS of 93% at five years. At present, it is not considered safe to omit radiation in the treatment of early-stage cHL due to an unacceptably high relapse rate (Meyer *et al*, 2012). Early-stage unfavourable disease is also treated with combined modality treatment, but with four cycles of ABVD chemotherapy and 30 Gy of involved field radiotherapy (von Tresckow *et al*, 2012).

The standard management of advanced stage cHL involves combination chemotherapy only. In the UK, the United States, and much of the rest of the world ABVD (6-8 cycles) is generally used. ABVD delivers high response rates but also has the advantages of preserving fertility and a lower risk of secondary haematological malignancy when compared with other regimens. It is recognised however, that on-going controversy exists as to the best chemotherapeutic

regimen to choose in advanced stage patients. The GHSG HD9 trial (Engert *et al*, 2009) compared patients receiving eight alternating cycles of ABVD and an alternative regimen, COPP (cyclophosphamide, vincristine, prednisolone and procarbazine) with patients receiving eight cycles of escalated BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, prednisolone, and procarbazine). Both groups received involved field radiotherapy to sites of initial disease bulk and residual masses. In this study, 10 year freedom from treatment failure (FFTF) was 82% and 10-year OS was 86% in the BEACOPP arm as compared with 64% 10-year FFTF and 75% 10-year OS in the COPP-ABVD arm. These outcomes have led to the adoption of escalated BEACOPP as the standard of care for advanced-stage cHL in Germany and some parts of Europe (Eichenauer & Engert, 2011; Borchmann *et al*, 2011). The study has been criticised for use of COPP-ABVD as opposed to ABVD in the comparison arm, therefore no true comparison with current gold-standard therapy was performed. In addition, the study reported a rate of secondary acute myeloid leukaemia (AML) of 3% at 10 years in the BEACOPP escalated arm as compared with 0.4% in the COPP-ABVD arm. BEACOPP escalated has also been shown to be associated with infertility (Behringer *et al*, 2005). In addition, it was impossible to accurately predict in the HD9 study, even using International Prognostic Score (IPS), the group of approximately 10% of patients who required treatment at the intensity of BEACOPP escalated, as compared with ABVD, to achieve cure.

A recent Italian study (Viviani *et al*, 2011) compared OS in patients treated at baseline with 6-8 cycles of ABVD and treatment escalation to salvage therapy (see below) only when required for relapsed or progressive disease, with patients treated with 8 cycles of BEACOPP escalated at baseline. Greater FFTF was seen in the BEACOPP escalated arm (7-year, 84% vs. 73%, $p = 0.004$).

However, once the planned treatment was delivered in all patients (including salvage for those in whom it was required), there was no difference in OS (89% vs. 84% at 7-years, p = 0.39), suggesting that the same outcome could be achieved whilst sparing two-thirds of patients unnecessary higher intensity treatment.

For these reasons, ABVD is still currently thought to deliver the best balance between treatment efficacy and toxicity, and is therefore regarded as the standard of care for advanced-stage cHL (Connors, 2011a; Lim & Johnson, 2011). Future refinements to the treatment are likely to be based on the use of novel agents (see below) or on the better targeting of intensive therapy to those that need it, using improved prognostic tools (see below) (Connors, 2011b).

For patients who have primary refractory disease, or who relapse, further treatment is required to achieve disease control and aim for cure. This is delivered in the form of "salvage therapy" which generally consists of multiple courses of a non-cross-reacting high-dose multi-agent chemotherapy, consolidated by an autologous or allogeneic haemopoietic stem cell transplant. There is no consensus on a gold-standard for salvage chemotherapy, and the decisions, particularly those regarding consolidation, depend on patient fitness, availability of a stem cell donor and other patient-specific features. It is recognised that outcomes are particularly poor in patients with primary refractory disease (5-year OS 26%) (Josting *et al*, 2000), or who relapse within 12 months (5-year OS 43%) (Josting *et al*, 2002).

As has been alluded to in the discussions above, both chemotherapy and radiotherapy for cHL are associated with both short and long-term toxicities. Short-term toxicities include cytopenias, neutropenic sepsis, hair loss, altered

taste and pulmonary toxicity associated with bleomycin. Late toxicities of therapy range from those affecting quality of life (such as fatigue) to those which may be life-threatening (such as cardiovascular disease or secondary malignancy). Some of the late effects of treatment for cHL are listed in Table 1.5.

Table 1-5 Late effects of therapy for Hodgkin lymphoma

	Toxicity
"Minor"	Endocrine dysfunction (hypothyroidism, hypo-a-menorrhoea, decreased libido) Long-term immunosuppression Viral infections (Herpes simplex, Varicella zoster, Human papillomaviruses)
More Serious	Lung fibrosis from radiation plus bleomycin Myocardial damage from anthracyclines and radiation Sterility in men and women Growth abnormalities in children and adolescents Opportunistic infections Psychological problems Psychosocial disturbances Fatigue
Potentially life-threatening	Acute myeloid leukaemia / myelodysplastic syndrome Non-Hodgkin's lymphoma Solid tumours (lung, breast, and colon cancers, sarcomas) Heart failure & myocardial infarction

Studies have examined the risk of second cancer in some detail. Acute myeloid leukaemia risk peaks at 5-9 years following treatment at a rate of approximately 1-3% (Standardised Incidence Ratio, SIR, 47.1, 95% confidence interval (CI), 28.4-73.6) (Swerdlow *et al*, 2011). There is an increased risk of a number of malignancies including lung cancer, colon cancer, melanoma and sarcomas, when compared with untreated populations. Some groups are particularly vulnerable to second malignancy; female patients under the age of 20 years who receive radiotherapy to a field which includes breast tissue have an standardised incidence ratio (SIR) of breast cancer of 18.2 (95% CI, 8.3 to 33.6) (Swerdlow *et*

al, 2011) and such patients enter mammography screening programmes from the age of 25 years. The overall 20-year cumulative risk of a second cancer in this study was 13% for patients treated with chemotherapy only and 18% for patients treated with combined modality therapy.

Cardiovascular disease is emerging as a particular concern in survivors of cHL, as the effect becomes more apparent with longer follow-up. Long-term survivors of cHL are more likely to die from cardiovascular disease than their cHL, and this risk increases with time; at 10 years post-diagnosis, survivors have a relative risk of death from cardiovascular disease of 6.3 when compared with age matched controls, (Figure 1.5) (Aleman *et al*, 2003).

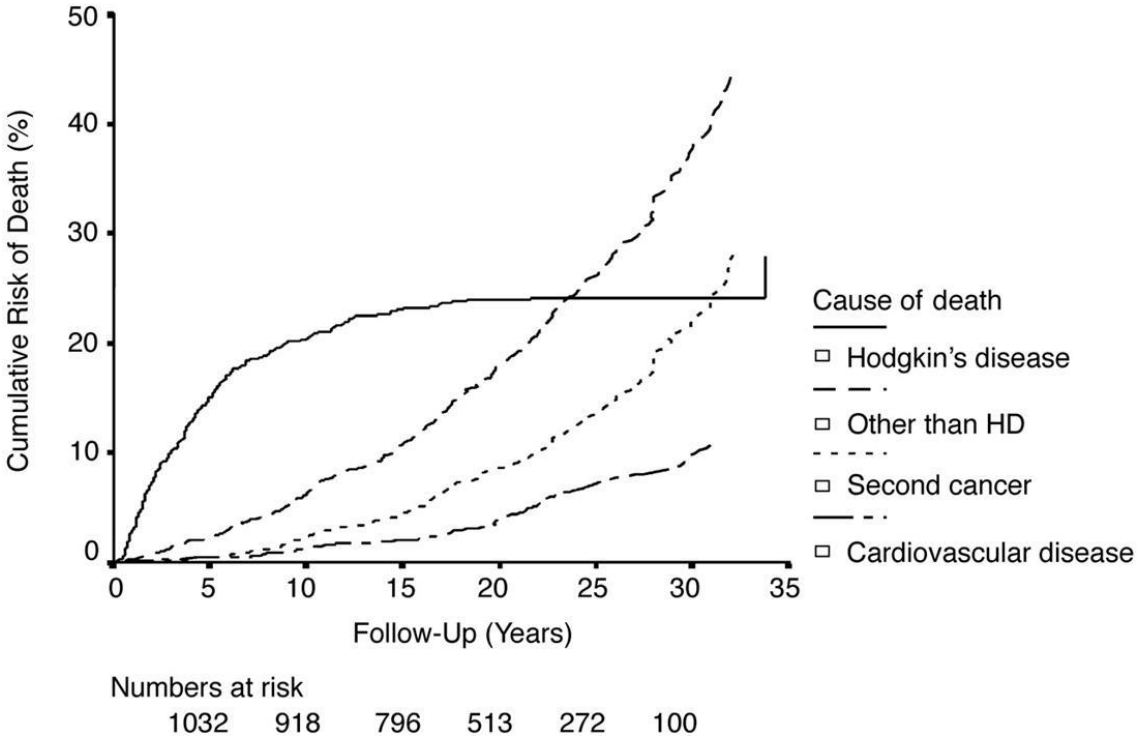


Figure 1-5 Acturial risk of death by cause (major disease categories), following a diagnosis of Hodgkin lymphoma

HD, Hodgkin lymphoma. Reproduced with permission from (Aleman *et al*, 2003), Copyright American Society of Clinical Oncology.

Balancing treatment efficacy and cure versus morbidity & mortality of late complications of treatment has led researchers to explore response-adapted therapy. This approach aims to identify disease which is not responding to therapy at an early stage, in order that treatment intensification can be considered with the aim of avoiding primary refractory disease or early relapse and the attendant poor survival. Prospective studies using ^{18}F-FDG PET to assess metabolic response after 1 or 2 cycles of chemotherapy have been performed, and show clinical utility as a prognostic tool. A pivotal study from a joint Italian and Danish group (Gallamini *et al*, 2007) demonstrated the predictive power of a ^{18}F-FDG PET after two cycles of ABVD; 2-year PFS of 95% was seen in patients with a negative PET vs. 12.8% 2-year PFS in those with a positive PET ($p < 0.001$). In this study, treatment was not changed on the basis of the PET-scan result. Instinctively, it is felt that treatment intensification in the group of PET-positive patients would improve outcome, but this hypothesis has yet to be proven in clinical studies. On-going clinical trials are addressing this question (National Cancer Research Initiative Lymphoma Clincal Studies Group, 2013a; German Hodgkin Study Group, 2013) and much work regarding standardisation of PET scanning will be required before this can be regarded as the standard approach.

Some authors (Connors, 2011b; Younes, 2009; Jona & Younes, 2010) believe that the maximal benefit that can be achieved with standard cytotoxic chemotherapeutic approaches has been reached and that further improvements in outcome will rely on the use of alternative agents which exploit the biology of the tumour. In contrast to diseases such as chronic myeloid leukaemia there is no single molecular target in cHL. However, the expression of markers on the

cell surface, or molecular pathways utilised by the cell (such as NF-κB, see Section 1.3.4) can potentially be exploited.

Agents currently being explored in clinical trials include the anti-CD20 molecule, rituximab, which has demonstrated benefit in phase I and II clinical studies (Kasamon *et al*, 2012; Younes *et al*, 2012), particularly in patients with EBV+ve cHL. Larger phase III studies are in progress (German Hodgkin Study Group, 2013).

One of the most exciting agents to emerge in recent years is brentuximab vedotin, a monoclonal anti-CD30 antibody conjugated to the anti-microtubule drug monomethyl auristatin E. Phase I studies in heavily pre-treated patients have demonstrated overall response rates of 50% with CR rates of 35% (Younes *et al*, 2010). On this basis, the drug has received a licence for use in relapsed and refractory disease, where it is currently being used as a bridge to transplant (Younes *et al*, 2010). Phase III clinical studies are examining the role of the agent in primary therapy (National Cancer Research Initiative Lymphoma Clincal Studies Group, 2013b).

1.4.3 Clinical Prognostic Scoring Systems

The purpose of any prognostic system is the prediction of clinical outcome to allow the identification of groups of patients who may benefit from reduced treatment, who will respond well to standard treatment, or who will fare badly with standard treatment and may well benefit from more intensive approaches. Clinical staging as described in Section 1.4.1, when used in isolation, lacks sensitivity in such prediction. In the recent HD10 (Engert *et al*, 2010) and HD11 (Eich *et al*, 2010) German Hodgkin Study Group (GHSG) studies of favourable and

unfavourable early-stage cHL respectively, clinical stage alone was not able to predict the 7% (favourable) or 15% (unfavourable) of early-stage patients who would relapse. It is estimated that of advanced stage patients assessed using clinical staging only, one third will fail to be cured with standard therapy and another third are likely to be over-treated (Borchmann *et al*, 2012; Hasenclever & Diehl, 1998); clinical staging alone offers no way to discriminate these groups. For these reasons, factors other than clinical stage are taken into account when estimating prognosis and more refined tools have been developed.

In advanced stage cHL, a clinical scoring system, the International Prognostic Score (IPS) (Hasenclever & Diehl, 1998) has been developed. This model, based on the multivariate analysis of a number of clinical measures in nearly 5000 patients with advanced stage cHL treated using the combination chemotherapy regimen of ABVD, or ABVD-like regimens, identified seven independent factors associated with poor outcome and led to the development of a scoring system detailed in Table 1.6.

Table 1-6 International prognostic score for advanced-stage Hodgkin lymphoma

Factor	Adverse Criteria	Score
Serum albumin	< 40 g/L	1
Haemoglobin	< 105 g/L	1
Sex	Male	1
Clinical stage	Stage IV disease	1
Age	≥ 45 years	1
White cell count	≥ 15 x 10^9/L	1
Lymphocyte count	≤ 0.6 x 10^9/L	1

Also known as the Hasenclever score (Hasenclever & Diehl, 1998).

Table 1-7 Clinical outcome by IPS in the original model

Score	5-year FFP (%)	5-year OS (%)
0	84	89
1	77	90
2	67	81
3	60	78
4	51	61
≥ 5	42	56
Grouped 0-3	70	83
Grouped ≥ 4	47	59

Adapted from (Hasenclever & Diehl, 1998). FFP, freedom from progression; OS, overall survival.

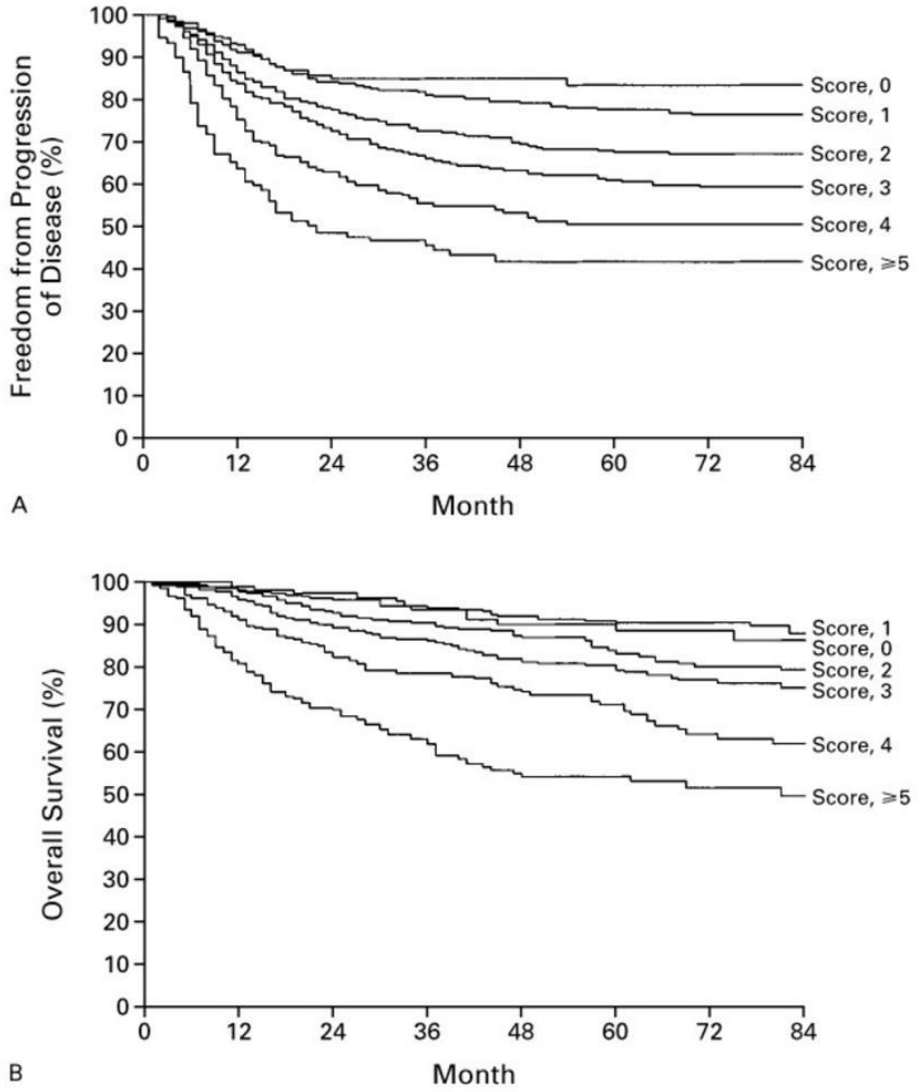


Figure 1-6 Use of the IPI to predict clinical outcome

A) Rates of Freedom from Progression of Disease; and B) Overall Survival in 1618 Patients with Advanced Hodgkin's Disease. Reproduced with permission from (Hasenclever & Diehl, 1998), Copyright Massachusetts Medical Society.

This scoring system was an improvement on clinical staging (see Figure 1.6) but still lacked the ability particularly to predict at baseline the 16% patients with score 0 who would progress, or indeed the 50% of score ≥ 4 patients who would continue in long-term remission (Table 1.7). As a result whilst the IPS is currently widely used in clinical practice, it is informative only, and is not used to select treatment. In addition, the scoring system was not validated for use in early-stage disease and, when used in this setting, has been shown to lack sensitivity (Franklin *et al*, 2000), therefore in early-stage disease no prognostic tools are available to identify those patients who will relapse and require further treatment, versus the majority of patients who will do well and may be over-treated.

1.4.4 Biological prognostic factors

Many of the features incorporated into the IPS reflect the biology of the underlying disease. It is apparent to pathologists and treating physicians that even within the stringent definitions of cHL as defined by the WHO, this is a heterogeneous disease. The underlying biology of the tumour is increasingly known to be important in determining outcome. Biomarkers, biological markers found in the body fluids or tissues of patients with malignancy, have gained attention in recent years for their power to predict outcome at time of diagnosis, and may be important in determining those patients who will benefit from earlier introduction of novel agents. In cHL, a number of biologically based methods have been shown to be prognostic.

TARC is produced by HRS cells and is elevated in the serum of patients with cHL at time of diagnosis (Niens *et al*, 2008). The level of TARC at time of diagnosis has been shown to correlate with clinical outcome (Weihrauch *et al*, 2005). In

this study, TARC levels at diagnosis were higher in those patients who went on to have progressive disease (PD) versus those who achieved a complete response (CR) ($p = 0.068$). In addition, a more rapid fall in TARC levels after just one cycle of chemotherapy is predictive of chemo-responsive disease (Plattel *et al*, 2011). The GHSG have recently shown that lower serum TARC at baseline is associated with negative PET after two cycles of chemotherapy, and with a CR to first-line therapy (Sauer *et al*, 2013).

Given that the symptoms of cHL are closely linked to the secretion of cytokines by the tumour, and that these levels likely reflect tumour bulk, other investigators have examined whether this would be helpful prognostically. Early studies demonstrated that elevated levels of IL-6 are associated with advanced-stage disease and adverse prognosis (Kurzrock *et al*, 1993). Additional studies have demonstrated that elevated serum IL-10 was significantly associated with early (< 12 months) treatment failure ($p = 0.0008$) (Rautert *et al*, 2008) and that IL-10 gene polymorphisms were associated with FFTF (Schoof *et al*, 2013). A combined plasma cytokine signature has been developed (Casasnovas *et al*, 2007), using levels of IL-1RA, IL-6 and soluble CD30 to prospectively predict EFS and OS. High levels of all 3 cytokines were associated with a 5-year EFS of 15% (95% C.I., 0% -37%) versus a 92% 5-year EFS (95% C.I., 88%-95%) in those with low levels of all three cytokines.

Given the importance of the tumour microenvironment in cHL (Section 1.3.7), there has been much focus in recent years on the prognostic significance of the populations of cells which serve to make up the bulk of the tumour. The presence of tissue-associated macrophages, as assessed by gene expression profiling (GEP) (Steidl *et al*, 2010a), has been shown to be associated with

primary treatment failure. The GEP findings were confirmed using IHC for the macrophage associated marker CD68 in formalin-fixed tissue to demonstrate that increased numbers of CD68-positive cells were associated with shorter progression-free survival (PFS) and increased risk of relapse. The same group went on to demonstrate by GEP of microdissected primary HRS cells from 29 cases target genes with expression levels which correlated with genomic copy number changes (Steidl *et al*, 2012). The authors identified a macrophage-like GEP signature, which included CSF1R. This was independently associated with poor outcome in an internal validation of 132 cases.

Studies aiming to validate the use of IHC for CD68 in a clinical setting (Sanchez-Espiridion *et al*, 2012; Harris *et al*, 2012) have not been able to demonstrate that it is independently predictive of outcome, and have highlighted technical issues which would require to be addressed before such a test could be used routinely, such as lack of reproducibility in scoring, even among experts.

A further study (Scott *et al*, 2013) used GEP to study formalin-fixed paraffin-embedded tumour tissue, which is more routinely available. From this large study of 290 patients a 23-gene outcome predictor was generated and validated in an independent cohort of 78 patients. The group demonstrated 63% 5-year OS in the high-risk groups and 92% 5-year OS in the low-risk groups ($p < 0.0011$). The found their predictor to be superior to the IPS and CD68 IHC in multivariate analyses.

As GEP is a technique not routinely available to treating clinicians the translational relevance of such studies remains to be proven.

It is known that much of the cellular infiltrate in cHL comprises cytotoxic and T_{regs} (Section 1.3.7). Low infiltration of FOXP3+ve T_{regs} in conjunction with high levels of TIA1 cytotoxic granule-associated RNA binding protein (TIA-1)-positive CTLs are associated with unfavourable outcome (Alvaro *et al*, 2005). The unfavourable outcome associated with low infiltration of FOXP3+ve T_{regs} seems counterintuitive to the suggestion that these cells suppress local anti-tumour or anti-viral responses (Section 1.3.7). Additionally, it has been shown that high levels of CTLs assessed by Granzyme B positivity are associated with unfavourable outcome (Oudejans *et al*, 1997). These features of tumour microenvironment are probably reflected in the improved OS and PFS of patients with an absolute lymphocyte count/ absolute monocyte count ratio of ≥ 1.1 (Porrata *et al*, 2011).

Genome-wide copy number analysis has also identified chromosomal changes associated with outcome. Array CGH was used to analyse microdissected HRS cells from 53 primary cases and identified a number of recurrent chromosomal changes (Steidl *et al*, 2010b). Gains of chromosome 16p11.2-13.3 were significantly associated with unresponsive disease and shortened disease-specific survival (DSS), the possible mechanism for this being the presence of the multidrug resistance gene ATP-binding cassette, sub-family C (ABCC1) nearby on chromosome 16p. Whilst illuminating in terms of underlying biology, this technology is not currently available to clinicians and is not impacting on treatment decisions at present.

The expression of CD20 has been reported to be associated with clinical outcome although these studies are somewhat contradictory, which may in part be accounted for by the localisation of expression. In the 5-10% of cases of cHL in

which the HRS cells themselves express CD20, this has been reported to be associated with reduction in FFTF and OS (von Wasielewski *et al*, 1997; Portlock *et al*, 2004). Reduced OS with CD20 expression on the HRS cell was confirmed in univariate and multivariate analysis in a third study, which also demonstrated a direct relationship of CD20 expression of the HRS cell and EBV-positivity of the tumour(Aldred *et al*, 2008). Other studies have failed to show any association (Tzankov *et al*, 2003; Rassidakis *et al*, 2002), or have shown an association in the opposite direction, i.e. CD20 expression in patients with chemoresponsive disease (Greaves *et al*, 2013). More recently, given the importance of tumour microenvironment in determining outcome, authors have shown that higher levels of CD20, not on the HRS cell, but in the surrounding cellular milieu, are associated with improved OS (Chetaille *et al*, 2009; Canioni *et al*, 2009).

A number of other features of the tumour, including B cell lymphoma 2 (bcl-2) expression (Sup *et al*, 2005), spleen tyrosine kinase (Syk) and FYN oncogene related to SRC, FGR, YES (Fyn) expression (Martin *et al*, 2011) and HLA Class II expression (Diepstra *et al*, 2007) have been shown to be associated with outcome.

Lastly, it has been shown that the EBV status of the tumour is important in determining outcome. In independent studies (Jarrett *et al*, 2005; Keegan *et al*, 2005), investigators demonstrated that EBV+ve cHL in older adults (defined as > 50 years or > 45 years in the two different studies) was associated with a significantly poorer outcome. In contrast, in younger patients and children, EBV+ve disease was associated with a survival advantage, but in neither study did this reach significance. EBV status of the tumour is often reported to clinicians but does not yet impact on treatment decisions.

There are weaknesses with the prognostic studies to date: some are contradictory and some have not adjusted for other recognised prognostic factors. An additional weakness of all the biologically based methods explored to date is that none has been validated prospectively in independent studies.

1.5 Epidemiology of HL

The epidemiology of cHL is unique among malignant diseases and suggests clues to the aetiology of this disease. cHL has a bimodal age incidence in developed countries, with a peak seen in young adults (age 15-35 years) where it is one of the commonest cancers, and a second peak in older adults, over the age of 50 years. This is in contrast to NLPHL, which does not demonstrate this bimodal pattern (Figure 1.7).

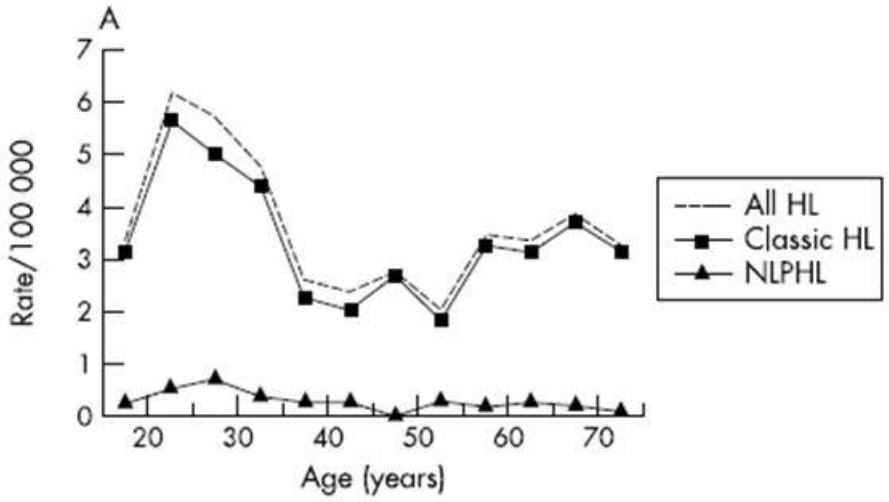


Figure 1-7 Age-specific incidence rates of Hodgkin lymphoma

Demonstrating the bimodal incidence curve for classical Hodgkin lymphoma. HL, Hodgkin lymphoma; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma. Incidence per 100,000 person years. Adapted from (Jarrett *et al*, 2003) with permission, Copyright BMJ Publishing Group.

Males have a higher risk of cHL compared to women (Glaser & Jarrett, 1996; Landgren & Caporaso, 2007) and account for approximately 60% of cases diagnosed (Information Services Division, 2010). In addition, males also have higher rates of EBV+ve cHL (Glaser *et al*, 1997; Cartwright & Watkins, 2004).

Like NHL, Hodgkin lymphoma is becoming more common. Scottish data (Information Services Division, 2010) demonstrate incidence increasing by 43.8% in men and 15.8% in women, between 2001 and 2011, and this finding has been reported in multiple series worldwide.

As has been mentioned, overall, approximately one-third of cases of cHL are associated with EBV (EBV+ve cHL) (Jarrett & MacKenzie, 1999; Jarrett, 2002; Jarrett, 2006; Kuppers, 2009b). Childhood cHL is almost always EBV+ve (Jarrett *et al*, 1991; Flavell *et al*, 2000), as is cHL in developing countries. In contrast, in developed countries, the majority of cases are young-adult cHL, most commonly NS-type, which is less likely to be EBV+ve. In older adult cHL (in adults > 50 years of age), the proportion of cases associated with EBV increases. Within developed countries, EBV+ve disease is associated with socio-economic deprivation (Flavell *et al*, 1999). On the basis of these findings, a "4-disease model" has been proposed (Figure 1.8). In this model, four entities are defined based on age at diagnosis and EBV status of the tumour. The majority of cases are EBV-ve cases with a peak in young adulthood. There are three groups of EBV+ve cases: EBV+ve cases in childhood; EBV+ve cases in young adults occurring following infectious mononucleosis (IM); and EBV+ve cases in older adults.

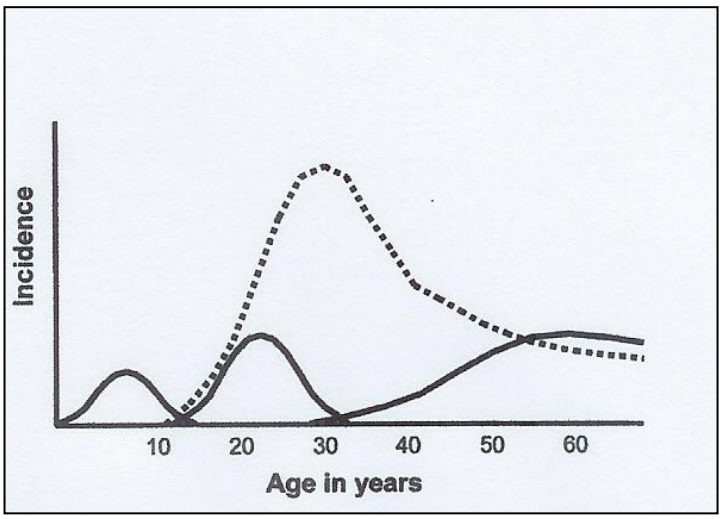


Figure 1-8 Four disease model of classical Hodgkin Lymphoma.
There are three groups of EBV+ve cases (solid lines) and one group of EBV-ve cases (dashed line). Reproduced with permission from (Jarrett, 2002), copyright Oxford University Press.

One of the first clues towards the role of EBV in the aetiology of cHL was an observation of an increased risk of cHL occurring after IM (Gutensohn & Cole, 1980). These cases associated with delayed exposure to EBV probably account for the peak of EBV+ve disease in young adults. Primary infection with EBV (see Section 1.6.2) occurs most commonly in childhood, when it is generally asymptomatic. Early exposure to EBV, or surrogates of early exposure to EBV such as oral exposures, shared bedrooms in childhood or early nursery care are protective for development of cHL (Alexander *et al*, 2000; Glaser *et al*, 2005; Cozen *et al*, 2009). Delayed exposure to the virus resulting in infection in the late teenage years or early adulthood is associated with a clinical syndrome of fever, pharyngitis, lymphadenopathy, variable hepato-splenomegaly, fatigue and debility which is recognised as IM. The pattern of clinical disease seen with IM (clinical disease resulting from late exposure to a pathogen) may echo that which is seen with EBV+ve cHL. The peak of young-adult cHL follows the peak of IM by approximately five years (Hjalgrim, 2012), and large cohort studies have

demonstrated a 3-fold increase in risk in individuals with a history of IM (Hjalgrim *et al*, 2000). This has been verified in case-control studies which have again demonstrated a 3-fold increase in risk in individuals with a personal or family history of IM (Alexander *et al*, 2003; Alexander *et al*, 2000). This risk is particularly pronounced for EBV+ve cases (Hjalgrim *et al*, 2003; Alexander *et al*, 2000).

Further epidemiological evidence of the EBV association with cHL was the altered patterns of antibodies to EBV in cHL patients as compared with controls. No increased risk was seen with simple EBV seropositivity, indicating prior infection (Lehtinen *et al*, 1993; De Sanjose *et al*, 2007); instead elevated antibodies to EBV early antigen (EA) and EBV nuclear antigen (EBNA) were observed in cases as compared with controls. This abnormal EBV serology has now been shown to be associated with risk of developing EBV+ve, but not EBV-ve cHL (Levin *et al*, 2012). In addition, higher numbers of EBV-infected B lymphocytes are noted in the pre-treatment blood of patients with EBV+ve cases of cHL as compared with EBV-ve cases (Khan *et al*, 2005). Both of these pieces of evidence suggest that risk of developing EBV+ve cHL may be associated with control of EBV viral infection.

That inherited genetic factors play a role in susceptibility to cHL is suggested by the striking racial and ethnic differences in the incidence of cHL and the numerous reports in the literature of familial clustering of disease. It is recognised that cHL is more common in Caucasian populations, and less common in people of Asian origin (Cartwright & Watkins, 2004). This is not thought just to be due to variation in environmental factors as migrants of Asian origin to high – incidence areas continue to have a lower incidence than the local population

(Landgren & Caporaso, 2007), and thus genetic factors are thought to play a role.

Population based studies have described a 7-fold excess risk of disease in siblings of young adults with cHL (Grufferman *et al*, 1977) and a 4-fold increase in deaths due to cHL in first and second degree relatives of patients with cHL (Kerzin-Storrar *et al*, 1983). Recent Swedish registry data suggest that familial clustering is stronger for cHL than any other malignancy with a standardised incidence ratio of 5.5 in siblings of patients with cHL (Altieri & Hemminki, 2006). Even stronger evidence that this risk is due to an inherited factor, as opposed to a common environmental exposure, comes from a large twin study which demonstrated a 99-fold increase in risk in monozygotic, but not dizygotic, twins of patients with cHL (Mack *et al*, 1995). Much of this genetic susceptibility has now been shown to be due to HLA type (discussed in Section 1.7.2). The challenge is now to determine how extrinsic environmental factors, such as EBV, interact with these genetic factors.

1.6 Epstein-Barr Virus (EBV)

1.6.1 The biology of EBV

EBV was the first virus associated with human malignancy when viral particles were observed in cell lines derived from primary Burkitt's lymphoma in 1964 (Epstein *et al*, 1964). EBV is a gammaherpesvirus member of the genus herpesviridae, which preferentially infects B lymphocytes(Rickinson & Kieff, 2007). It is ubiquitous with 95% of the world's population being infected (Henle *et al*, 1969). EBV is an ancient virus; robust evidence now demonstrates that an EBV arose in a common primate precursor around 12 million years ago and has

co-evolved synchronously with humans since (Ehlers *et al*, 2010; McGeoch *et al*, 2006).

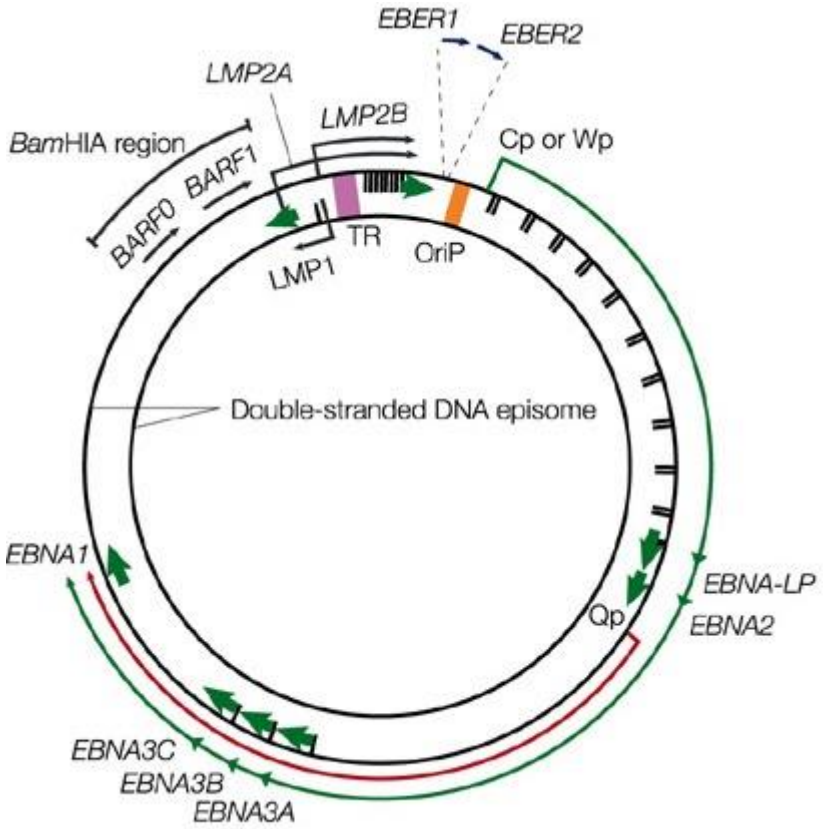


Figure 1-9 EBV genomic structure

Diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The origin of plasmid replication (OriP) is shown in orange. The large green solid arrows represent exons encoding each of the latent proteins, and the arrows indicate the direction in which the genes encoding these proteins are transcribed. The latent proteins include the six nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C, and EBNA-LP) and the three latent membrane proteins (LMPs 1, 2A and 2B). *EBNA-LP* is transcribed from a variable number of repetitive exons. *LMP2A* and *LMP2B* are composed of multiple exons, which are located on either side of the terminal repeat (TR) region, which is formed during the circularization of the linear DNA to produce the viral episome. The blue arrows at the top represent the highly transcribed non-polyadenylated RNAs *EBER1* and *EBER2*; their transcription is a consistent feature of latent EBV infection. The long outer green arrow represents EBV transcription during a form of latency known as latency III (Lat III), in which all the EBNAs are transcribed from either the Cp or Wp promoter; the different EBNAs are encoded by individual mRNAs that are generated by differential splicing of the same long primary transcript. The inner, shorter red arrow represents the *EBNA1* transcript, which originates from the Qp promoter during Lat I and Lat II. Reproduced and adapted from (Young & Rickinson, 2004) with permission. Copyright Nature Publishing Group.

EBV is a large (184 kilobase) double-stranded DNA virus (see Figure 1.9). The encapsulated virus particle carries its DNA in a linear form. In the cell, the virus does not integrate into the host genome, but instead exists as circular episomes. Infection with EBV can either be lytic or latent. The number of EBV episomes (and hence genomes) present in any one cell can vary from one to 100 of copies in latent infection to many thousand in active lytic infection.

EBV displays one of the characteristic features of the herpesviridae, namely life-long persistence of infection in an immune host (Rickinson & Kieff, 2007). EBV exists as two strains, 1 and 2 differing by 16-47% in their coding sequences for the EBNAs. Type 1 infection is the dominant form in most of the world and accounts for 80-90% of EBV infections in the developed world. The B95-8 reference strain (National Center for Biotechnology Information (NCBI), 2008; Miller & Lipman, 1973) is the archetypal strain 1 virus. Type 2 infection is more prevalent in central Africa and New Guinea. Co-infection with type 1 and type 2 EBV is rare other than in immune compromised hosts.

The viral genome is known to have > 85 open reading frames (ORFs). A selection of the functionally more important, or better studied, is given in Table 1.8.

Table 1-8 Functionally important open reading frames in the EBV genome

Open reading frame	Protein or more common name
Latent genes	
BKRF1	EBNA1
BYRF1	EBNA2
BERF1	EBNA3A
BERF2	EBNA3B
BERF3/4	EBNA3C
BWRF1	EBNALP
BNLF1	LMP1
BNRF1	LMP2A/2B
BARF0	BARF0
EBER1/2	EBER1/2
Lytic genes	
Immediate early genes	
BZLF1	BZLF1
BRLF1	BRLF1
BILF4	BILF4
BMLF1	BMLF1
BRLF1	BRLF1
Early genes	
BMRF1	BMRF1
BMLF2	BMLF2
BALF2	BALF2
BALF5	BALF5
BORF2	BORF2
BARF1	BARF1
BXLF1	BXLF1
BGLF5	BGLF5
BSLF1	BSLF1
BBLF4	BBLF4
BKRF3	BKRF3
Late genes	
BLLF1	gp350/220
BXLF2	gp85
BKRF2	gp25
BZLF2	gp42
BALF4	gp110
BDLF3	gp100–150
BILF2	gp55–78
BCRF1	BCRF1
BHRF1	BHRF1

In addition to the viral proteins, the virus expresses two classes of RNA which are non-protein coding and not translated (Kieff & Rickinson, 2007). There are the two small EBERs and a group of alternatively spliced RNAs of the *Bacillus* amyloli restriction endonuclease (B*am*HI) A rightward transcripts (BARTs) segment of the EBV genome which gives rise to a large number of miRNAs. The function of these non-coding RNAs is poorly understood but they have been demonstrated to be important in oncogenesis.

The virus has different expression patterns depending on the stage of infection, which reflect the different requirements of the virus at different times to infect, persist within and reactivate from B-memory lymphocytes, whilst evading the immune system.

At time of lytic infection, the priority is the generation of daughter genomes, and the envelope proteins to coat these to release new virus. A large number of viral genes are expressed; there is replication of the genome and, ultimately, death of the infected cell.

During latent infection, the virus evades the immune response and downregulates much of its transcription to avoid immune recognition. Restricted sets of up to nine EBV proteins are expressed: the EBNAs: EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA-LP; and the LMPs: LMP1, LMP2A and LMP2B; together with the EBERs, comprise the different programmes of gene usage known as the “latency” patterns of gene expression (Table 1.9).

Table 1-9 EBV latency gene expression patterns

Latency Programme	Genes expressed	Function in normal latency	Occurrence in EBV-associated disease
0	EBERs	EBV viral persistence in memory B cells in peripheral blood	
I	EBERs, EBNA1	Maintains infection whilst allowing peripheral memory B cells to divide	Burkitt's lymphoma, PEL
II	EBERs, EBNA1, LMP1, LMP2A	Differentiate activated B cell In GC into memory B cell	Hodgkin lymphoma, NPC, Peripheral T/NK lymphoma
III	EBERs, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2A, LMP2B	Activates a resting B cell to become a proliferating lymphoblast (also pattern expressed in lymphoblastoid cell line (LCL))	PTLD HIV-associated NHL

PEL, primary effusion lymphoma; PTLD, post-transplant lymphoproliferative disease; NHL, non-Hodgkin lymphoma; NPC, nasopharyngeal carcinoma.

1.6.2 Natural history of infection

The life cycle of EBV consists of primary infection, expansion, establishment of *in vivo* latency and reactivation, replication and dissemination of infection to new hosts.

Infection with EBV usually occurs in early childhood, where primary infection is typically asymptomatic or sub-clinical. Exposure to the virus later in life, e.g. in adolescence or adulthood, leads to the development of symptomatic disease, IM, in up to 25% of cases (Crawford *et al*, 2006) (Section 1.6.4.4). Infection is usually by the oral route, but it is known that other mucosal exposures e.g. sexual contact also constitute a viable route of infection (Naher *et al*, 1992; Sixbey *et al*, 1986; Israele *et al*, 1991).

Primary infection most usually occurs via the oropharynx, which is also believed to be the site of virus replication (Sixbey *et al*, 1984). Whether the initial cells infected are the oropharyngeal epithelial cells or oropharyngeal mucosal

associated B lymphoid tissues remains controversial (Young & Rickinson, 2004). EBV lytic infection is difficult to study due to the lack of an in vitro model of lytic infection; therefore many questions regarding primary infection and oropharyngeal replication remain unanswered. During symptomatic infection, high levels of free virus are present in oral and pharyngeal secretions (Young & Rickinson, 2004). Oral virus shedding may persist for some time; a median of 180 days was reported in one study (Balfour, Jr. *et al*, 2013). At time of acute primary infection, up to 50% of circulating B lymphocytes may be infected with EBV (Hochberg *et al*, 2004). Subsequently there is a rapid reduction in numbers of infected cells in the weeks following acute infection (Hochberg *et al*, 2004). EBV DNA viraemia is cleared quickly (< 1 week) in most subjects, although viraemia is more persistent in a minority of individuals (median 95 days) (Balfour, Jr. *et al*, 2013). The factors determining this remain to be established. Much of the work studying primary infection has been in patients with IM. It is not known if the same processes occur in asymptomatic primary infection.

Almost immediately following primary infection, the virus becomes latent in B-memory lymphocytes where the virus remains for the lifetime of the host (Bornkamm & Hammerschmidt, 2001). Polymerase chain reaction (PCR) performed on limiting cell dilutions of B cells suggests a frequency of EBV infected B cells ranging from 1 in 10^4 in acutely infected hosts, to 1 – 50 per 10^6 B cells in steady state immune competent hosts (Kurth *et al*, 2000; Rickinson & Kieff, 2007; Khan *et al*, 1996). The frequency of infected cells within an individual is stable over time and appears to be tightly regulated (Khan *et al*, 1996).

There is agreement that memory B lymphocytes are the reservoir of latent infection, but the exact process by which this occurs is still the subject of some debate. EBV may exploit the normal GC reaction to establish latency: a model has been proposed whereby infection with EBV drives a naïve antigen-independent B cell towards an IgD-, CD27+ memory B cell phenotype, mimicking the GC reaction (Thorley-Lawson, 2001). Infected naïve B cells are the only cells that express all of the latent EBV genes (latency III), just as in LCLs (Joseph *et al*, 2000). The naïve infected B-cells clonally expand and some will undergo the GC reaction, where they express the latency II pattern of gene expression (Babcock *et al*, 2000). Post-GC, these cells differentiate into long-lived memory B lymphocytes. Some controversies to this model exist. The demonstration that most EBV infected B memory cells are antigen-selected would suggest that instead of only driving naïve cells through to B memory cell differentiation, the EBV infected Ig mutated B cell may have a selective advantage in a conventional GC reaction (Thorley-Lawson, 2005). Further evidence supports that naïve B lymphocytes may not be the only cell type infected (Ehlin-Henriksson *et al*, 2003) and studies of tonsillar tissue have demonstrates that EBV-infected B cells are only rarely detected in germinal centres (Araujo *et al*, 1999). The exact process of establishment of viral persistence *in vivo* in primary infection remains to be established.

Most infected memory B lymphocytes do not contain active replicating virus, but instead the virus is resting in a latent state. There is complete or near-complete down-regulation of EBV antigen expression in these cells (latency 0). This lack of viral protein expression allows the virus to evade the immune response.

Latent EBV infection is kept in check by the CTL response, discussed in Section 1.6.5. Viral reactivation occurs intermittently, probably as a result of normal B cell homeostasis allowing viral replication (Kuppers, 2003). Such reactivation is usually asymptomatic, and does not normally lead to clinical symptoms or signs. This periodic reactivation can result in infection of greater numbers of B memory cells or in the secretion of infectious virus into saliva where it may be the source of infection between contacts. Recent work (Shannon-Lowe & Rowe, 2011) has demonstrated that the normally inefficient infection of oropharyngeal mucosal epithelial cells can be increased by the formation of cellular synapses with EBV infected B memory lymphocytes at the baso-lateral surface of the epithelial cell. Whilst this does not explain the mechanism of primary epithelial infection, it does provide a means for secretion of virus and onward infection.

The natural history of EBV in the host is summarised in Figure 1.10. The CTL response is important in maintaining this equilibrium between viral latency and reactivation.

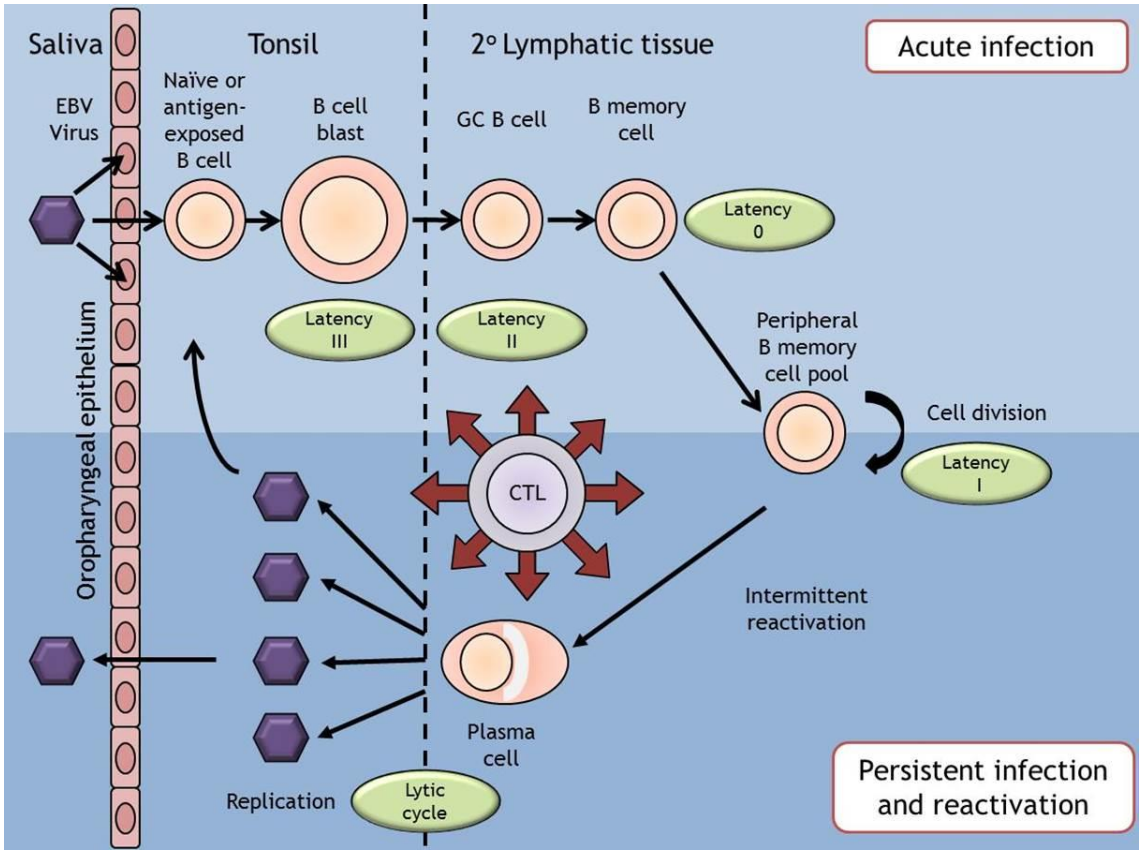


Figure 1-10 The natural history of Epstein-Barr virus infection

EBV virus is acquired most commonly via the oropharynx. Viral particles in saliva may infect epithelial cells of the oropharynx directly where lytic infection can occur. Virus particles infect naïve or antigen exposed (Ig V gene mutated) B cells in oropharyngeal lymph tissue. The cell is driven into blastic proliferation (latency III pattern). A number of B cells will be driven through the germinal centre reaction (latency II pattern) and become differentiated to immortalised B memory cells. The EBV persists in the peripheral B cell memory pool for the life of the host (latency 0 or I). Intermittently EBV reactivates, exploiting differentiation to plasma cells to manufacture large numbers of infectious virus (lytic cycle). These virus particles can be secreted into saliva to infect new hosts or can infect further B cells. The CTL response is central in controlling infection at all stages. CTL, cytotoxic lymphocyte; EBV, Epstein - Barr virus; GC, germinal centre.

1.6.3 The role of the EBV latent proteins in B cell transformation

EBV has the unique capacity to efficiently transform and immortalise resting B lymphocytes (Rickinson & Kieff, 2007; Henle *et al*, 1967). The immortalised cells have features of malignant cells and are known as lymphoblastoid cell lines (LCLs). They carry multiple copies of the viral episome and express a latency III pattern (Young & Rickinson, 2004). Whilst imperfectly matched to the expression

patterns of cHL and Burkitt's lymphoma, such LCLs do provide an *in vivo* model for studying EBV lymphomagenesis.

It is the latent infection programme of EBV which is capable of this immortalisation, and only a limited number of EBV viral genes are expressed: EBNA1, EBNA2, EBNA3A, 3B and 3C, EBNA-LP, LMP1, LMP2 and the EBERs (Bornkamm & Hammerschmidt, 2001). The minimal set of genes required for this process to occur is still not known, but experiments have demonstrated the functional importance in transformation of some of the proteins involved. A number of the EBV proteins and the EBERs have been shown to be directly oncogenic. EBNA2, EBNA3C and LMP1 when expressed individually in human B lymphocyte lines can induce an LCL-like phenotype, suggesting that these three proteins may be key in the transformation process (Wang *et al*, 1990).

EBNA1 is responsible for the maintenance of the viral genome as an episome during cell proliferation and is involved in the transcriptional regulation of the EBNAs. In a transgenic mouse model, EBNA1 has been shown to produce B cell lymphomas (Wilson *et al*, 1996). EBNA2 appears essential for transformation with EBNA2 deficient strains being incapable of transformation (Young & Rickinson, 2004). EBNA2 activates transcription of viral LMP1 and LMP2 and also a number of cellular genes including the oncogene c-myc (Kaiser *et al*, 1999; Kuppers, 2003). ENBA2 also interacts with EBNA-LP to induce cyclinD2 and the cell-cycle (Bornkamm & Hammerschmidt, 2001) and may be involved in mimicking Notch signal transduction (Hsieh *et al*, 1996). EBNA-LP has been shown to co-localise with a number of proteins associated with proliferation or apoptosis resistance such as p53, retinoblastoma and the promyelocytic leukaemia (PML) gene product (Szekely *et al*, 1993; Szekely *et al*, 1996). EBNA3A and EBNA3C are also

essential for transformation, appearing important in transcriptional activation of a number of genes implicated in oncogenesis such as cyclin A or histone deacetylase 1 (Radkov *et al*, 1999) and, through co-operation with RAS, in the over-riding of cell-cycle check-points (Parker *et al*, 2000; Parker *et al*, 1996)

LMP1 is the main protein in EBV-driven B cell transformation, is essential for immortalisation and is directly oncogenic (Wang *et al*, 1985). LMP1 transgenic mice are observed to develop B cell lymphoma (Kulwichit *et al*, 1998). LMP1 has a number of roles in the cell. It inhibits apoptosis through up-regulation of the anti-apoptotic proteins A20 and bcl-2 and directly activates the NF- κB pathway (Mosialos *et al*, 1995). Indirect activation of the NF- κB pathway is also observed as one of several downstream signals which result from the structural and functional resemblance LMP1 has with CD40. This member of the TNFR superfamily normally functions to transduce the signal from CD40 ligand, resulting in a signal which provides growth, anti-apoptotic and differentiation signals to the B cell (Uchida *et al*, 1999; Kilger *et al*, 1998). This mimicry is thought to be one of the key ways in which LMP1 is oncogenic to B cells. LMP1 is also capable of activating PI3K, JAK-STAT and other signalling pathways which have been shown to be significant in the oncogenesis of cHL (Young & Murray, 2003), and can upregulate production of IL-10 (Nakagomi *et al*, 1994).

LMP2 is not an essential protein for transformation but does contain a domain which can replicate the tonic BCR signal and has been shown in vitro to be capable of rescuing and transforming GC B lymphocytes with no functional Ig-containing BCR (Bechtel *et al*, 2005; Chaganti *et al*, 2005; Mancao *et al*, 2005).

The RNAs encoded directly by EBV may also play a role in oncogenesis. The EBERs are important in determining the transformation efficiency of EBV (Yajima

et al, 2005) and have been demonstrated to downregulate p53 and STAT1 and therefore increase the ability of the cells to survive an apoptotic signal (Liu *et al*, 2010).

The specific contribution of these mechanisms to EBV-associated malignancies is not well understood, and current evidence would suggest that they are likely to be different in the various EBV-associated malignancies (Ambinder, 2007).

1.6.4 EBV-associated diseases

EBV infection is associated with a number of different epithelial and lymphoid malignant diseases. Although infection with EBV is usually asymptomatic, EBV is recognised as a carcinogen by the International Agency for Research on Cancer (IARC Working Group on the Evaluation of Carcinogenic Risk to Humans, 2009) on the basis of its association with a number of cancers, including cHL. In 2010, it was estimated that 1213 cases of cancer in the UK were directly attributable to EBV (Parkin, 2011).

1.6.4.1 Malignant disease - cHL and EBV

Approximately one third of cases of cHL are associated with EBV, where the virus is believed to play a causal role (Jarrett, 2011). Evidence for the involvement of EBV in the oncogenesis of cHL comes from a number of strands of evidence.

In EBV+ve cHL, EBV is detected in all the HRS cells seen (Figure 1.11). In addition, it has been demonstrated the virus present is clonal, proving that infection occurred prior to clonal expansion and supporting a causative role (Gulley *et al*, 1994; Weiss *et al*, 1989; Weiss *et al*, 1987; Jarrett *et al*, 1991).

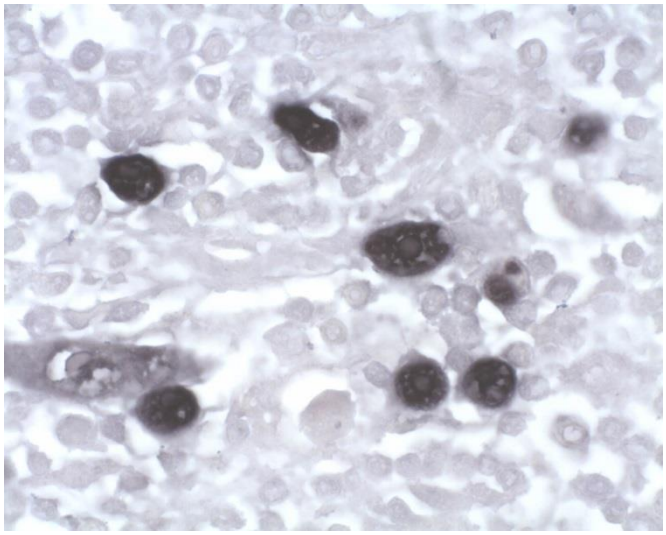


Figure 1-11 Lymph node biopsy, EBV EBER in situ hybridisation

x1000 original magnification. Positive staining in the nuclei of all the tumour cells can be seen. Reproduced from (Farrell & Jarrett, 2011) with permission, copyright Blackwell Publishing Ltd.

HRS cells express the limited latency II pattern of EBV expression (Section 1.6.1), which includes EBNA1, LMP1, LMP2, the EBERS and BART RNAs. As discussed in Section 1.6.3, these gene products are involved in oncogenesis and cell transformation. Functional studies suggest that these same mechanisms may be involved in HRS cell survival and proliferation (Jarrett, 2002). Strong corroborative evidence for the importance of EBV specifically in rescuing HRS cells from an apoptotic death includes the demonstration that cHL cases with crippling mutations of Ig genes are restricted almost entirely to EBV+ve cases (Brauninger *et al*, 2006).

Additional survival mechanisms specific to cHL are also reported. EBNA1 can act to upregulate CCL22, attracting T_{reg} cells to the tumour, thus suppressing an EBV-specific immune response (Baumforth *et al*, 2008). Both LMP1 and LMP2 may also promote the downregulation of the B cell phenotype by the HRS cell (Vockerodt *et al*, 2008), in particular via the activation of Notch1 (Anderson & Longnecker, 2009).

EBV is known to harness the cellular methylation machinery to counteract the cellular response to methylate viral genes and repress their expression. This has recently been shown to be another way in which EBV may contribute to oncogenesis in cHL; EBV infection of GC B- lymphocytes altered expression of DNA methyltransferases in a pattern identical to that seen in HRS cells (Leonard *et al*, 2011), an effect thought to be due to LMP1 expression (Tsai *et al*, 2002).

EBV thus has a plausible role in cHL pathogenesis, although its direct role in pathogenesis is difficult to absolutely prove in the absence of an EBV-prevention strategy. However, a number of pieces of evidence strongly support the causative association of EBV in cHL pathogenesis: the epidemiology of cHL; the clonal nature of the infection in the malignant cells; the biologically plausible transforming role for the latent proteins expressed in the HRS cell; the ability of EBV to rescue B-cells with crippling Ig mutations; the observation of TNFAIP3 and Iκ-Bα mutations in predominantly EBV-ve cases (See Section 1.3.4); and the temporal association of cHL with another EBV-associated condition, IM.

1.6.4.2 Other EBV-associated malignant diseases

EBV-associated malignancies are recognised in immune competent hosts and in those with immune compromise(IARC Working Group on the Evaluation of Carcinogenic Risk to Humans, 2009), and worldwide account for an estimated 200,000 cancers per year (Cohen *et al*, 2011). The malignancies with an established EBV association are summarised in Table 1.10.

Table 1-10 EBV-associated malignancies

	Disease	Proportion associated with EBV
Immune competent host	classical Hodgkin lymphoma	~ 35% (varies, see text)
	Burkitt's lymphoma	> 95% endemic Burkitt's lymphoma; > 20% sporadic Burkitt's lymphoma
	Nasopharyngeal carcinoma	~ 100% (mainly seen in those of Chinese and other Asian ethnic origin)
	NK / T cell lymphoma	> 80% (increased incidence in Asia and Central/South America)
	Gastric carcinoma	5-10%
	EBV+ve DLBCL of the elderly †	100%
Immune suppressed host	Post-transplant lymphoproliferative disease (PTLD)	> 90%
	AIDS-associated NHL	100% CNS lymphoma; 50% other NHL
	HIV-associated cHL	> 90%
	Primary effusion lymphoma (PEL)	70-80%
	Leiomyosarcoma	100%
	NHLs associated with inherited immune compromise	> 90%

† Elderly adults with no recognised immune impairment. There is some suggestion that immune senescence may play a role in this condition. DLBCL, diffuse large B cell lymphoma; NHL, non-Hodgkin lymphoma; cHL, classical Hodgkin lymphoma; AIDS, acquired immune deficiency syndrome.

In immune competent hosts, EBV is associated with a number of malignant diseases other than cHL, including the B cell malignancy Burkitt's lymphoma, nasopharyngeal carcinoma (NPC) and others (Table 1.10). Although the association is lower, the higher numbers of patients presenting with gastric carcinoma make this the commonest EBV-associated malignancy worldwide (Cohen *et al*, 2011).

The cell mediated response, particularly the CTL response, in central in maintaining equilibrium between stable asymptomatic viral persistence and viral reactivation (discussed in Section 1.6.4.3). Clinical situations resulting in a reduction of T cell mediated immunity can result in clinically significant EBV-associated diseases. Examples of causes of T cell immune-suppression associated with EBV-driven malignancy include: iatrogenic reduction of T cell immunity with immunosuppressive drugs such as methotrexate, cyclosporin, or mycophenolate; following haematopoietic stem cell or solid organ transplant; HIV/AIDS; and inherited immune deficiencies such as x-linked lymphoproliferative syndrome (XLP).

Acquired immune deficiency syndrome (AIDS) is associated with a recognised increase in EBV-associated NHL, particularly primary CNS lymphoma, plasmablastic lymphoma and primary effusion lymphoma (PEL) (as a result of co-infection with Kaposi's sarcoma herpesvirus). Patients with HIV infection are also at increased risk of cHL, which is almost always EBV+ve, but only when CD4 levels are reasonably normal, mirroring more the "immune competent" host, and probably reflecting the importance of the CD4 cell in forming the cHL microenvironment.

Other diseases associated with a reduction in T cell mediated immunity such as the XLP are also associated with symptomatic EBV disease. XLP is an inherited disease of young males; most cases being caused by mutations in the XLP or SH2D1A gene, which encodes the adaptor molecule Signalling Lymphocytic Activation Molecule (SLAM)-associated protein (SAP) (Rezaei *et al*, 2011). When these boys acquire primary EBV infection in childhood, a rapidly progressive illness, starting with symptoms akin to IM and developing into

haemophagocytosis, takes hold. Subsequently, widespread lymphoid proliferations and hepatic infiltration lead to death in two-thirds of cases. Survivors remain prone to EBV-driven NHL. Children with other forms of immune suppression such as Wiskott-Aldrich syndrome (WAS) and chronic variable immune deficiency (CVID) are also prone to EBV-associated NHL.

Studies have demonstrated that a number of factors can impact on whether or not EBV-associated malignancy develops. Co-infection with other pathogens at time of primary infection is thought to impact on risk of developing disease; higher EBV viral loads have been reported in children co-infected with Plasmodium falciparum (Moormann *et al*, 2005) or HIV (Slyker *et al*, 2013), and the incidence of PTLD is higher in individuals with active cytomegalovirus (CMV) infection (Manez *et al*, 1997). More subtle levels of immune suppression may also be linked to the development of EBV+ve cHL. Varicella zoster virus (VZV) reactivation resulting in shingles is a surrogate of subtle immune suppression, and is a recognised risk factor for development of cHL (Karunanayake *et al*, 2009; Tavani *et al*, 2000). Patients with EBV+ve cHL are more likely to have had shingles in the year prior to their diagnosis than patients with EBV-ve disease (Jarrett *et al*, 2005). Such sub-clinical immune suppression, possibly arising as a result of immune senescence (shingles being more common with increasing age), may account for the greater predominance of EBV+ve cHL in older adults, and may suggest an aetiological overlap with EBV+ve DLBCL of the elderly (Nakamura *et al*, 2008). These findings reinforce the importance of the immune response in the development of EBV-associated malignancies.

1.6.4.3 EBV-specific T cell immunotherapy

The T cell immune response to EBV has been exploited to treat EBV-associated malignancies. A number of studies have used LCL-stimulated cultures of T cells to treat cHL (Bollard *et al*, 2004), PTLD (Haque *et al*, 2002; McAulay *et al*, 2009; Heslop *et al*, 2010) and NPC (Straathof *et al*, 2005a). The clinical trials of these cells in the setting of cHL have had limited success which may be due to the different latency pattern expressed or the inability of the infused T cells to be effective locally due to the immune suppressive microenvironment of the tumour (Peggs, 2006).

1.6.4.4 Non-malignant diseases – IM

In addition to the EBV-associated malignancies described above, EBV has also been implicated in the pathogenesis of a number of non-malignant conditions.

IM, or glandular fever, is a self-limiting disease characterised by painful lymphadenopathy, pharyngitis, fever, malaise and occasionally splenomegaly and hepatomegaly. Disease severity can range from asymptomatic disease to severe disease leaving individuals essentially bedridden. The median duration of illness is approximately 10 days but symptoms can last up to two months in some individuals.

During IM, high titres of infectious EBV viral particles are shed into the oropharynx from epithelial cells and local mucosal associated lymphoid tissue. Latently infected B cells begin to appear in the local lymph nodes, the tonsils of Waldeyer's ring and the cervical lymph nodes, accounting for the associated lymphadenopathy (Kurth *et al*, 2000). Peripheral blood morphology demonstrates "atypical mononuclear" cells which are the oligoclonal CTLs responding to the

virus, rather than the infected cells themselves. The disease is associated with the development of a heterophile antibody, which can be detected clinically using the non-specific Paul-Bunnell test for sheep red cell agglutination, also known as the "monospot". Confirmation is with specific serological testing initially for EBV viral capsid antigen (VCA)-IgM and then for the subsequent development of VCA-IgG. Disease severity has been reported to be related to the magnitude of the CD8+ve lymphocytosis and the level of EBV viraemia (Balfour, Jr. *et al*, 2013).

The benign disease oral hairy leukoplakia is associated with EBV. Areas of white, thickened tissue appear on the lateral surface of the tongue and cannot be scraped off. This disease is associated with reduced T cell immunity, most commonly in the context of HIV infection, and the lesions demonstrate productive infection of EBV (Slots *et al*, 2006).

An extremely rare complication of primary EBV infection in previously healthy individuals is chronic active EBV (CAEBV). This condition, which is distinct from chronic fatigue syndrome, has a duration of greater than 6 months and is associated with high peripheral blood viral load and altered antibody responses (Macsween & Crawford, 2003). End-organ involvement directly by EBV can result in pneumonitis, hepatitis, bone marrow hypoplasia, or uveitis (Cohen, 2000; Okano *et al*, 2005; Kimura *et al*, 2001). There is a high-risk of development of haemophagocytic syndrome and T cell and NK-cell malignancies with attendant high mortality rate (> 40%) (Macsween & Crawford, 2003).

A number of autoimmune conditions are associated with EBV, where an aberrant immune response to the virus may be the pathogenic mechanism. Some of these associations remain highly controversial, such as the links with rheumatoid

arthritis, diabetes mellitus or systemic lupus erythematosus (Thorley-Lawson & Gross, 2004; Rickinson & Kieff, 2007). Other links, such as the association between multiple sclerosis (MS) and EBV, are stronger with more evidence in support of an aetiological role (Ascherio & Munger, 2007; Habek *et al*, 2008). Whilst genetic, and indeed HLA susceptibility has been suggested (Qiu *et al*, 2013; Link *et al*, 2010; Chao *et al*, 2008), environmental triggers may be important. There is evidence that primary EBV infection is followed by an elevated risk of developing MS (Levin *et al*, 2010), and that EBV-specific antibodies are present in the cerebrospinal fluid of patients with MS (Bray *et al*, 1992).

1.6.4.5 EBV vaccination

The possibility of vaccinating against EBV, ultimately to prevent EBV-associated malignancies, has had a resurgence of interest particularly following the widespread clinical use of the human papillomavirus vaccine to prevent cervical carcinoma (Frazer, 2004). To date, vaccines have generally stimulated a humoral immune response to e.g. gp350, which has not been effective in preventing either infection or clinical illness in the form of IM (Moutschen *et al*, 2007; Allen, 2009; Moss *et al*, 1998). The ability of any vaccine to prevent EBV-associated malignancies has not been studied, although it is hypothesised that any vaccine which could decrease the level of persistent infection may help prevent EBV-associated malignancy even if sterile immunity cannot be achieved. The importance of the T cell immune response in maintaining control of EBV infection would suggest that any EBV vaccine will have to stimulate a CTL response for it to be effective. At present there is no EBV vaccine available.

1.6.5 Immune response to EBV

Given that life-long infection with an oncogenic virus in 95% of the world's population does not result in more disease is testimony to the ability of the human immune system to keep this virus in check.

1.6.5.1 Innate immune response

In the first few days of EBV infection, before the adaptive immune response can react, the innate immune response acts to minimise the impact of the infection. Relatively little is understood of this, but the first innate immune response to EBV observed is the production of the antiviral cytokines interferon (IFN)-α and IFN-β. Further to this, an innate immune response involving the NK cell responds to the very early phases of acute infection (Williams *et al*, 2005). NK cells lyse virally infected cells and secrete further antiviral cytokines including IFN-γ. The importance of the NK cell response can be appreciated in the context of XLP. The SAP gene product (Section 1.6.4.2) associated with this disease is expressed by NK cells, acting as a cell activation molecule. NK cells from XLP patients do not develop properly and are unable to kill EBV-infected B cells (Parolini *et al*, 2000), emphasising the importance of this early response in disease control.

1.6.5.2 Adaptive immune response - humoral

EBV also stimulates an adaptive, both serological and cell-mediated, immune response. As with other infections, acute infection is associated with generation of specific IgM class antibodies to a number of components of the virus, particularly those proteins expressed in the lytic phase of infection; viral capsid antigen (VCA); immediate early (IE) and early antigen (EA); and membrane antigen (MA) comprising gp350 and gp85. Subsequently class-switching to

specific IgG antibodies occurs, and in addition, IgG antibodies to EBNA become detectable (Moss *et al*, 2001). These antibodies persist for the lifetime of the host, barring any other cause for immune paresis. These antibodies are used in the diagnosis of current or prior EBV infection (Henle *et al*, 1974). The clinical significance of these antibodies cannot be known for certain but it is thought that their importance is secondary to that of the cell-mediated immune response. Evidence in support of the secondary importance of the humoral immune response includes the observation that suppression of T cell immunity has been shown to lead to an increase in levels of EBV-specific antibody, but these are insufficient to prevent clinically significant disease in the face of low specific T cell numbers. In addition, trials of EBV vaccines which successfully stimulate a neutralising gp350 antibody response do not necessarily prevent infection (Khanna *et al*, 1999a).

1.6.5.3 Adaptive immune response - cell-mediated

EBV simulates a particularly vigorous cell-mediated immune response. This response is central in controlling both acute lytic and persistent latent EBV infection. The importance of the T cell immune system in controlling EBV infection can be seen in primary symptomatic infection. The name “infectious mononucleosis” derives from the large number of atypical mononuclear cells seen on a peripheral blood film. Initially thought to represent the infected cells, these are now known to be mainly CD8+ve EBV-specific CTLs. These have been shown to be present at levels 5 to 20 times greater than normal (up to 50% of CD8+ T cells) and are oligoclonal with a skew in their T cell receptor (TCR) usage (Callan *et al*, 1996; Maini *et al*, 2000). In the initial response at time of primary infection, the response to lytic proteins (principally BRLF1, BZLF1, BMRF1, BMLF1 and BALF2) predominates with responses to epitopes derived from EBV

latent proteins usually accounting for no more than 2.5% of circulating CD8+ve T lymphocytes (Hislop *et al*, 2002). The magnitude of some of the acute lytic responses can be great; studies using major histocompatibility (MHC) tetramers (Section 1.6.5.5) (Callan *et al*, 1998) have demonstrated that as many as 12% of circulating T cells during acute infection are specific *for a single peptide epitope* (Section 1.6.5). As the primary infection resolves, the numbers of these CTLs gradually falls from approximately 50% of circulating T cells to approximately 2% of circulating T cells.

CTLs specific to latent proteins appear later during infection, and persist at steady levels within the host. The CD8 responses to latent proteins develop in parallel to developing viral persistence, and are thought to be crucial in immune surveillance and maintenance viral latency. The main latent specificities detected are to EBNA3-derived epitopes (Rickinson & Moss, 1997; Steven *et al*, 1996) with responses also seen to LMP2-derived epitopes. CD8 responses to EBNA1 were initially not detected; a glycine-alanine repeat domain in this protein prevents HLA class I presentation, most likely through decelerating its own synthesis via mRNA interference (Apcher *et al*, 2010) or via proteasomal degradation (Yin *et al*, 2003). This viral evasion strategy is thought to contribute to an escape from CD8+ve T cell immunity, however both CD8 and CD4 T cell immune responses to EBNA1 have since been observed at low frequency and are thought to arise either through processing of partial defective EBNA1 molecules, via a non-proteasomal pathway (Lee *et al*, 2004; Daskalogianni *et al*, 2008) or possibly inter-cellular antigen transfer (Taylor *et al*, 2006).

The population of EBV-specific CD8+ CTL memory cells (or effector memory cells) thus consists of cells specific to both lytic and latent specificities. There is

no evidence that the CD8+ memory T cell responses detectable in viral persistence are different between those who had asymptomatic primary infection or those who suffered IM (Callan, 2004). These cells continue to be capable of direct cytolytic cell killing and of IFN-γ secretion (Bharadwaj *et al*, 2001; Hislop *et al*, 2001).

EBV restricted CD4+ve Th responses are present at low levels at time of acute infection and then are maintained at a low level (Precopio *et al*, 2003), approximately one-tenth of the CD8+ve CTL response. CD4+ve Th cells function to augment antibody responses and to initiate and maintain CD8+ve T cell memory. The functional importance of these responses in EBV are less well understood, but CD4+ve T cells specific to epitopes from both lytic and latent proteins are observed, and have been mapped (Long *et al*, 2005; Long *et al*, 2011a). EBV-specific CD4+ve T cells have been observed to have a Th1 phenotype. Of note, they recognise a different spectrum of EBV antigens as compared with CD8+ve CTLs, with an immunodominance hierarchy skewed towards latent proteins, particularly EBNA1, EBNA3B, EBNA3C and LMP1 (Marshall *et al*, 2003; Paludan & Munz, 2003). Recent availability of class II MHC tetramers have permitted the more detailed study of CD4 EBV-specific responses; during acute IM responses to EBNA2, BZLF1, BMRF1, BARF1, and BFRF1 were observed (Long *et al*, 2013) and declined rapidly to the low levels previously seen with chronic infection. Responses to EBNA1, previously observed to be amongst the most immunodominant antigens in CD4-restricted EBV-specific responses (Leen *et al*, 2001), were seen, but not during acute infection. The appearance of CD4+ve Th cells specific for EBNA1 epitopes was delayed, probably due to lack of release of EBNA1 from EBV-infected cells (Long *et al*, 2013). Most EBV-specific Th cells are Th1 polarised and secrete IFN-γ (Amyes *et*

al, 2003), although some have been observed to secrete IL-10 (Marshall *et al*, 2003), suggesting a role in stimulating a T_{reg} response. The functional importance of CD4+ve Th cells in EBV immune response remains to be fully determined.

Any immune suppression in the lifetime of the host is associated with a fall in the CD8+ve and CD4+ve EBV-specific T cells and a concomitant rise in numbers of EBV infected B cells. The T cell immune response to the virus during the lifetime of the host is summarised in Figure 1.12.

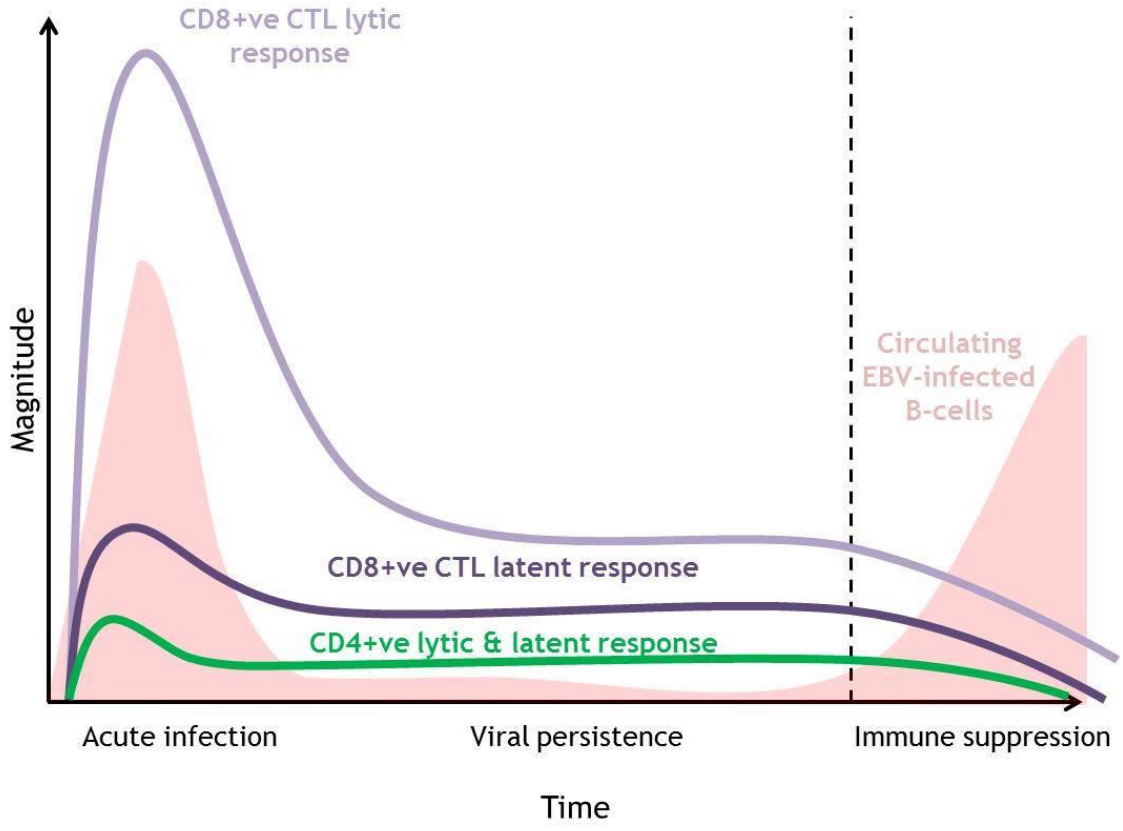


Figure 1-12 The T cell response to EBV during the lifetime of the host

During acute infection, there is a high level of viral replication and large numbers of EBV-infected B cells (pink shaded area). During the acute infection, the CD8+ve cytotoxic T cell (CTL) response to peptides derived from proteins expressed in the lytic phase of infection (pale purple line) predominates. Later, CD8+ve CTLs to latent protein specificities (dark purple line) appear, but at a lower level. The CD4+ve T-helped cell immune response is of a much lower magnitude compared with the CTL responses. During persistent latent infection, the magnitude of the CTL response falls but CD8 memory CTL responses particularly are maintained and act to help maintain good control of viral latency. Any immune suppression in the lifetime of the host is associated with a fall in the CD8+ve and CD4+ve EBV-specific T cells and a concomitant rise in numbers of EBV infected B cells.

The nature of antigen-presentation to T cells means that the CD8+ve and CD4+ve T lymphocyte response generated depends on the antigens available at any given time, the ability of those antigens to be presented by the HLA molecule, and the host's HLA type (Section 1.7.1). The epitope specificities and profiles of the EBV-specific CTL response in acute and persistent infection have been mapped (Hislop *et al*, 2007; Khanna & Burrows, 2000; Moss *et al*, 2001). Given the > 85 ORFs of the virus, one might anticipate a broad response to many EBV proteins. However, most individuals have a much more restricted response which focuses on a few specificities. What is in fact observed is a marked hierarchy in strength and frequency of response, referred to as immunodominance, such that certain peptide epitopes via certain HLA-restrictions tend to raise responses more commonly, and also elicit responses of greater magnitude (Hislop *et al*, 2007). Such responses during acute infection tend to have specificities for proteins from the lytic cycle and include the immunodominant HLA-B*08-restricted BZLF1-derived peptide RAKFKQLL and the HLA-A*02 restricted BMLF1-derived GLCTLVAML (Callan *et al*, 1998). The magnitude and specificity of the EBV-specific CTL response are thus directly dependent on the class HLA-phenotype of the individual.

Responses to peptides derived from latency programme proteins appear later in acute infection and then persist in viral latency. In most individuals, the responses to peptides from proteins expressed in latency are strikingly focused on epitopes from the immunodominant EBNA3A, EBNA3B and EBNA3C. Subdominant responses to epitopes from LMP2 may be seen, but much less commonly observed are responses seen to epitopes from EBNA1, EBNA2, EBNA-LP or LMP1 (Murray *et al*, 1992; Khanna *et al*, 1992; Hislop *et al*, 2007). The patterns of immunodominance are established early in the individual and,

although the magnitude of the response falls, the pattern persists (Khanna & Burrows, 2000; Woodberry *et al*, 2005). Responses can be detected for many years, likely for the lifetime of the host (Hislop *et al*, 2007), and there is some evidence that, akin to CMV, the proportion of the CD8+ve immune response specific to EBV may actually increase with age (Ouyang *et al*, 2003; Khan *et al*, 2004). Not all specificities raised at time of initial infection persist to the same degree; undefined differences in epitope specificity and TCR gene usage may play a role in which specificities are long-lived (Greenough *et al*, 2010), phenotypically reflected in differences in PD-1 and IL7-R expression(Sauce *et al*, 2009).

The pattern of immune dominance to latent protein specificities has important implications for the CTL response in EBV+ve cHL given its limited latency patterns of proteins (Table 1.9). Such hierarchies of immune dominance likely reflect the immune escape resulting from viral latency, and may be why class I HLA type may be significant. The CTL response to this limited set of proteins is important in responding to persistent infection, and probably goes some way to explaining why the virus is never fully cleared. When occasional viral reactivation occurs, the full spectrum of CD8 and CD4 memory available to an individual host is brought to bear on the reactivating virus, and, in a healthy host, control is rapidly re-achieved.

1.6.5.4 Adaptive immune response – cytokines

Levels of several cytokines are noted to be elevated in response to acute primary EBV infection. These include IFN-α, IFN-ß and IFN-γ which then initiate a cascade of responses including IL-15 and IL-12. Further cytokines which may be important include TNF, IL-1 and IL-6.

Interestingly, in studies of subjects with IM, only IL-6 levels correlated with severity of illness. Plasma IL-6 was also noted to be higher in subjects with a higher EBV viraemia. IFN-γ did not correlate with severity of symptoms or viraemia (Balfour, Jr. *et al*, 2013).

In addition to the cytokines produced by the human immune system in response to EBV, the virus also expresses proteins with cytokine functions, most notably BCRF1, which is a functional homologue to IL-10 (Hsu *et al*, 1990) and thus may drive a T_{reg} response; and BARF1 which acts as a soluble receptor for colony stimulating factor 1 (CSF-1), preventing monocyte proliferation and the further release of IFN-γ (Cohen & Lekstrom, 1999).

1.6.5.5 Measuring the EBV-specific CTL response

A number of different assays are capable of detecting and enumerating antigen-specific T cell responses in peripheral blood. Investigators have examined these assays for sensitivity and specificity, and have compared their advantages and disadvantages for different purposes (Hobeika *et al*, 2005; Karlsson *et al*, 2003; Kim *et al*, 2007; Letsch & Scheibenbogen, 2003). These methods are summarised in Table 1.11. For the purpose of this project, the ability to screen for novel, unrecognised CTL responses with maximal sensitivity meant that IFN-γ enzyme-linked immunospot (ELISPOT) was the most appropriate method. This is described further in Chapter 2.

Table 1-11 Comparison of assays of T cell responses

Method	Advantages	Disadvantages
IFN-γ ELISPOT	• High sensitivity and specificity. • Does not require a known antigen to be able to detect response, therefore is suitable technique for screening. • Cellular responses measured at the single cell level. • Large number of antigens can be screened with comparatively lower cell numbers. • Lowest "limit of detection" – ability to enumerate CTLs at low numbers.	• Non-specific background reactivity can be seen and may be a problem rendering assay uninterpretable.
IFN-γ ELISA	• Detects bulk response to antigen.	• Cannot detect responses at a single cell level, therefore does not enumerate antigen-specific cells.
MHC Tetramer	• Good for confirming any detected responses. • High sensitivity and specificity.	• Requires known epitopes – not generally possible to use as a screening method. • Costly compared to other methods. • Cannot detect functional responses to any particular antigen i.e. cognate cells which do not display functional cytotoxicity may be recognised.
Intracellular cytokine analysis by flow cytometry	• Greater dynamic range – better at quantifying magnitude of response in responding cells. • Cellular responses measured at the single cell level. • Ability to phenotype the responding cells. • High sensitivity and specificity.	• Less able to detect low level responses. • Very labour intensive and requires large number of cells for screening of large numbers of antigens.
Chromium release assay	• Good method for confirming any particular response detected.	• Require the use of irradiation, with recognised hazards to health, cost, practical aspects regarding use, short half-life and training. • Method less suitable for screening large numbers of antigens. • High inter-test variability. • High background release may prevent interpretation of result. • Cannot detect responses at a single cell level, therefore does not enumerate antigen-specific cells. • Cells cannot be used in downstream assays.

CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot; MHC, major histocompatibility compatibility.

1.7 The HLA system and HL

The HLA system, encoded by the MHC genes, defines "self" and "non-self" recognition by the immune system. First described in the 1950s by Jean Dausset, for which he won the 1980 Nobel prize in Physiology or Medicine (Nobelprize.org, 2013), the HLA system is central in antigen presentation for recognition by the T cell immune system.

Perhaps unsurprisingly given its role in self/non-self discrimination, the HLA system is recognised to be associated with at least 40 diseases, particularly those with an autoimmune aetiology (Ghodke *et al*, 2005) such as ankylosing spondylitis (Allen *et al*, 1999; Bowness *et al*, 1999), rheumatoid arthritis (Bax *et al*, 2011), type 1 diabetes mellitus, coeliac disease (Lie & Thorsby, 2005) and Behçet's disease (de Menthon *et al*, 2009). In addition, as the HLA system presents pathogen-derived antigen for immune recognition, it is again unsurprising that associations between HLA-type and susceptibility to infectious diseases, such as malaria (Hill *et al*, 1991) and HIV (McNeil *et al*, 1996) have been described.

The physiology and genetics of the HLA system will be described first, followed by a description of the studies demonstrating the association between class I HLA and cHL, and lastly, how this may be of significance in EBV+ve cHL.

1.7.1 The HLA system

1.7.1.1 Structure and function

The HLA molecules are encoded by a region on the short arm of chromosome 6 (6p21.3) called the MHC. This region of the genome is very gene-dense and is divided into three "class" sub-regions based on the function of the genes. The

class I and class II regions contain the HLA genes (Figure 1.13). Every human has three class I genes: HLA-A, B and C on each copy of chromosome six. The class I genes are co-dominant, i.e. all are expressed, and thus every individual has 6 class I alleles.

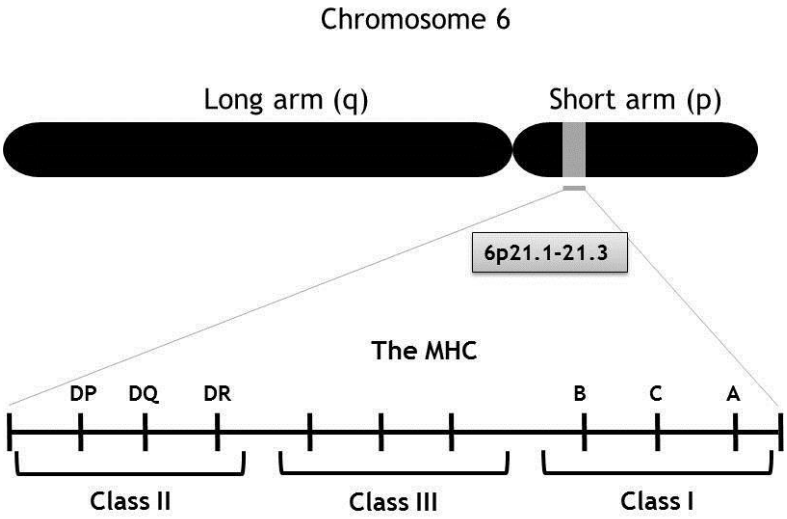


Figure 1-13 The major histocompatibility complex.

The approximate position of the complex on the short arm of chromosome six is indicated, along with the three class regions. The approximate location of the class I genes, HLA-A, HLA-B and HLA-C are given as are the three main class II genes, HLADP, -DQ and –DR.

HLA class I genes are expressed by all nucleated cells in the human body, and also on platelets. HLA class II genes are expressed only on professional antigen-presenting cells of the immune system such as B lymphocytes, dendritic cells and macrophages. The class III region does not contain HLA genes, but many of the genes within this region have an immune function and include TNF-α and the complement genes C2 and C4. A more detailed map of the MHC is given in Figure 1.14.

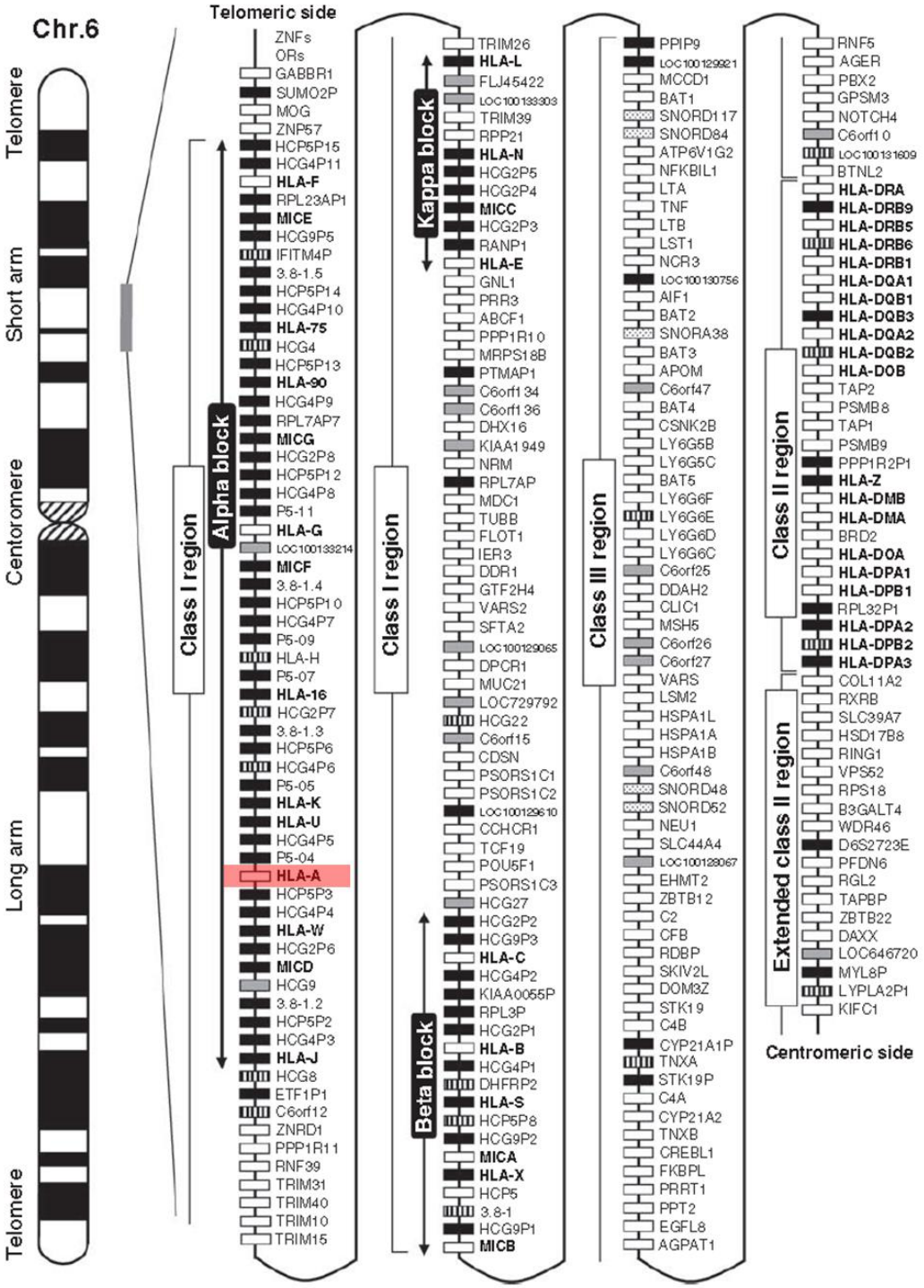


Figure 1-14 Gene map of the human leukocyte antigen (HLA) region.

The regions separated by arrows show the HLA sub-regions extended class I, classical class I, class III, classical class II and extended class II regions from telomere (left and top side) to centromere (right and bottom side). White, grey, striped and black boxes show expressed genes, gene candidates, non-coding genes and pseudogenes, respectively. HLA-A is highlighted in red. Reproduced and adapted from (Shiina *et al*, 2009) with permission. Copyright Nature Publishing Group.

The function of both class I and class II HLA molecules is to present peptides derived from pathogens to T cells, initiating or re-activating a cellular immune response. HLA class I molecules present endogenously derived peptides including non-self peptides e.g. those derived from viruses or altered self peptides. CD8+ve CTLs recognise peptides bound to HLA class I. Class II molecules present peptides derived from extracellular proteins which are internalised. Class II presents peptides for recognition by CD4+ Th cells, thus stimulating T cell help and initiation of a humoral immune response.

HLA class I genes encode a membrane-bound glycoprotein, the variable α-polypeptide chain. The α-chain is non-covalently bound to a constant ß2-microglobulin molecule (encoded on chromosome 15) to form the HLA class I molecule (Figure 1.15).

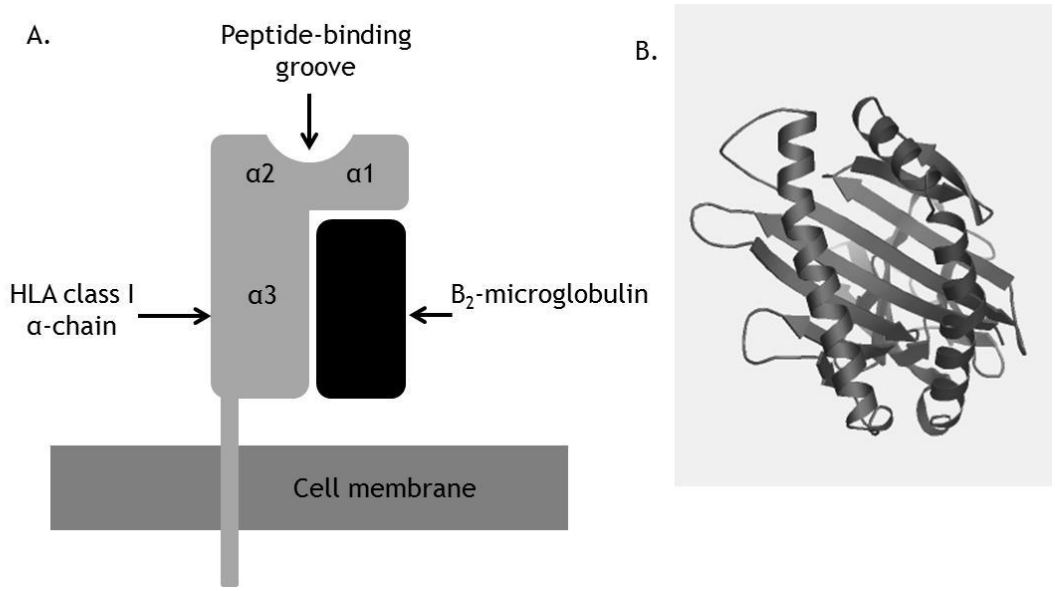


Figure 1-15 HLA class I molecule

A. The HLA molecule comprises the HLA class I coded α-chain non-covalently bound to a molecule of ß2-microglobulin. The α3 domain of class I HLA is constant. The α1 and α2 domains of the protein are variable and together form the peptide binding groove, which faces out from the cell to present the peptide to the T cell receptor for recognition.
B. View of the HLA-binding groove of HLA-A*01:01 from above. Figure drawn using the sequence of HLA-A*01:01 (Uniprot Consortium, 2008) using Swissprot protein modelling programme (Bordoli *et al*, 2009).

The three-dimensional structure of HLA class I has been determined. The first two domains, α1 and α2 fold to form a ß-pleated sheet bound by two α-helices, forming a groove (Figure 1.15). The α1 and α2 regions of the HLA-molecule are highly variable leading to the different class I HLA types. The greatest variation is seen in the regions which form the binding groove (coded by exons 2 and 3), and which ultimately determine which antigenic peptides can bind.

In an infected cell, viral proteins are digested into small peptides which are transported to the endoplasmic reticulum (ER) via the transmembrane pump transporter associated with antigen processing (TAP). A peptide which "fits" the binding groove of a given HLA class I molecule is bound into the forming HLA molecule by the enzyme tapasin, stabilising the structure. Class I bound peptides are usually 8 – 10 amino acids (AA) in length, with the majority being 9 AA in length. The HLA molecule with bound peptide leaves the ER and is transported to the cell surface via the Golgi apparatus. On the cell surface, the HLA-peptide complex is free to interact with a cogent T cell, via the TCR, initiating the process of cell killing. There are thought to be roughly 10^5-10^6 HLA class I molecules per cell and, although not all HLA molecules are occupied at any one time, potentially tens of thousands of class I-bound peptides could be available at the cell surface. In addition, it has been shown that "naked" HLA, i.e. empty HLA molecules without bound peptide, can bind exogenously provided peptide (Kubitscheck *et al*, 1992). Such "naked" HLA in alternative conformations can reach the cell surface via the conventional ER route, or can be endocytosed into the cell via endosomes for recycling to the cell surface (Jondal *et al*, 1996). As few as 100 copies of a peptide per cell have been shown to be able to stimulate a CTL response (Marsh *et al*, 2000).

The selection of any given peptide for presentation in the binding groove of the HLA molecule depends on the affinity within binding pockets of the HLA molecule for AA (known as anchor residues) within the peptide. This affinity is defined by the ability of the cell to digest the viral protein and by the physical and chemical properties (size, polarity and acid/basic properties) of the AA within the resultant peptides. Thus the ability of any single peptide to bind to a particular HLA molecule is a function of both the viral protein and the HLA molecule present, and the resultant peptide presented by the HLA molecule is referred to as an epitope.

For many years it has been possible to systematically study the epitopes presented from any given antigen and their HLA-restriction. Investigation into which epitope from a given antigen stimulates a T cell response usually starts with studying the T cell response. The most reliable approach is to use libraries of synthetic peptides which fully span every protein in an organism/ virus. Once a response is detected, the HLA-restriction is identified, and the epitope is then characterised using smaller peptides until a minimum core sequence epitope can be defined. Functional studies as described in Section 1.6.4.5 can then be used to further analyse the response stimulated e.g. for immunodominance, strength of response etc. Using these techniques, HLA-restrictions to EBV peptides have been described. Small numbers of healthy donors of known HLA type have generally been used in these experiments; therefore HLA-restrictions through common alleles are described more frequently.

The properties of the HLA class I binding groove mean that certain patterns of the characteristics of peptides (e.g. anchor residues, binding motifs etc.) can be used to study HLA-binding affinities (Chelvanayagam, 1996). A number of

epitope prediction software programmes use these properties to predict which antigen-derived epitopes might bind to a particular HLA molecule. These are summarised in Table 1.12.

Table 1-12 HLA-binding peptide prediction software used in this project

Software	Method	Reference
SYFPEITHI	Uses a database of more than 7000 known epitopes and their physio-chemical characteristics, such as anchor points and allele-specific binding ligands. Epitopes that would be predicted to bind in the peptide groove of an HLA molecule with a functional binding strength are identified from full-protein sequences.	http://www.syfpeithi.de.home.htm (accessed 19/11/08) (Rammensee, 2008)
RANKPEP	Predicts peptides predicted to bind to Class I and Class II HLA molecules from protein sequence or sequence alignments using Position Specific Scoring Matrices (PSSMs) derived from tables listing the observed sequence-weighted frequency of all AAs in every column of a sequence alignment. In addition RANKPEP takes into account whether the C-terminus of the epitope resulted from cleavage by the proteasome.	http://imunax.dfci.harvard.edu/Tools/rankpep.html (accessed 19/11/08) (Bioinformatic Cancer Vaccine Center Harvard University, 2008)
ProPred	Predicts peptides predicted to bind to Class I and Class II HLA molecules from protein sequence or sequence alignments using Position Specific Scoring Matrices (PSSMs) derived from tables listing the observed sequence-weighted frequency of all AAs in every column of a sequence alignment.	http://www.imtech.res.in/raghava/propred1, (accessed 19/11/08) (Singh & Raghava, 2003; Singh & Raghava, 2008)
BIMAS	Ranks potential 8 to 10 AA length peptides based on a predicted half-time of dissociation from HLA class I molecules, based on coefficient tables of AA binding strength deduced from the published literature	http://www.bimas.cit.nih.gov/molbio/hla_bind/, (accessed 19/11/08) (NIH BioInformatics and Molecular Anaysis Section, 2008; Parker *et al*, 1994)

These software programmes were used in this study to predict EBV-restricted epitopes; this is discussed further in Chapter 4. Note that none of the methods

available in 2008 used the ability of peptide to be processed via the TAP system in the endoplasmic reticulum in their algorithm.

1.7.1.2 HLA inheritance

One of the striking features of HLA genes is their remarkable level of polymorphism when compared to other somatic genes. At each of the genes (in the case of class I, A, B and C), there are multiple possible variants, called alleles. The nucleotide differences at gene level are translated into protein sequencing differences, and thus differences in the binding affinity for different peptides. The HLA region is the most variable part of the human genome studied; at the most recent review by the HLA informatics group of the World Health Organization (WHO), there were 2244 HLA-A, 2934 HLA-B and 1788 HLA-C alleles at the DNA level, resulting in 1612, 2211 and 1280 different proteins respectively (Robinson *et al*, 2013), each of which can bind many thousands of epitopes. As discussed above, each individual has six class I HLA alleles (two each of A, B and C) which together these define an individual's HLA phenotype. This variability means that, at the MHC region, humans are enormously diverse and are, outside the case of identical twins, genetically unique.

The extraordinary diversity at HLA allows, at a population level, the capacity to respond to a wide range of pathogens. That the bulk of variation in the HLA molecule is in the peptide binding groove of HLA suggests that HLA molecules have evolved as a result of natural selection (Little & Parham, 1999). Populations which have more limited HLA diversity e.g. due to small founding populations, such as the Native peoples of North and South America, face difficulties when faced with new antigenic challenges such as the introduction into the New World of measles by the conquistadors (Black, 1992). An HLA

diverse population, whilst still encountering morbidity and mortality in the face of an epidemic, is likely to have a greater proportion of individuals surviving such an event (de Vries *et al*, 1979).

The evolution of HLA is therefore assumed to be predicated on maintenance of diversity. HLA class I heterozygotes are more frequent in populations than would be expected by chance. Heterozygosity increases the number of possible epitopes which can be presented, referred to as heterozygote advantage (Little & Parham, 1999). There is even some evidence that mate selection in humans may be influenced by HLA haplotype, with individuals less likely to choose a partner with a similar HLA type than would be expected by chance alone (Ober *et al*, 1997; Wedekind *et al*, 1995).

The HLA molecules first appear in the jawed vertebrates, demonstrating the importance to a species of an adaptive immune system (Parham, 1999). It is likely that human HLA molecules derived from a common ape ancestor, most likely the HLA-A locus. There is evidence that the common chimpanzee (*Pan troglodytes*) HLA-A shares features with human HLA-A*03, suggesting divergence from an allele in a common ancestor approximately 5 million years ago (Little & Parham, 1999). The class I B and C alleles are thought to have arisen through duplication events (and class II from duplication of class I alleles) and have then undergone divergence through point mutation and recombination. Class I allele lineages, also known as “supertypes”, can be defined based on this sequence divergence.

HLA-class I genes are also remarkable in that they are often inherited as “blocks” of genes which do not undergo recombination during meiosis – a phenomenon referred to as “linkage disequilibrium” (LD) (Yunis *et al*, 2003). A

combination of alleles encoded by different HLA loci on the chromosome, when found together at higher frequencies than would be expected by chance, is referred to as a haplotype. Single-nucleotide polymorphisms (SNPs) are associated with HLA haplotypes such that HLA-alleles can be imputed from knowledge of the SNPs (de Bakker *et al*, 2006; Miretti *et al*, 2005; Leslie *et al*, 2008). Such haplotypes also encompass the non-HLA genes which sit between the classical HLA genes. In some studies of disease association, the haplotype association has been demonstrated to be in an intermediate gene such as TNF-α (Abraham *et al*, 1993; Abdou *et al*, 2010). Investigators undertaking HLA disease association studies must be careful to take haplotype effects into account.

Some haplotypes are more common in certain populations, an example in Caucasian populations being the most ancient "HLA-A1, C7, B8, DR3" haplotype, in which A*01 and B*08 sit in LD. It is suggested that in ages when infectious disease was the main determinant of individual survival such haplotypes may have carried some evolutionary advantage in dealing with common primitive pathogens, and have been maintained by positive selection. However, such haplotypes may be disadvantageous in modern times. For instance, the "HLA-A1, B8, C7, DR3" haplotype has been described as the "autoimmune haplotype" for its association with numerous autoimmune diseases including type I diabetes mellitus, Graves' disease, SLE and many other conditions (Candore *et al*, 2002; Price *et al*, 1999).

Such selective pressure has probably been instrumental in determining which alleles are present in a particular population. Allele frequencies vary in different populations around the world. The HLA-B*53:01 gene, which is associated with resistance to malaria, is more common in West African populations where it has

probably increased in frequency as a result of positive selection (Hill *et al*, 1991). Similar ancient and interacting pressures are likely to have shaped the allele frequencies around the world. The geographical patterns of HLA diversity suggest significant evolution as man has spread around the globe (Abi-Rached *et al*, 2011), and, secondarily, may account for some of the ethnic differences seen in incidences of some viral-associated malignancies such as NPC and cHL.

An international database has been devised to hold information on allele frequencies in different populations (Gonzalez-Galarza *et al*, 2011). This information tells us that, for example, the most common HLA-A allele in Scottish & UK populations is HLA-A*02 at 42-54% of the population. HLA-A*02 is in fact one of the commonest HLA-A alleles around the world suggesting that at some point the allele held a selective advantage. HLA-A*01:01 is a relatively common gene in those of Celtic heritage, present at 29-33% in the Scottish & UK population and up to 44% in Northern & Southern Irish populations vs. 11% in Chile and 2% in Hong Kong Chinese (all allele frequency data, accessed 16/06/2013 (Gonzalez-Galarza *et al*, 2013)).

1.7.1.3 HLA nomenclature

HLA nomenclature, as defined by the WHO Nomenclature Committee for Factors of the HLA System (Marsh *et al*, 2010; Nunes *et al*, 2011) is used throughout this thesis. Briefly each HLA allele name has a unique number corresponding to up to four sets of digits separated by colons (Figure 1.16). All alleles receive at least a four digit name, which corresponds to the first two sets of digits; longer names are only assigned when necessary. The first two digits describe the type, which corresponds to the serological allotype. The next two digits define the subtypes, defined by different AA sequences. The next two digits define variants with

silent or non-coding differences in nucleotide sequence. The fourth set of digits defines variants in the untranslated regions of the gene (Figure 1.16). Letter suffixes may be used to define variants which are expression-based e.g. L for low-level of expression, N for null etc. Where HLA is discussed in the following text, the resolution of typing reflects that performed in the studies described.

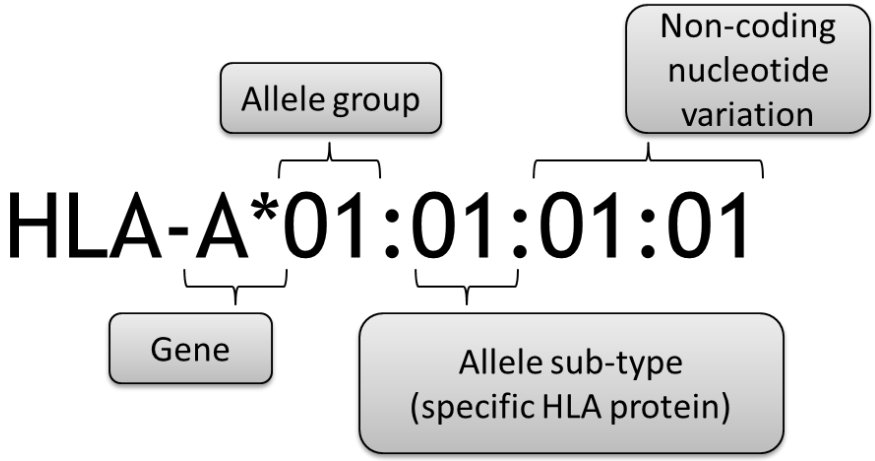


Figure 1-16 International HLA nomenclature
As defined by WHO Nomenclature Committee for Factors of the HLA System (Marsh *et al*, 2010).

1.7.2 The Association between class I HLA and cHL

The ethnic differences in incidence of cHL and the elevated risks seen in family members (particularly identical twins, Section 1.5), suggest that there is an inherited component to the risk of cHL. It is now apparent that there are strong associations between HLA class I genes and EBV+ve cHL.

cHL was the first disease for which any HLA association was described in 1967 (Amiel, 1967). Although earlier HLA-typing methods were limited by available technology, further studies (Dausset & Hors, 1975; Hors & Dausset, 1983;

Svejgaard *et al*, 1975; Falk & Osaba, 1974; Hansen *et al*, 1977) demonstrated that HLA-A*01 and HLA-B*08 were most closely associated with increased risk.

This work was furthered by family segregation studies. The first of these, (Marshall *et al*, 1977), did not demonstrate any clear association with disease, although HLA-B*08 was seen more often in first-degree relatives of cases. It must be noted that the initial HLA-association studies were published in the era before the EBV-association of cHL was recognised and, as such, EBV status of disease is not reported. Further familial studies demonstrated excess of HLA-B*35 and HLA-B*37 in cases within families (Greene *et al*, 1979). A greater than expected HLA identity between siblings with cHL was described and an association with HLA-A*01 reported (Hors *et al*, 1980). Similar haplotype concordance was observed between cHL-affected siblings in a study of 16 families (Berberich *et al*, 1983). In addition, 8 of 16 families in this series had the A*01, B*08 haplotype.

Population based studies (Hors & Dausset, 1983; Falk & Osaba, 1974; McDevitt & Bodmer, 1974; Harris *et al*, 1978; Bjorkholm *et al*, 1975) continued to demonstrate consistent associations with HLA-A*01, and to a lesser extent B*08, with A*03 and A*11 being less prevalent in cases as compared with controls.

Interestingly, some of the earlier studies (Hornmark-Stenstam *et al*, 1978; Falk & Osoba, 1971; Hafez *et al*, 1985) also observed a survival advantage associated with particular HLA types; remarkably consistently these studies demonstrated that although HLA-*01 was associated with risk of disease, it was also associated with improved survival. Some of these were retrospective and may have been affected by survivor bias.

More recently, data analysed in the modern era with PCR-based HLA-typing better diagnostic accuracy and availability of EBV-status of disease, have confirmed the strong association between HLA genotype and EBV+ve cHL, suggesting that HLA-restricted CTL responses to EBV may play a key role in determining risk of developing disease. Genotyping studies across the HLA region have shown that microsatellite markers and SNPs in the HLA class I region are strongly associated with EBV+ve cHL (Diepstra *et al*, 2005; Niens *et al*, 2006). The markers associated with disease were shown to be in LD with HLA-A*01 (associated with risk) and HLA-A*02 (protective) (Niens *et al*, 2007).

Further evidence pointing to an association between class I HLA and EBV+ve cHL has been the study of HLA-associated genetic markers and risk of IM. As has been discussed, prior IM is associated with elevated risk of cHL. Studies have demonstrated that the SNPs and microsatellite markers in the class I HLA region associated with symptomatic IM are the same as in EBV+ve cHL (McAulay *et al*, 2007).

A recent large Scottish and Scandinavian case series analysed 934 patients with cHL for whom EBV status and class I HLA typing was available. In this study (Hjalgrim *et al*, 2010), HLA-typing was performed to 4-digit resolution, but the statistical analysis performed at 2-digit level. Increased risk of disease was associated with HLA-A*01 (OR per allele, 2.15; 95% CI, 1.60-2.88) and decreased risk with HLA-A*02 (OR per allele 0.70; 95% CI, 0.51-0.97). The effects were independent of each other, but dependent on allele copy number, resulting in an almost 10-fold greater odds of developing EBV+ve cHL in HLA-A*01 homozygotes when compared with HLA-A*02 homozygotes.

GWAS studies have corroborated the association with the HLA class I region with remarkably consistent ORs. In a recent GWAS study, the SNP rs2734986 in HLA-A was associated with EBV+ve cHL (OR, 2.45; 95% CI = 2.00 - 3.00, $p = 1.2 \times 10^{-15}$) but not with EBV-ve cHL (Urayama *et al*, 2012).

The class I HLA link with EBV+ve cHL is biologically plausible. There is involvement of a pathogen (EBV) in the disease, to which the CTL immune response in known to be crucial. HLA class molecules, whilst known to be downregulated in EBV-ve cHL, presumably as a means of immune escape, are observed to be expressed at high levels on the surface of HRS cells in EBV+ve disease (Oudejans *et al*, 1996), thus suggesting that a CTL response to the malignant cell is still possible.

Several groups have also observed associations between class II HLA and cHL, particularly EBV-ve cHL or NSHL. Such risk alleles include HLA-DPB*03:01 (Bodmer *et al*, 1989; Taylor *et al*, 1999; Alexander *et al*, 2001), and class II associations have been verified in SNP studies (Moutsianas *et al*, 2011) and in GWAS studies (Cozen *et al*, 2011; Urayama *et al*, 2012). No association between Class II and EBV+ve cHL has been described and class II HLA is not discussed further.

A criticism frequently levelled at HLA association studies is that the association observed may not be with the HLA allele in question, but may be with another gene in LD with the allele. This can only be answered by explaining the underlying mechanism of any association.

1.7.3 Exploring possible mechanisms for the association between class I HLA and EBV+ve cHL

The association between HLA class I, most particularly HLA-A*01:01 and HLA-A*02:01, and EBV+ve cHL suggests a critical role for the CTL response to EBV in determining risk of disease.

EBV is an intensively studied virus. Over the last 20 years, a number of groups have interrogated the EBV-specific CTL responses. Generally, these studies were concerned with systematically mapping T cell epitopes and HLA-restrictions, and did not specifically seek or examine HLA-A*01:01 or HLA-A*02:01-restricted responses.

That numerous immunodominant HLA-A*02:01-restricted peptides have been described is of interest given the protective effect of this allele. In contrast, in spite of intensive study, no confirmed HLA-A*01:01-restricted CTL responses to EBV latent or lytic proteins have been described (Hislop *et al*, 2007; Straathof *et al*, 2005b; Long *et al*, 2011b; Moss *et al*, 2001). HLA-A*01:01 is able to efficiently present peptides from viruses other than EBV. For instance, immunodominant peptides from CMV and Influenza virus raise very efficient CTL responses via this allele (Currier *et al*, 2002; DiBrino *et al*, 1993; Elkington *et al*, 2003; Wills *et al*, 1996). Thus, there is no suggestion that HLA-A*01:01 is in any way deficient in terms of antigen presentation capability.

This raises the possibility that absent or weak CTL responses specifically to EBV might lead to the elevated risk of disease. The stage in the disease process when the HLA-A*01:01 associated risk is exerting its effect is unknown. It may be that the CTL response to the proliferating tumour is critical, or, alternatively, it may

be that control either of initial infection or of viral persistence may be important.

The HLA-A*01:01 associated risk is unexplained; individuals who carry HLA-A*01:01 will also have potentially 4 or 5 other class I HLA alleles through which they may be able to raise an EBV-specific CTL response. It is not clear whether the increased risk associated with HLA-A*01:01 is simply because there are no EBV-specific responses restricted through this allele, or whether HLA-A*01:01 exerts qualitative or inhibitory changes to the EBV-specific immune response.

The interaction between HLA-A*01:01 and HLA-A*02:01, specifically the intermediate risk of disease seen in heterozygotes is also of interest. The reason for the elevated risk of disease in HLA-A*02:01/A*01:01 heterozygotes is unclear, since these individuals should be capable of mounting effective, even immunodominant, CTL responses to EBV.

1.8 Summary

The pathophysiology of EBV+ve cHL is gradually being elucidated; however many fundamental questions remain. EBV is ubiquitous, infecting much of the world's population, but causing malignancy in only a small minority. The question of why some individuals in the face of a common and normally well-tolerated persistent infection develop EBV+ve cHL, whilst others do not, has not been answered.

The cellular re-programming of the HRS cell is only part of the story; the central role of the tumour microenvironment in cHL tells us that this cancer, more than most lymphoid malignancies, manipulates the immune response. The recent findings of the associations of class I HLA allotype with risk of developing EBV+ve cHL only reinforce that immune response, particularly CTL response, is crucial in

disease pathogenesis. Such associations, however, do not explain the mechanisms at work.

Clinically, the majority of patients with cHL do well, and are long-term survivors of their disease. Two main clinical challenges persist. First, as most patients survive their disease, focus moves to minimising the life-changing or life-shortening late effects of therapy. Secondly, and more crucially, to say that cHL is a "done deal" in that the majority of patients survive does an injustice to the 15-20% of patients who will succumb to their disease. Given the epidemiology of cHL, this means that every day young adults are dying of this disease. Current prognostication is not sufficiently accurate to target therapies, and there is no good evidence that such targeted approaches will improve outcome. New approaches are required both to identify and successfully treat such high-risk patients.

1.9 Aims and Objectives

This project aims to address a number of specific objectives regarding the role of class I HLA, specifically HLA-A*01:01 and HLA-A*02:01 in EBV+ve cHL, namely:

Does the presence of an HLA-A*01:01 allele modify the magnitude of the CTL response to HLA-A*02:01-restricted epitopes? (Chapter 3)

Can any HLA-A*01:01-restricted EBV-specific CTL responses be detected or are any inhibitory cytokine responses detected? (Chapter 4)

Are HLA-A*01:01 and A*02:01 alleles a factor in determining clinical outcome in EBV+ve cHL? (Chapter 5)

Chapter 2. Materials & Methods

2.1 Materials

Materials used in the project are described in the relevant methods section. Suppliers and addresses are given in Appendix 1. Plastic ware is listed in Table 2.1 and named in full at first use. Thereafter, for brevity, the shortened name listed in the table is used.

Table 2-1 Plasticware

Item	Abbreviation used in text (where applicable)	Supplier
1.5 ml sterile screw-cap polypropylene microcentifuge tube	1.5 ml screw-cap tube	Elkay
15 ml conical polypropylene tube	15 ml tube	Falcon, BD Biosciences
50 ml conical polypropylene tube	50 ml tube	Falcon, BD Biosciences
5 ml round-bottom polystyrene tube (Falcon #2054)	5 ml tube	BD Biosciences
50 ml LeucoSep tube		Greiner Bio-One
50 ml reagent reservoir - Costar™	reagent reservoir	Corning Inc.
48-well flat-bottom plate (Multiwell™)	48-well plate	BD Biosciences
96-well round-bottom sterile plate (Nunclon™ surface)	96-well plate	Sigma
Corning 25 cm^3 flask	25 cm^3 flask	SLS
Corning 250 cm^3 culture flask	250 cm^3 culture flasks	SLS
NUNC™ 1.8 ml cryotube	cryovial	Fisher Scientific Inc.
Pastette		Fisher Scientific Inc.
Pipettes		Gilson Scientific Ltd.
Pipette tip - 10 µl aerosol resistant tips (ART Reach™)		Sigma
Pipette tip - 20 µl, 200 µl, 1000 µl		Rainin
VACUETTE® tube		Greiner Bio-One

2.1.1 Culture media and solutions

A list of the culture media, buffers and solutions used is given in Table 2.2.

Table 2-2 Culture media, buffers and solutions

Culture media, buffers and solutions	Acronym used in text	Purpose	Recipe
Complete culture medium 10% FBS†	10% CCM	General cell culture	RPMI 1640 (Invitrogen) 500 ml FBS (Invitrogen) 50 ml Penicillin/Streptomycin (Invitrogen) 20 ml L-glutamine (Invitrogen) 5 ml Total volume 575 ml
Complete culture medium 20% FBS†	20% CCM	Culture of lymphoblastoid cell lines	RPMI 1640 (Invitrogen) 500 ml FBS (Invitrogen) 100 ml Penicillin/Streptomycin (Invitrogen) 20 ml L-glutamine (Invitrogen) 5 ml Total volume 625 ml
Dulbecco's Phosphate buffered saline	PBS	Isotonic buffer	Sterile water (Invitrogen) 100 ml 10 x Dulbecco's phosphate buffered saline (Invitrogen) 11 ml
Freezing medium	FM	Viable freezing of cells	FBS (Invitrogen) 9.2 ml DMSO (Fisher) 0.8ml
Mini-Macs (Modified magnetic activated cell separation) Buffer	MMB	For cell separation and cell counting	10 x PBS (Invitrogen) 50 ml EDTA (supplier) 2 ml Bovine serum albumin (supplier) 2.5 g Fill to 500 ml with distilled water Filtered using a 500ml 0.45 µm Corning filter flask (Sigma) and dispensed into 50 ml aliquots stored at 4°C.

† By convention "10% CCM" is made by adding 50 ml FBS to 500 ml RPMI, and “20% CCM” by adding 100 ml FBS to 500 ml RPMI, which results in a slightly lower percentage serum. The medium is filtered using a 500 ml 0.45 µm Corning filter flask (Sigma) and dispensed into 50 ml aliquots stored at 4oC. FBS, foetal bovine serum; PBS, phosphate buffered saline; DMSO, Dimethyl sulfoxide; EDTA, ethylendiamine tetra-acetic acid.

2.1.2 Equipment

Large items of equipment used in the project are detailed in Table 2.3 and mentioned in the text where relevant.

Table 2-3 Large items of equipment

Item	Supplier
Heraeus Multifuge 3 S-R	DJB Labcare Ltd.
Jencons Sealpette	vWR
Pipetteboy-acu	Integra Biosciences
Allegra 6R Centrifuge	Beckmann Coulter
Incubator	Thermo Scientific
1500 series freezer -70°C	MVE System
Freezer -20°C	Haier
Vapour-phase nitrogen freezer	New Brunswick Scientific
FC500 Flow cytometer	Beckmann Coulter
AID (Autoimmun Diagnostika) Plate reader	Oxford Biosystems/ Cadama Medical Ltd.

2.2 Safety Measures

All of the work carried out in this project was performed according to the required health and safety considerations, per University of Glasgow guidelines. Control of substances hazardous to health (COSHH) assessments were performed for all procedures. All work was performed in a Containment Level 2 laboratory; cell cultures, viruses and human samples were handled in a Class 2 Microbiological Safety Cabinet (MSC). Gowns were worn over laboratory coats for all work undertaken in the MSCs. These gowns were autoclaved regularly, including after each occasion when virus or virus-infected cell lines were handled. All metal surfaces were sprayed with 70% alcohol (Fisher Scientific Inc.) before and after use. Liquids containing biological material were discarded into 2% Virkon (Fisher Scientific Inc.) and other material coming into contact with biological substances was disinfected in 1% Virkon (Fisher Scientific Inc.). All waste material leaving the laboratory was autoclaved at 120°C and 100 kilo Pascal above atmospheric pressure for 20 minutes (min) prior to appropriate collection. All sharps waste was disposed of into standard sharps containers conforming to UN 3291 and BS 7320 standards.

2.3 Samples from Healthy Donors

2.3.1 Subjects and Ethical Approval- The SHARE Study

In order to study the HLA-restricted immune responses of healthy individuals, as discussed in the Introduction, a source of healthy human T and B lymphocytes was required. Healthy adult volunteers (age ≥ 18 years) were recruited to a study designated SHARE (Study of Healthy Adult Responses to EBV). Ethical approval for this study was obtained via the national Integrated Research Application System (IRAS), and granted via the West Glasgow Research Ethics Committee 2 (REC reference: 08/S0709/149). In addition, ethical approval was obtained from the University of Glasgow, Faculty of Veterinary Medicine Ethics and Welfare Committee, so that recruitment to the study could take place within the University of Glasgow. Ethical approval was also obtained from the clinical governance committee of the Scottish National Blood Transfusion Service (SNBTS) and various stakeholders within the SNBTS, for use of donors as detailed below. A questionnaire was administered to all recruits to the study, collecting basic demographic data, postcode, smoking status, and history of previous IM; this is included as Appendix 2.

2.3.1.1 Recruitment

The subjects were recruited in a number of ways. Firstly, healthy adults were invited to visit the Clinical Research Facility (CRF) at the Western Infirmary, Glasgow, where research nurses administered the questionnaire and collected blood samples. This recruitment mechanism was open to the public. Secondly, participants were recruited from within the University of Glasgow. Permission was granted by Dr. Graham Caie, University of Glasgow Clerk of Senate and Vice Principal, and the Heads of Faculties for recruitment e-mails to be sent to staff

and students. These were sent via distribution lists to all staff and students, with the exception of medical students. Volunteers recruited in this way were able to participate via the CRF, or approach me directly. Recruitment sessions were also held on the Garscube Estate (University of Glasgow). Thirdly, recruitment e-mails were sent out to our collaborators and contacts within the local haematology community. Interested individuals requested a pack for further information. These individuals then arranged for local sampling, completed the questionnaire and returned the samples to the Leukaemia Research Fund (LRF) Virus Centre by pre-paid post in an appropriate biological transport container (Safebox™, Royal Mail). All participants recruited via these three routes completed the questionnaire and, as their HLA type was unknown, gave an initial blood sample (detailed below) for HLA typing and viral serology.

Participants with HLA types of interest were then approached as detailed below for a further sample of 50 millilitres (ml) whole blood.

Volunteers of known HLA type were also recruited from the SNBTS platelet-donor population. These donors are special in the donor community in that they altruistically donate apheresed platelets every few weeks. An advantage of this donor population was that their extended HLA type was known, thus enabling donors with specific HLA types to be targeted. A list of platelet donors with the HLA types relevant to the study was generated by John Tomkins, Senior Biomedical Scientist, Histocompatibility and Immunogenetics Service, SNBTS, Gartnavel General Hospital, Glasgow. Donor identities were pseudonymised and donors were identifiable to us only by their national donor (PROGESA) number. The donors with HLA types of interest were then approached by the clinical and nursing staff of the Old Athenaeum Donation Centre, Nelson Mandela Place,

Glasgow where platelet donations take place. As HLA type was already known, no initial sample was required. These donors were required only to complete the questionnaire, and donate a 50 ml whole blood sample. On some occasions, samples could not be transported to the LRF Virus Centre on the same day as they were collected, and so were kept overnight at room temperature (RT) at the Old Athenaeum Donation Centre prior to next day transportation. This information was recorded. SNBTS donors were not approached for further specimens.

2.3.1.2 Consent, Pseudonymisation and Recall

All subjects gave written, informed consent to participate in the study, and completed the questionnaire. Subjects without prior HLA-typing information were made aware that they may be approached for an additional blood sample. Consent forms and the top sheet of the questionnaire both contained patient-identifiable data and were kept securely and separately by the laboratory clinical co-ordinator, Mrs. Shauna Crae. Participants were allocated a non-sequential "SHARE number" at time of recruitment and staff processing the samples did not have access to the participant-identifiable data. On arrival of the initial sample at the laboratory, the participant was also allocated a sequential "LRF patient number", a unique identifier which enabled data storage and unblinding by authorised personnel for recalls. All non-identifiable data were stored in a computer database held by the LRF Virus Centre (Section 2.3.1.3). Access to the database is limited to authorised personnel. Data were held in compliance with the Data Protection Act.

Donors with HLA types relevant to downstream investigations were identified by me and decoding of donor numbers was performed by Mrs Crae or Prof. Jarrett.

Depending on the route of recruitment, donors were then contacted by CRF staff or by Prof. Jarrett. Donors were able to state a preference for mode of recall at time of initial recruitment, for example e-mail, telephone or letter. Donors were also offered the choice of donating their 50 ml blood sample at the CRF, and thus maintaining anonymity, or contacting me to arrange for a blood sample to be taken by me at a location convenient to the donor. Clinically suitable areas with appropriate facilities were used for all venepunctures.

2.3.1.3 The LRF Virus Centre Database

The LRF virus centre uses a computer database system to record details of all participants in studies (patients and healthy volunteers) and samples held. There are separate, linked databases for all participant information, events associated with any participant, diagnosis, samples and freezer location. This enables good research governance, and timeous and accurate retrieval of participant information and sample information. Participant names are not held on the database; instead each participant is allocated a unique "LRF patient number" which acts as an identifier on the system. All details described in the descriptions of the SHARE study and HLA & Outcome study (Chapter 5) were derived from records held on this database.

2.3.1.4 Description of Cohort

In total 172 individuals were recruited to the study. The recruitment target of 200 subjects in an HLA-unscreened population was not necessary as, through the more targeted recruitment of the platelet donors, the study achieved its recruitment targets in terms of number of donors of required HLA type (Table 2.4).

Table 2-4 Recruitment by HLA Class I genotype

HLA type	Target number to recruit	Number recruited
A*01:01/A*01:01 homozygotes	10	14
A*01:01/A*02:01 heterozygotes	15	30
A*02:01/A*02:01 homozygotes	15	18
A*02:01/A*x heterozygotes†	15	26

† (where x is any allele other than A*01:01 or A*02:01)

Of the 172 subjects recruited to the study, 64% (n = 108) volunteers were female and 36% (n = 61) male. Information on sex was unavailable for three volunteers. The median age of the cohort was 39 years (range 18-68 years). Twenty six individuals (15.1%) reported a previous history of glandular fever and nineteen individuals (11.0%) stated in the questionnaire that this had been confirmed by a blood test. In these individuals, the episode of IM was a median of 18.5 years prior to entry into the study (range 1-39 years). The median age of individuals at time of IM was 19 years (range 5-30 years). Forty-four individuals (25.6%) had been smokers at some point, fifteen of whom (8.7% of total cohort) were current smokers.

Deprivation category information, using the Carstairs 5–point score for deprivation, was available for 137 volunteers. First described by Carstairs and Morris in 1981 (Carstairs, 1981b; Carstairs, 1981a), Carstairs scores are a measure of access to societal resource, derived by combining selected variables taken from small area Census data, reflecting economic status of populations at a small geographical (postcode) level. The most recent update of the score, using 2001 census data (McLoone, 2004) was used. Four pieces of information (overcrowding, male unemployment, low social class and car ownership) were standardised and scored to define the Scottish postcodes into quintiles by deprivation (DEPCAT5). The proportion of individuals in each category, versus the expected Scotland-wide population proportion is given in Figure 2.1. As can

be seen, there is a slightly higher proportion of individuals in Carstairs index category 1 (least deprived), as might be expected from a recruitment strategy targeting University and NHS employees.

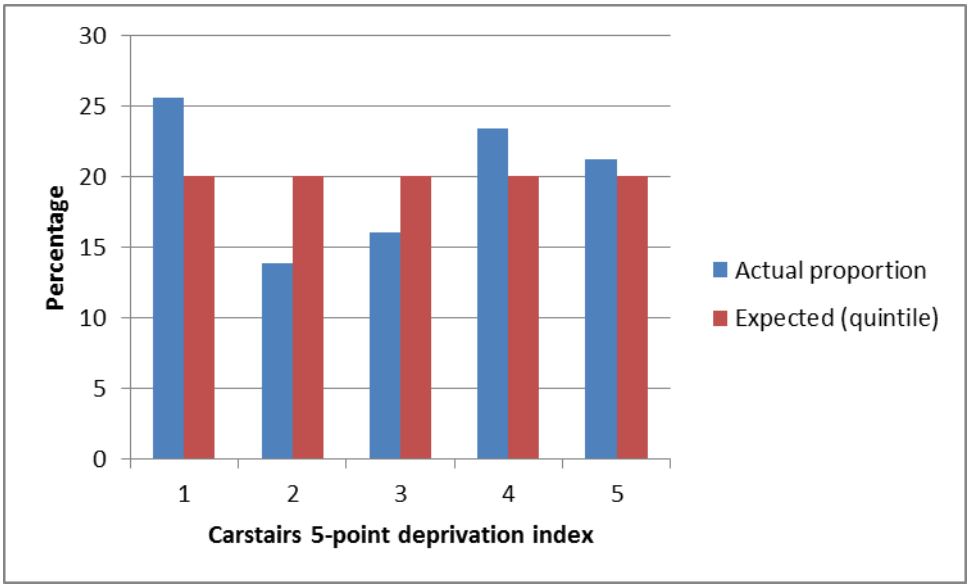


Figure 2-1 Carstairs deprivation index score of individuals in study
Shown versus expected proportions.

A number of possible biases as a result of the recruitment strategy were observed. Although not systematically recorded, we were aware of a small number of individuals who volunteered for this study because they had a relative with HL. As this study was selecting individuals by HLA type, and examining CTL response in healthy individuals, it was not felt that this would impact on the CTL responses assessed.

Previous IM, confirmed by a blood test, was reported by 11.8% of recruits. Data on overall population prevalence of IM infection are not available, as it is not a notifiable disease. Previous studies have reported an annual incidence of 45-50 cases per 100,000 population per year (Newell , 1957; Heath, Jr. *et al*, 1972)

and in a previous case-control study of IM and HL (Alexander *et al*, 2003) 4.7% of the healthy adult controls reported prior IM. That almost 12% of subjects in our study population reported previous IM is higher than would be expected. This potential bias may be important as it is known that individuals who have suffered previous IM are at an elevated risk of cHL for a number of years and, as discussed in the Introduction, it is suggested that CTL response may be important in determining this risk. Since this information was recorded systematically for all cases, prior IM was included as a covariate in some analyses of CTL response.

All adults recruited in to the study were healthy at time of recruitment. In addition, at time of recall for donation of the "large sample", donors were again asked to verbally confirm that they were in good health. This was important as any illness e.g. viral infection may impact on the results of the analyses. One donor contacted us during the study period to inform us that since donating they had been diagnosed as suffering from multiple sclerosis (MS) and may have been symptomatic at time of donation. MS is an inflammatory disease of the central nervous system characterised by demyelination. It is increasingly being recognised that EBV, and immune response to EBV may be important in disease pathogenesis (Cepok *et al*, 2005; Habek *et al*, 2008; Jilek *et al*, 2012; Levin *et al*, 2010); for this reason, samples from this donor were excluded from the study.

2.3.2 Peripheral Blood Sample Collection

Samples were collected by venepuncture into VACUETTE® tubes (Greiner Bio-One). For the initial "small" sample, whole blood samples were taken into a gel separation serum tube (5 ml) and into a tube containing ethylenediamine tetra-

acetic acid (EDTA) as an anti-coagulant (5 ml). For the "large" specimen, approximately 50 ml of whole blood was collected into 9 ml tubes containing EDTA as an anti-coagulant.

2.3.3 Sample Processing

2.3.3.1 Processing of initial "small samples"

Samples were allowed to equilibrate to RT. The time taken from sample collection to the beginning of sample processing was recorded. All blood centrifugation steps were performed using a Heraeus centrifuge, with brake on, unless stated otherwise. Serum was obtained by centrifuging the gel-separated sample at 1500 x gravity (g) for 15 min and 500 μl aliquots were subsequently dispensed into 1.5 ml sterile screw-cap polypropylene microcentifuge tubes (Elkay). Aliquots were labelled with LRF patient number, sample number and date, and stored at -70^{o}C. Samples were not heat inactivated.

Plasma was obtained by centrifuging the anti-coagulated sample at 1500 g for 15 min. Aliquots of 500 μl plasma were dispensed, labelled and stored as above.

The buffy coat, visible after centrifugation, was removed by Pastette (Fisher Scientific Inc.) into a 15 ml conical polypropylene tube (Falcon, B.D. Biosciences). DNA was extracted from the buffy coat, as described below.

2.3.3.2 Processing of 50 ml "large" samples

Peripheral blood mononuclear cells (PBMCs) were obtained by the separation of anti-coagulated whole blood using the LeucoSep centrifuge tube system (Greiner Bio-one). LeucoSep tubes were prepared by adding 15 ml of Lymphoprep density medium (Greiner Bio-one) at RT to each of four 50 ml LeucoSep tubes. These

were centrifuged at 1000 g for 30 seconds (s) to drive the medium through the membrane. Samples of approximately 50 ml anti-coagulated whole blood were allowed to equilibrate to RT. The blood was divided between 4 x 50 ml conical polypropylene tubes (Falcon, BD Biosciences) and the volume in each was made up to 30 ml with Hank's Balanced Salt Solution (HBSS) (Gibco, Invitrogen). These were then poured into each of the four prepared LeucoSep tubes which were then centrifuged at 800 g for 15 min with the brake off (0). The PBMC layer was removed using a Pastette (Fisher Scientific Inc.) and transferred to a 50 ml conical polypropylene tube (Falcon, BD Biosciences). The volume was made up to 20 ml with minimacs buffer (MMB) if required. Aliquots (2 x 100 μl) were removed for flow cytometric analysis. The PBMCs were then used in downstream experiments, or taken through to B cell separation.

2.3.4 Cell counting and analysis of cell viability

Cells were counted at a 1 in 5 dilution (50 μl Trypan blue 0.4% (Sigma), 30 μl MMB and 20 μl cells) using a haemocytometer (Neubauer 1/400 mm^2, Hawksley) and a hand tally counter (ENM). Only viable cells, i.e. those not staining with Trypan blue, were counted. For all cell counts, 4 sets of 16 squares on the cytometer were counted, and the mean taken to give number of cells x 10^4 per ml. This was adjusted for the dilution factor.

2.3.5 DNA extraction

DNA extraction was performed using the Nucleon DNA extraction method (GE Healthcare). Cells were lysed by the addition of Reagent A to the buffy coat fraction at 4 x the volume of the buffy coat (approximately 4 ml). Tube contents were mixed on a rotary mixer (Beckman Coulter) for 5 min, and then centrifuged

at 1300 g for 5 min. The supernatant was removed carefully using a Pastette (Fisher Scientific Inc.), leaving a soft pellet. This pellet was re-suspended in 2 ml buffer B and briefly vortexed (HATi Rotamixer, HTZ), as per kit instructions. Sodium perchlorate (500 μl, supplied as part of the Nucleon kit) was added and the contents of the tube mixed by inversion seven times, to facilitate protein removal. Chloroform (2 ml, Sigma) was added to this tube and the contents of the tube again mixed by inversion seven times. Resin beads (300 μl, supplied as part of the Nucleon kit) were added and, without further mixing, tubes were centrifuged at 1300 g for 3 min. The supernatant was removed into 5 ml cold 100% analar-grade ethanol (Fisher Scientific Inc.). The DNA was precipitated by gentle inversion, spooled out on the tip of a pipette tip and placed in a sterile screw cap tube. Excess ethanol was aspirated. The DNA pellet was left to air-dry for 10- 30 min, 100 μl tris-EDTA (TE) (Microzone) was added and the sample left overnight to re-suspend, in a water bath (Grant) at 37^{o}C. The DNA was then stored at 4^{o}C before quantification and storage at -70^{o}C.

2.3.6 DNA quantification

DNA was quantified using the fluorimetry-based Qubit system (Invitrogen) with the Quant-it dsDNA high-sensitivity assay kit for double-stranded DNA (Invitrogen). Following quantification as per kit instructions, DNA was diluted with TE (Microzone) to an appropriate concentration for storage at -70^{o}C. DNA samples (1 μg) were sent to Dr. Little for HLA typing (detailed below).

2.3.7 HLA Typing

HLA typing was performed by Dr. Ann-Margaret Little, initially at The Anthony Nolan Trust Histocompatibility Laboratories (Fleet Road, Hampstead, London

NW3 2QG) and subsequently at NHS Greater Glasgow & Clyde (Tissue Typing and Immunology laboratory, Paul O'Gorman Building, Gartnavel Hospital). Molecular typing of class I HLA genes was performed to an intermediate resolution i.e. four-digit typing defining variations in AA sequence which may affect peptide binding affinity (Nunes *et al*, 2011) (See also Section 1.7.1.3). The method employs PCR using allele-specific primers (Gen-probe) and subsequent bead-based sequence specific oligonucleotide (SSO) assay (Luminex). Assignment of the most likely allele from the allele strings generated was performed manually by Dr. Little using Quicktype for Lifematch software (Gen-probe).

2.3.8 B cell Separation

PBMCs were re-suspended in residual buffer and washed by filling the 50 ml tube with MMB and then centrifuging at 300 g for 10 min. The supernatant was removed by pouring off into a clean 50 ml tube, care being taken not to disrupt the cell pellet. The supernatant was further centrifuged at 500 g for 5 min and the supernatant removed as before. Cell pellets were re-suspended in residual buffer, diluted in 10 ml MMB and then combined in the first tube. The second tube was rinsed with 5 ml MMB, which was then added to the cell suspension (total volume 25 ml). An aliquot of 100 µl was removed for flow cytometry and a cell count performed as described. The cell suspension was divided between five or six 5 ml round-bottomed, polystyrene tubes (Falcon #2054, BD Biosciences) and centrifuged at 300 g for 10 min. Following removal of the supernatant, cells were re-suspended in MMB to a final concentration of 1 x 10^7 cells per 60 µl. Immunoglobulin constant region (FCR) blocking agent (Miltenyi Biotec) (20 µl per 1 x 10^7 cells) and CD20 microbeads (Miltenyi Biotec) (20 µl per 1 x 10^7 cells) were added to the cell suspension. Following mixing, reactions were incubated at 4°C

for 15 min, with periodic re-mixing. After incubation, the cells were washed and the pellets re-suspended in 300 μl MMB. One MS (medium capacity) cell-separation column and one LS (large capacity) cell-separation column (Miltenyi Biotec) were prepared by placing in MiniMacs Separation Magnet Units (Miltenyi Biotec), inserting a 50 μm sterile cup Filcon filter (BD Biosciences) and flushing with 500 μl (MS) or 3 ml (LS) MMB. The prepared cells were placed on the Filcon filter in the LS column, without introducing bubbles, and allowed to flow through. The column was washed three times with 3 ml MMB. The flow-through was collected and retained ("negative fraction 1"). The LS column was removed from the magnet; 5 ml MMB were added and plunged through to collect the CD20-positive fraction ("positive fraction 1"). Positive fraction 1 was pelleted at 300 g for 7 min, re-suspended in 500 μl MMB and pipetted on to the prepared MS column. The column was washed three times with 500 μl MMB and the flow-through retained ("negative fraction 2".) The MS column was removed from the magnet; 1 ml of MMB was added and plunged through to collect the CD20-positive fraction ("positive fraction 2").

The "positive fraction 2" cells (CD20 positive B lymphocytes) were counted as described. An aliquot (20 μl) was removed for flow cytometry and the remaining cells were then used in EBV-infected cell frequency experiments or frozen viably.

The negative fractions 1 and 2 (B cell deplete PBMC (BD-PBMC) were combined and a cell count performed as described. An aliquot (100 μl) was removed for flow cytometry. The cells were then used fresh in ELISPOT assays (for optimisation experiments, see Section 2.8.3) or frozen viably for use at a later date.

2.4 Tissue Culture

2.4.1 Viable Freezing of PBMCs

PBMCs or BD-PBMCs obtained as above were frozen in aliquots of 1 or 2 x 10^7 cells. Following counting as described in Section 2.3.4, the cell suspension was centrifuged at 300 g for 5 min, and re-suspended at 1 or 2 x 10^7 / ml in freezing medium. Cells were frozen in volumes of 1 ml in NUNC™ 1.8 ml cryotubes (Fisher Scientific Inc.), hereafter referred to as "cryovials". Cells were taken to -70°C using the NALGENE™ (Fisher Scientific Inc.) system for controlled rate freezing. This commercial system employs a container, the base of which is filled with isopropyl alcohol (Fisher Scientific Inc.). The rate of freezing achieved is close to the ideal of -1°C / min, and allows the preservation of cell viability. The following day, cells were transferred to long-term storage in liquid nitrogen tanks at -200°C.

2.4.2 Viable Freezing of Cell Lines

All cell lines used or created in the study, detailed below and including LCLs, were frozen using the same method and conditions as for freezing of PBMC.

2.4.3 Recovery of viably frozen cells

The required aliquot of cells was identified using the LRF Virus Centre database – (Section 2.3.1.3). Using the usual safety procedures involved with liquid nitrogen, the cryovial was removed from the liquid nitrogen tank into a metal flask. The cryovial was defrosted slowly in water at 37°C. The defrosted vial was submerged briefly in 70% alcohol to sterilise the exterior surface before opening. The cells were then added drop wise to 10 ml 10% complete culture medium (CCM) at 37°C, washed by spinning at 300 g for 5 min and re-suspended in fresh

CCM, before counting as described. If required, cells were centrifuged again and re-suspended at the required concentration for use.

2.4.4 Cell lines

The human cell line 721.221 is an EBV-transformed LCL which does not express MHC Class I, i.e. it is negative for HLA-A, -B and –C. Professor John Trowsdale and Dr. Louise Boyle, Cambridge Institute for Medical Research, University of Cambridge, provided us with viably frozen 721.221 cells. In addition to the 721.221 parental line, cells transduced to express only HLA-A*01:01 or HLA-A*02:01 were provided. Transductions and checks on transduction efficiency were performed by Dr. Boyle. On receipt, cells were expanded and master stocks stored. Subsequent working stocks were derived from these cells.

The B95-8 cell line is a primate lymphocyte cell line derived from the peripheral blood lymphocytes of a cotton-top tamarin. These cells release high titres of EBV, providing a source of infectious virus. The B95-8 cell line used in this work was gifted by Prof. Alan Rickinson of the School of Cancer Sciences, College of Medical and Dental Sciences, University of Birmingham.

The P3HR1 cell line used in the production of slides for EBV VCA IgG serology was provided by Prof. Dorothy H. Crawford of the Centre for Infectious Diseases, University of Edinburgh.

2.4.5 Generation of infectious EBV virus

B95-8 cell cultures were used as a source of EBV for generation of LCLs. EBV-containing culture supernatant, rather than ultra-filtered pelleted virus, was used. The B95-8 cells described above were cultured in 10% CCM in Corning 250

cm^3 culture flasks (SLS). The cultures were expanded by splitting 1:2 twice weekly. When the culture reached 6 flasks, each containing 220 ml, an equal volume of medium was added, and Parafilm M (Sigma-Aldrich Company Ltd.) applied to the flask lid to deprive the cells of gas, induce cellular stress and cell death. After 3-4 days, a visual check was performed to ensure that the cells were dead or dying. The culture was decanted into 50 ml tubes and centrifuged at 300 g for 5 min at 4°C. The supernatants were retained and filtered through a 0.8 µm filter using a Costar filter bottle (Fisher Scientific Inc.) and vacuum pump (Millipore). Aliquots of 1 ml were frozen in 1.5 ml screw cap tubes. The EBV obtained was used only in the generation of LCLs and, as efficient transformation was achieved, titration of virus stocks was not performed. To minimise risk of contamination, all EBV-infected cell cultures were handled in a MSC reserved for working with cell lines, and only after all other non-viral work was complete for the day. To avoid cross-contamination, a separate incubator was used for all viral work, and dedicated pipettes were used.

2.4.6 Generation of donor LCLs

Donor PBMCs were selected and defrosted as per Section 2.4.3. Each infection required 2 x 10^6 cells. Cells were pelleted at 400 g for 10 min in a 15 ml tube. The pellet was re-suspended in 500 µl B95-8 viral supernatant. T cell immune suppression was achieved by the addition of cyclosporin A (Sigma-Aldrich, UK). Master stocks of cyclosporin A (1mg/ml) in absolute alcohol (Fisher Scientific Inc.) were stored at -20°C; the working stock (100 µg/ml) was made by dilution in 20% CCM, and kept at 4°C. Ten microlitres of this were added to the cells, and the total volume was made up to 1 ml (final concentration 1 µg/ml cyclosporin A). The cell suspension was transferred to one well of a 48-well flat-bottom plate (Multiwell™, BD Biosciences). The plate was incubated at 37°C in

humidified 5% carbon dioxide (CO_2). The cells were fed weekly by removal and addition of 300-500 µl 20% CCM and, when proliferating enlarging clumps were observed (usually at 2-4 weeks), cultures were split 1 in 2 with 20% CCM. Proliferating cells were fed twice weekly. Cells were transferred to a Corning 25 cm^3 culture flask (SLS) once cultures occupied 4-6 wells of the plate. When established, master stocks of each individual LCL were frozen viably, and working cultures continued for use in downstream experiments.

2.5 Flow Cytometric Analysis

All antibodies were purchased from Beckman Coulter. Cells (in volumes described in Section 2.3.3) were incubated with 10 µl antibody for 10 min (unless stated otherwise) at RT. CalLyse (100 µl, Invitrogen) was added to each tube, mixed well and incubated for a further 10 min at RT. Distilled water (2 – 3 ml) was added to each tube, and mixed well by vortexing (HATi Rotamixer, HTZ). The cells were centrifuged at 300 g for 5 min. The supernatant was removed and cells were resuspended in 500 µl of 1% paraformaldehyde (Sigma) to fix the cells. Samples were analysed using a Beckman FC500 flow cytometer. Five thousand events per sample were counted and analysed. Isotype controls were performed for every assay.

2.5.1 Flow cytometry method for B cell depletion protocol

The standard panel described below (Table 2.5) was used to analyse each "large" sample. An aliquot of 20 µl of cells was taken from the CD20 positive fraction into a 5 ml tube and 100 µl MMB added. Aliquots of 100 µl were taken from PBMC pre-fractionation and CD20 negative fractions respectively to 5 ml tubes, and 500 µl added to each. Cells were then aliquoted as per Table 2.5 (100 µl cells per tube), and 10 µl of each antibody added.

Table 2-5 Flow cytometric analysis of B cell separations – standard antibody panel

Cell Fraction	Antibodies
PBMC pre-fractionation	CD3FITC/CD20PE CD3FITC/HLA-DR PE CD8FITC/CD4PE/CD3ECD
CD20 negative fraction	CD3FITC/CD20PE CD3FITC/HLA-DR PE CD8FITC/CD4PE/CD3ECD
CD 20 positive fraction	CD3FITC/CD20PE

ECD, electron coupled dye; FITC, fluorescin isothiocyanate; PE, phycoerthyrin.

2.5.2 Flow cytometry antibodies used in specific experiments

In addition to the standard panel described above, additional antibodies were used in specific experiments. The antibodies are listed in Table 2.6 and the methods for these experiments described in Sections 3.2.5 and 4.2.4.5.

Table 2-6 Flow cytometry antibodies used in specific experiments

Cell Fraction	Antibodies
Optimisation of B-Deplete PBMC	CD3FITC/HLA-DR PE CD3FITC/CD20PE CD8FITC/CD4PE/CD3ECD CD16FITC/56PE/3ECD CD15FITC/45PE/33ECD
CTL Degranulation	CD107PE

ECD, electron coupled dye; FITC, fluorescin isothiocyanate; PE, phycoerthyrin.

2.6 Viral Serology

2.6.1 Serology for EBV

EBV serotyping (VCA IgG)was performed using an immunofluorescence method (Henle *et al*, 1974). Known positive and negative sera were used as controls. Briefly, 100-250 µl serum were heat inactivated at 56^{o}C for 20 min. Sera samples at a 1:8 dilution in PBS (20 µl) were placed on prepared multi-well slides on which P3HR1 cells (Section 2.4.4) were fixed. Slides were incubated for 90 min at RT in a moist chamber then washed twice with PBS for 5 min in a slide holder and drained on a tissue. The fluorescin isothiocyanate (FITC)-conjugated, anti-

human IgG secondary antibody (DAKO) was prepared as a 1:50 dilution in PBS, and 15 µl added to each spot on the slide. This was incubated for 45 min at RT in a moist chamber. Slides were washed as before and mounted with PBS:Glycerol (50:50) (Fisher) and a coverslip. Slides were read under a fluorescent microscope at x 25 objective; positive cells displayed a strong fluorescent green staining. For the purposes of this study, titres were not recorded, and results were recorded as either positive or negative.

2.6.2 Serology for CMV

CMV serotyping was performed at the West of Scotland Specialist Virology Laboratory, Gartnavel General Hospital (collaborators Dr. William F. Carman and Dr. Jane McOwan). Serotyping used the CMV IgG chemiluminescent microparticle immunoassay (Abbot Diagnostics) on the Abbot platform. Samples (100-250 µl serum) were prepared by heat inactivation at 56^{o}C for 20 min, prior to analysis. The automated platform loaded samples and performed the immunoassay. Both low- and high-level positive controls and negative controls of CMV-specific IgG were provided with the assay. Results were recorded as either positive or negative.

2.7 Peptides

2.7.1 EBV peptides

A list of peptides derived from EBV viral proteins and known to be presented by particular HLA specificities was synthesised from the published literature (see review (Hislop *et al*, 2007) and references in Table 2.7). The AA sequences of these peptides, their HLA-restriction, and the protein from which they are derived are listed in Table 2.7. The AA sequences of EBV proteins were taken

from the B95-8 reference sequence (Human herpesvirus 4) (National Center for Biotechnology Information (NCBI), 2008). The one-letter abbreviation for AA is per the International Union of Biochemistry and Molecular Biology guidelines (IUPAC-IUB Joint Commission, 1984) (see Appendix 3.)

2.7.2 CMV and Influenza peptides and the CEF pool

Peptides derived from CMV and influenza virus proteins, known to be presented by particular HLA specificities, were used as controls in the experiments (Kern *et al*, 2000; Elkington *et al*, 2003; Collins *et al*, 1999; DiBrino *et al*, 1993; Suhrbier *et al*, 1993). The AA sequences of these peptides, their HLA-restriction, and the protein from which they are derived are listed in Table 2.8. Where pools of peptides were used, their compositions are detailed in the relevant results Chapters.

The CEF (CMV, EBV, 'flu) pool is a combination of 23 peptides derived from proteins expressed by CMV, influenza and EBV. These peptides are restricted through a range of HLA-specificities. This broad range of specificities covers the most common human HLA-types and three common viruses, and thus most individuals would be expected to raise a CTL response to at least one of the constituent peptides in this combination. The pool was first described by HIV vaccine researchers (Currier *et al*, 2002) and is now recognised by the international committee for the standardisation of ELISPOTS (Janetzki *et al*, 2005) as a gold standard, positive control for quality control (QC) where ELISPOTs are used in clinical e.g. vaccine studies. The AA acid sequences of these peptides, their HLA-restriction, and the protein from which they are derived are listed in Table 2.9.

Table 2-7 EBV peptides used in the study

AA sequence	HLA restriction	Protein	Cycle	Abbreviation	Reference
FMVFLQTHI	A2	EBNA1	Latent	FMV	(Stuber et al, 1995)
HPVGEADYFEY	B3501	EBNA1	Latent	HPV	(Blake et al, 1997)
RPQKRPSCI	B7	EBNA1	Latent	RPQK	(Blake et al, 2000)
IPQCRLTPL	B7	EBNA1	Latent	IPQ	(Blake et al, 2000)
DTPLIPLTIF	A2/B51	EBNA2	Latent	DTP	(Schmidt et al, 1991)
SVRDRLARL	A2	EBNA3A	Latent	SVR	(Burrows et al, 1994)
RLRAEAQVK	A3	EBNA3A	Latent	RLR	(Hill et al, 1995)
YPLHEQHGM	B3501	EBNA3A	Latent	YPL	(Burrows et al, 1994)
RPPIFIRRL	B7	EBNA3A	Latent	RPP	(Hill et al, 1995)
VPAPAGPIV	B7	EBNA3A	Latent	VPA	(Rickinson & Moss, 1997)
QAKWRLQTL	B8	EBNA3A	Latent	QAK	(Burrows et al, 1994)
FLRGRAYGL	B8	EBNA3A	Latent	FLR	(Burrows et al, 1990b)
IVTDFSVIK	A11	EBNA3B	Latent	IVT	Gavioli (Gavioli et al, 1993)
LLDFVRFMGV	A2.01	EBNA3C	Latent	LLD	(Kerr et al, 1996)
RRIYDLIEL	B27	EBNA3C	Latent	RRI	(Brooks et al, 1993)
EENLLDFVRF	B44	EBNA3C	Latent	EEN	(Burrows et al, 1990a)
QPRAPIRPI	B7	EBNA3C	Latent	QPR	(Hill et al, 1995)
SLREWLLRI	A2	EBNA-LP	Latent	SLR	(Lee *et al*, 2000)
YLLEMLWRL	A2	LMP1	Latent	YLL	(Khanna et al, 1998)
YLQQNWWTL	A2	LMP1	Latent	YLQ	(Khanna et al, 1998)
TLLVDLLWL	A2	LMP1	Latent	TLL	(Khanna et al, 1998)
LLLIALWNL	A2	LMP1	Latent	LLL	(Duraiswamy et al, 2003)
LLVDLLWLL	A2	LMP1	Latent	LLVD	(Duraiswamy et al, 2003)
TVCGGIMFL	A2	LMP2	Latent	TVC	(Straathof *et al*, 2005b)
LIVDAVLQL	A2	LMP2	Latent	LIV	(Straathof *et al*, 2005b)
GLGTLGAAI	A2	LMP2	Latent	GLG	(Meij et al, 2002)
FLYALALLL	A2	LMP2	Latent	FLY	(Lautscham et al, 2003)
LLSAWILTA	A2	LMP2	Latent	LLS	Lee (Lee *et al*, 2000)
LTAGFLIFL	A2	LMP2	Latent	LTA	(Lee et al, 1996)
LLWTLVVLL	A2.01	LMP2	Latent	LLWT	(Lee et al, 1996)
CLGGLLTMV	A2.01	LMP2	Latent	CLG	(Lee et al, 1993)
FTASVSTVV	A68	LMP2	Latent	FTA	(Straathof *et al*, 2005b)
MGSLEMVPM	B3501	LMP2	Latent	MGS	(Straathof *et al*, 2005b)
LLWAARPRL	A2	BARF0	Lytic	LLWA	(Kienzle et al, 1998)
GLCTLVAML	A2.01	BMLF1	Lytic	GLC	(Scotet et al, 1996)
TLDYKPLSV	A2.01	BMRF1	Lytic	TLD	(Hislop et al, 2002)
RPQGGSRPEFVKL	B7	BMRF1	Lytic	RPQG	(Pudney et al, 2005)
LVSDYCNVLNKEFT	B18	BRLF1	Lytic	LVSD	(Pepperl et al, 1998)
ATIGTAMYK	A11	BRLF1	Lytic	ATI	(Pepperl et al, 1998)
RALIKTLPRASYSSH	A2	BRLF1	Lytic	RAL	(Pepperl et al, 1998)
YVLDHLIVV	A2.01	BRLF1	Lytic	YVL	(Saulquin et al, 2000)
DYCNVLNKEF	A24	BRLF1	Lytic	DYC	(Pepperl et al, 1998)
RVRAYTYSK	A3	BRLF1	Lytic	RVR	(Pudney et al, 2005)
EPLPQGQLTAY	B3501	BZLF1	Lytic	EPL	(Pepperl et al, 1998)
APENAYQAY	B3501	BZLF1	Lytic	APE	(Redchenko & Rickinson, 1999)
RAKFKQLL	B8	BZLF1	Lytic	RAK	(Bogedain et al, 1995)
ILIYNGWYA	A2	GP110	Lytic	ILI	(Khanna & Burrows, 2000)
VLTLLLLLV	A2	GP350	Lytic	VLT	(Bharadwaj et al, 2001)
VLQWASLAV	A2	GP350	Lytic	VLQ	(Khanna *et al*, 1999b)
QLTPHTKAV	A2	GP350	Lytic	QLT	(Khanna *et al*, 1999b)
LIPETVPYI	A2	GP350	Lytic	LIP	(Khanna *et al*, 1999b)
LMIIPLINV	A2	GP85	Lytic	LMI	(Khanna *et al*, 1999b)
TLFIGSHVV	A2	GP85	Lytic	TLF	(Khanna *et al*, 1999b)
SLVIVTTFV	A2	GP85	Lytic	SLV	(Khanna *et al*, 1999b)

Table 2-8 CMV and influenza peptides used in the project

Epitope AA sequence	HLA restriction	Protein	Virus	ECF pool	Abbreviation
CVETMCNEY	A1	IE1	CMV		CVE
DEEEAIVAY	A1	IE1	CMV		DEE
ELKRKMIYM	B8	IE1	CMV		ELK
ELRRKMMYM	B8	IE1	CMV		ELRR
QIKVRVDMV	B8	IE1	CMV		QIK
VLEETSVML	A2	IE1	CMV		VLA
IPSINVHHY	B35	pp65	CMV		IPS
NLVPMVATV	A2	pp65	CMV	*	NLV
QEFFWDANDIYRIFA	B44	pp65	CMV	*	QEF
RKTPRVTGGGAMAGA	B7	pp65	CMV	*	RKT
YSEHPTFTSQY	A0101	pp65	CMV		YSE
GILGFVFTL	A2	Matrix 1	Influenza A	*	GIL
CTELKLSDY	A1	NP	Influenza A	*	CTE
ELRSRYWAI	B8	NP	Influenza A	*	ELRS
ILRGSVAHK	A3	NP	Influenza A	*	ILR
KTGGPIYKR	A68	NP	Influenza A	*	KTG
SYRWAIRTR	B27	NP	Influenza A	*	SYR
VSDGGPNLY	A1	PB1	Influenza A	*	VSD

Table 2-9 Composition of the CEF control peptide pool

Epitope amino acid sequence	HLA restriction	Protein	Virus	Abbreviation
NLVPMVATV	A2	pp65	CMV	NLV
QEFFWDANDIYRIFA	B44	pp65	CMV	QEF
RKTPRVTGGGAMAGA	B7	pp65	CMV	RKT
CTELKLSDY	A1	NP	Influenza A	CTE
ELRSRYWAI	B8	NP	Influenza A	ELRS
GILGFVFTL	A2	Matrix 1	Influenza A	GIL
ILRGSVAHK	A3	NP	Influenza A	ILR
KTGGPIYKR	A68	NP	Influenza A	KTG
SYRWAIRTR	B27	NP	Influenza A	SYR
VSDGGPNLY	A1	PB1	Influenza A	VSD
EENLLDFVRF	B44	EBNA3C	EBV Latent	EEN
FLRGRAYGL	B8	EBNA3A	EBV Latent	FLR
IVTDFSVIK	A11	EBNA3B	EBV Latent	IVT
QAKWRLQTL	B8	EBNA3A	EBV Latent	QAK
RLRAEAQVK	A3	EBNA3A	EBV Latent	RLR
RPPIFIRRL	B7	EBNA3A	EBV Latent	RPP
RRIYDLIEL	B27	EBNA3C	EBV Latent	RRI
YPLHEQHGM	B3501	EBNA3A	EBV Latent	YPL
ATIGTAMYK	A11	BRLF1	EBV Lytic	ATI
DYCNVLNKEF	A24	BRLF1	EBV Lytic	DYC
GLCTLVAML	A2.01	BMLF1	EBV Lytic	GLC
RAKFKQLL	B8	BZLF1	EBV Lytic	RAK
RVRAYTYSK	A3	BRLF1	EBV Lytic	RVR

The individual peptides were synthesised as described in Section 2.7.3, and pooled at working stock concentration as described in Section 2.7.4.

The prediction methods used to derive putative HLA-A*01:01-restricted EBV peptides, and the peptides sequences generated are described in Chapter 4.

2.7.3 Source of synthetic peptides

All peptides used in the study were synthetic peptides generated by Alta Bioscience (Birmingham, UK). Solid phase synthesis (EpiScan) was used to generate peptides of the specified sequences. Peptides were synthesised with C-terminal free acids as this is the natural state of the peptide following digestion of the protein, prior to assembly with the HLA molecule. Peptides were supplied as 2 mg dry product in individual tubes, with a specification sheet detailing purity and quantity. QC of purity was performed by high-performance liquid chromatography (HPLC), and these data were supplied with the peptides.

2.7.4 Preparation of peptides

Due to the relatively small numbers of AAs in the short peptides used in this study, the peptides varied in their polarity, i.e. some were hydrophobic, and some hydrophilic. In order to ensure uniform solubility of peptides, it is recommended that the initial reconstitution of all peptides is in dimethyl sulfoxide (DMSO). To ensure maximum purity of peptides was preserved, with minimal contamination, HPLC-grade DMSO (Fisher Scientific Inc.) was used throughout. Aseptic technique was used in reconstituting all peptides. The lyophilised peptides were dissolved in DMSO to a master concentration of 10 mg/ml and stored at -20^{o}C. Working stocks of peptide (100 µg/ml in 1 x PBS) were stored at -20^{o}C. As peptides degrade with multiple freeze-thaws, aliquoted

volumes were those required for single experiments. For large sequences of experiments using the same peptides, the peptides were stored at working stock in 96-well sterile single-use round-bottom plates (Nunclon™, Sigma) with the peptides in the positions for their final use. These plates were sealed with PCR film (Thermo Scientific) to minimise evaporation.

Peptides were used at a final concentration of 10 µg/ml (once cells in CCM were added) unless stated otherwise. Where pools of peptide were used, these were made from master stocks of peptide, diluted with 1 x PBS to a concentration for each individual peptide of 100 µg/ml.

2.7.5 Optimisations with synthetic peptides

2.7.5.1 Quantitation of peptides in solution

Short-chain peptides vary in their solubility. Even when DMSO was used to reconstitute lyophilised peptides, differences were observed in the speed and ease of dissolving the solid peptides in solution. For this reason, and because responses between peptides were to be compared, efforts were made to measure the concentration of peptides in master stock solutions. The expected concentration of the peptides in master stock solutions was 10 mg/ml based on 2 mg dry peptide reconstituted in 200 µl DMSO.

Spectrophotometric absorption at 280 nm using a Nanodrop (ThermoFisher) was performed. The absorption curves at 280 nm were different for every peptide measured. The spectrophotometric absorption at 280 nm relies on the presence of aromatic AAs in proteins. Due to the short length of the peptides used, not every peptide contained aromatic AAs, and even small differences in the number of aromatic AAs made a large difference to the absorption and thus, the derived

concentration. The Nanodrop was therefore not a suitable technique for assessing peptide concentration in solution.

Quant-iT™ technology (Invitrogen) is a fluorescent dye-based assay employing a detergent/dye based preparation which binds to the hydrophobic regions and the detergent coating on a protein. When actively bound, fluorescence is enhanced, and can be measured using the Q-bit™ fluorometer. As before, there was wide variability in peptide measurement, with strikingly low levels recorded. After discussion with the company's technical service staff, it became apparent that the presence of hydrophobic pockets in the structure, and not merely hydrophobic AAs, are required to detect the protein. As the peptides are generally too short to form such pockets, and vary in their content of hydrophobic AAs, this was not a suitable technique for assessing peptide concentration in solution.

Lastly, a Biuret assay was employed. This gold-standard assay of protein concentration detects the presence of peptide bonds, using a chemical technique whereby copper ions bind to the nitrogen of the peptide bonds in an alkaline solution. Results are compared against a standard curve generated from replicate dilutions of bovine serum albumin of known concentrations. This was felt to be a suitable technique due to the ubiquitous presence of peptide bonds, even in short-chain peptides. Where the assay was successful, concentration of the peptide was measured at 3.9-14 mg/ml. However, in more than half of the peptides tested (31 of 56), cloudiness was observed in the solution, rendering it unsuitable for analysis. The likely reason for this is that hydrophobic peptides can produce a cloudy solution when added to the biuret reagent, and this gives a

falsely high reading. The Biuret method was therefore not a suitable technique for assessing peptide concentration.

In order to resolve this issue, advice from leaders in the peptide and T cell immunology fields was sought. Although most papers using peptides in ELISPOT techniques refer to protein concentration, little is mentioned of the methods used for assessing the quantification of peptides in solution. Advice was sought from Dr. John E. Fox, Director of Alta Bioscience (the company from which we purchased the peptides). The only reliable way of quantifying peptide is to accurately weigh the dry peptide at synthesis using matrix-assisted laser desorption and isolation (MALDI), and HPLC. Whilst this is done for single peptides in, for example, pharmaceutical use, it is too expensive and impractical to use for large panels of peptides as in this study. Experts in the ELISPOT field were consulted: Dr. Josephine Cox, Maryland, USA International AIDS vaccine Initiative & committee for the international standardisation of ELISPOTS; Dr. Graham Taylor, Birmingham, UK and lead author on a number of papers using ELISPOT to assess vaccine response, and Dr. Maher Gandhi, Queensland Institute of Medical Research and lead author on a number of papers using ELISPOT to assess EBV responses. All felt that peptides cannot be accurately quantified in solution using standard biochemical techniques. They agreed that for clinical trials work, quantification of dry peptide by the manufacturer as described is desirable, although still does not provide information on concentration in solution. However there was consensus that at the concentrations used in standard ELISPOT assays (1-10 μg/ml), peptides were in excess, and that accurate quantification of peptide concentration in solution was not required, and was not practiced by the majority of laboratories using ELISPOT outside the context of a clinical trial. A concentration of 10 mg peptide

per ml in master stock is assumed from this point, based on the assumption of full solubility of the supplied dry peptide in DMSO.

2.7.5.2 Optimisation of peptide concentration for use in ELISPOTs

To assess whether the peptides in solution were indeed at excess, a dose-response experiment was performed, in which donors known to respond to particular peptides were exposed to these peptides at varying concentration. The results from three representative donors are shown in Figures 2.2, 2.3 and 2.4.

In these experiments, a consistent response was detected only at or above 5 µg/ml. At this concentration, there was a clear discrimination between peptides driving a detectable response and those for which no response was detectable.

In general, responses increased with increasing peptide concentration, suggesting that peptides present in the assay were not “in excess”. Some peptide responses (e.g. to the B*07:01-restricted CMV peptide RKT) in some donors were maximal at 10 µg/ml, whereas responses to other peptides increased at higher concentrations. However, it was apparent that the ELISPOT assay is exquisitely sensitive, and was capable of detecting responses to immunodominant peptides in some donors at peptide concentrations as low as 0.625 µg/ml. As maximal sensitivity was important, in addition to the ability to compare responses, a concentration of 10 µg/ml was selected for use in subsequent assays. This was a good compromise between maximal response and minimal sensitivity.

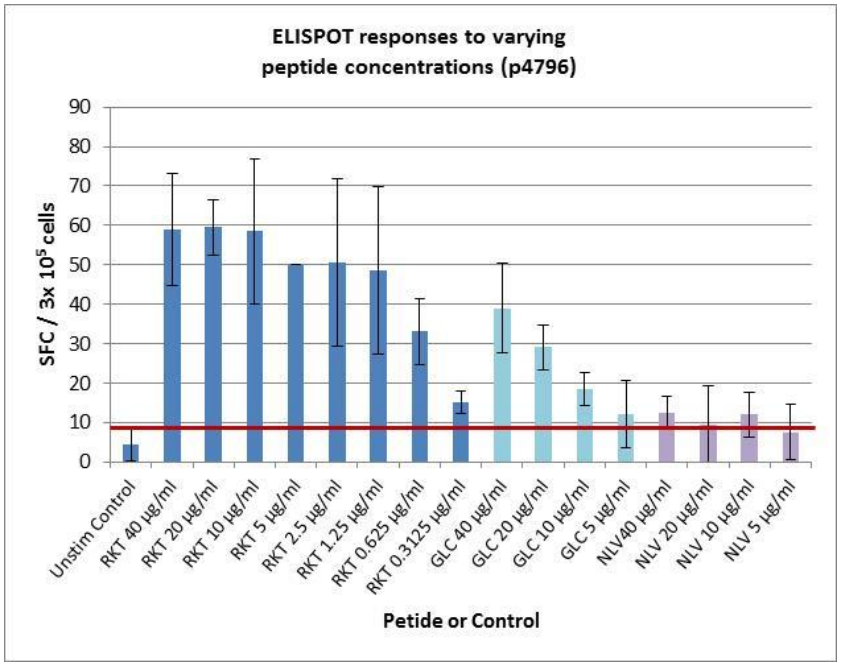


Figure 2-2 Effect of varying peptide concentration, donor p4796

PBMCs from donor p4796 (HLA-A*02:01 & B*07:01 heterozygote) exposed to three peptides: (HLA-B*07:01-restricted CMV peptide RKT, A*02:01-restricted EBV peptide GLC and A*02:01-restricted CMV peptide NLV) at 0.3125 – 40 µg/ml. Results are expressed as the mean of duplicates in spot-forming cells (SFC) per 3 x 10^5 cells; error bars demonstrate two standard deviations. The threshold of detection of a positive response (red line) is set at the mean of the highest negative control, plus two standard deviations.

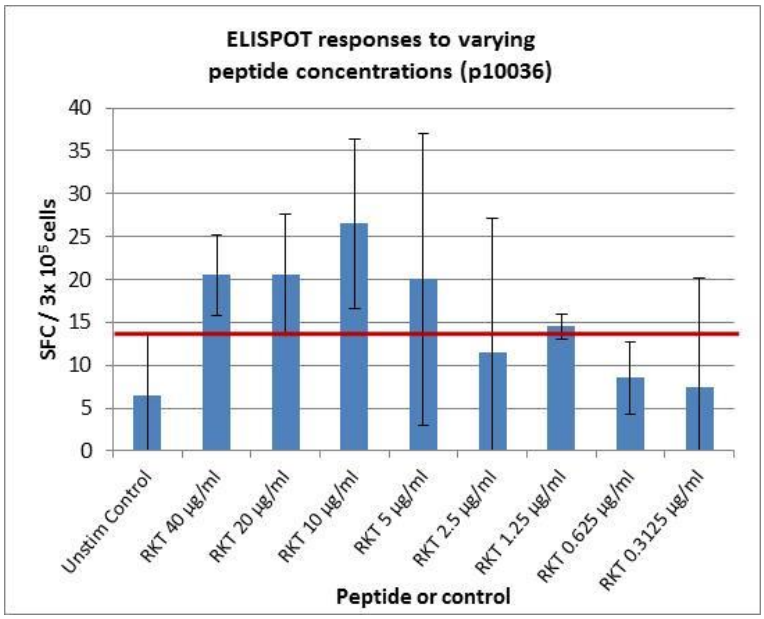


Figure 2-3 Effect of varying peptide concentration, donor p10036

PBMCs from donor p10036 (B*07:01 heterozygote) exposed to a known reacting peptide (HLA-B*07:01-restricted CMV peptide RKT) at 0.3125 – 40 µg/ml. Results are expressed as the mean of duplicates in spot-forming cells (SFC) per 3 x 10^5 cells; error bars demonstrate two standard deviations. The threshold of detection of a positive response (red line) is set at the mean of the highest negative control, plus two standard deviations.

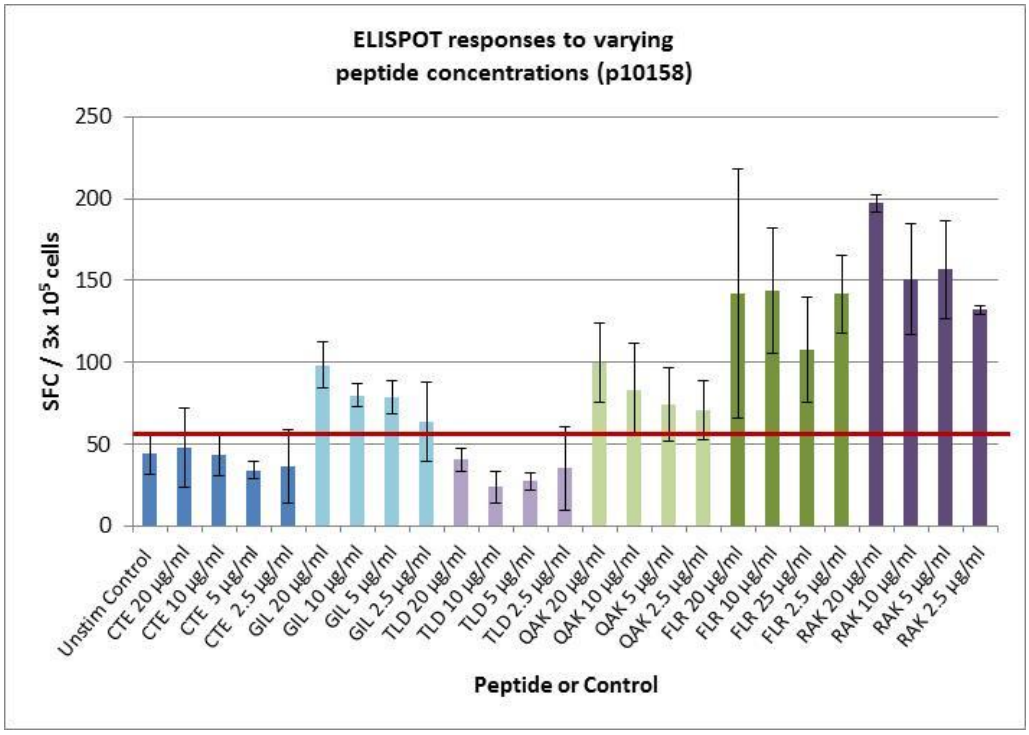


Figure 2-4 Effect of varying peptide concentration, donor p10158

PBMCs from donor p10158 (HLA-A*02:01/HLA-A*01:01 heterozygote, B*08:01 heterozygote) exposed to different peptides: A*01:01-restricted flu peptide CTE, A*02:01-restricted flu peptide GIL, A*02:01-restricted EBV peptide TLD, B*08:01-restricted EBV peptide QAK, B*08:01-restricted EBV peptide FLR and B*08:01-restricted EBV peptide RAK at concentrations of 2.5-20 µg/ml. Results are expressed as the mean of duplicates in spot-forming cells (SFC) per 3 x 10^5 cells; error bars demonstrate two standard deviations. The threshold of detection of a positive response (red line) is set at the mean of the highest negative control, plus two standard deviations.

2.7.5.3 Optimisation of use of peptides in pools

DMSO, the solvent used for peptide reconstitution is known to be toxic to cells when used at high concentration and for prolonged exposure times. One of the limitations on the number of peptides that can be used in a peptide pool is the final concentration of DMSO. At concentrations of > 2% for more than 2 hours, DMSO can be sufficiently toxic to PBMC to abolish detectable functional T cell responses (Kloverpris *et al*, 2010). It was crucial therefore that DMSO concentration be kept below this level, effectively limiting the pool size to fewer than 20 peptides. To ensure that responses were detectable in such pools,

single peptides eliciting known responses were combined with non-reactive peptides in pools of up to 25 peptides. Reliable responses were detected for these peptides in PBMCs from three donors. In this project, the maximal number of peptides in any pool was thirteen (see Table 3.2 in Chapter 3). There is some evidence that combining peptides into pools of greater than ten peptides can inhibit T cell proliferation assays, but not ELISPOT reactivities or cytokine secretion (Suneetha *et al*, 2009). To rule out the possibility of an effect in the HLA-A*01:01 experiments, described in Chapter 4, where maximal sensitivity was important, maximal pool size was restricted to five peptides.

2.8 ELISPOT Assay

The enzyme linked immunospot (ELISPOT) assay (Czerkinsky *et al*, 1988) was used for the detection of cells producing IFN-γ. This was performed using the IFN-γ ELISPOT kit (R&D Systems), which comprised the IFN-γ antibody-coated coated 96-well plate and the required reagents. Extensive optimisation was carried out as described in Sections 2.7.5 and 2.8.3.

2.8.1 ELISPOT Assay Method

2.8.1.1 Day 1

Peptides used in ELISPOT experiments are described in more detail in the relevant Chapters. The 10 µl aliquots of peptide previously prepared in sterile 96-well round-bottom plates (Section 2.7.4) were defrosted at RT. Peptides were used at a final concentration of 10 µg/ml, unless stated otherwise. Aliquots (10 µl) of appropriate positive and negative controls were added to the corresponding wells. All peptide stimulations or controls were performed in duplicate at a minimum.

Negative controls were a) CCM only and b) DMSO (Fisher Scientific Inc.) at a 1:50 concentration in CCM, corresponding to the highest concentration of DMSO in the peptide preparations. The non-specific positive control was PHA (Phytohemagglutinin PHA-P lyophilised, Sigma Aldrich) reconstituted to a working concentration of 10 µg/ml in distilled water and kept at 4°C. Further specific positive controls included CMV and influenza peptides of known HLA restriction, and the internationally recognised standard positive control for ELISPOT assays, the CEF pool. The CEF pool is fully described in Section 2.7.2. As an example, a typical plate illustrating position of controls and test wells is given in Figure 2.5.

PBMCs or BD-PBMCs had been prepared as described in Section 2.3.3 and suspended in 10% CCM to the desired concentration. Unless stated otherwise, a concentration of 3 x 10^6 cells / ml was used. Cells in suspension (100 µl) as described, were added to pre-aliquoted peptides and controls. The cells were incubated with the peptide or control for approximately 30 min at RT.

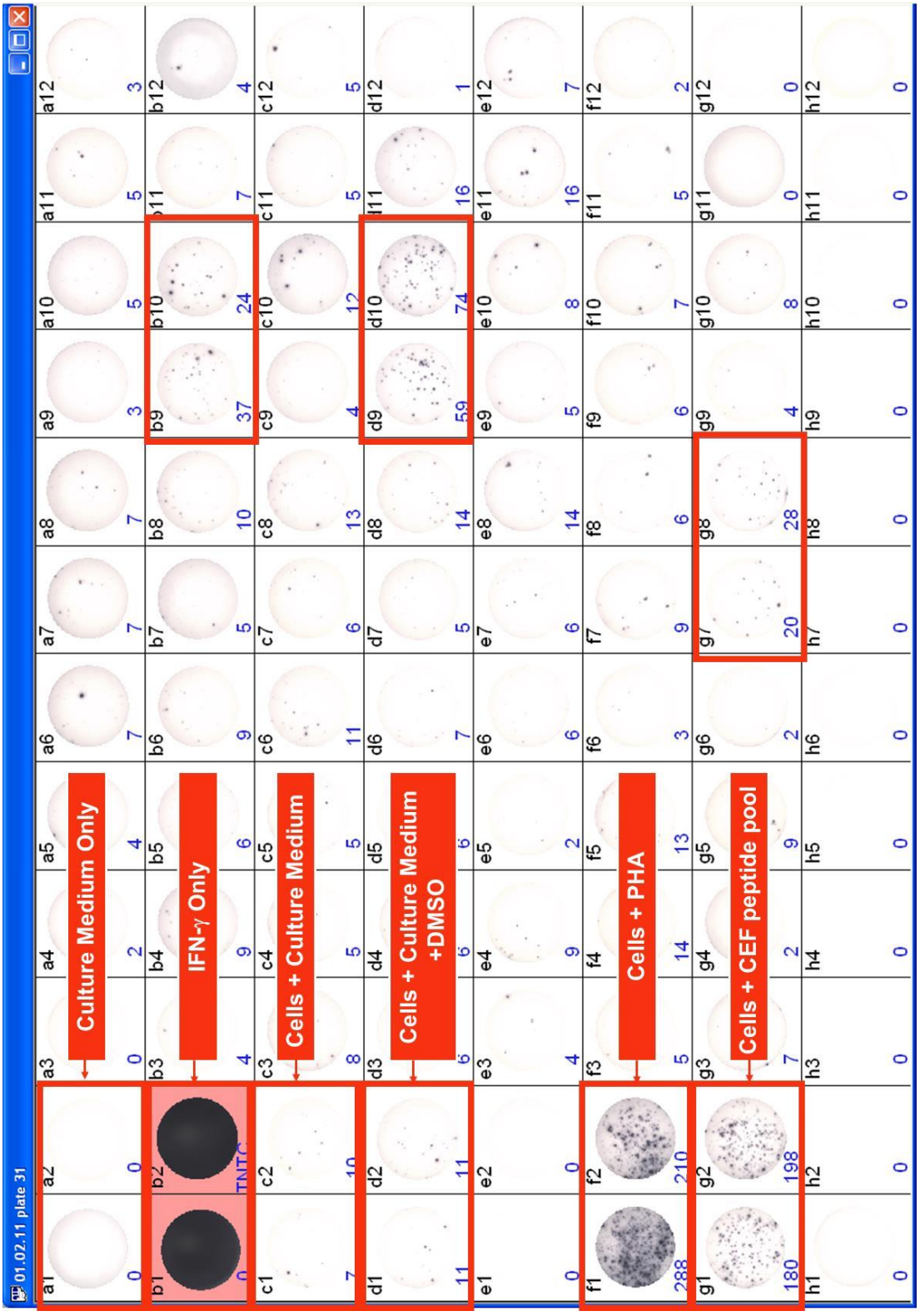


Figure 2-5 Example of typical ELISPOT plate layout

The positions of negative control wells (CCM alone, cells + CCM, cells + CCM + DMSO), positive control wells (IFN-γ alone, cells + PHA, cells + CEF pool) are shown. In this example, responses to 3 peptides, assayed in duplicate, are detected.

During this incubation step, the ELISPOT plate was prepared. All kit reagents, except detection antibody concentrate and dilution buffer 1, were brought to RT. The 96-well ELISPOT microplate (for brevity hereafter referred to as the ELISPOT plate) was prepared by filling all wells with 200 μl sterile 10% CCM and incubating for approximately 30 min at RT. Care was taken at all steps not to damage the membrane at the base of the well with the pipette tip. The CCM was then discarded from the plate. The plate was placed, bottom down, on a piece of aluminium foil approximately 10 x 15 cm in size. This was tucked round the base of the plate and remained *in-situ* until after removal of the chromogen solution. The purpose of this was to reduce the recognised possibility of "edge-effect", whereby the external wells of the 96-well plate produce unreliable results (Kalyuzhny & Stark, 2001). The cells in suspension (100 μl) were transferred from the 96-well round-bottom plate where they were incubating to the ELISPOT plate using a multichannel pipette (vWR).

The ELISPOT plate was covered with the lid, and the edges of the foil were then shaped round the plate. ELISPOT plates were incubated overnight (16 – 20 hours) at 37^{o}C in 5% CO_2. Care was taken not to disturb the plates during their incubation, to avoid "trails" made by moving cells producing IFN-γ, which might lead to aberrant cell counts.

2.8.1.2 Day 2

Cells were discarded into Virkon (Fisher Scientific Inc.) and the plates washed by manually filling each well completely with 250- 300 μl of the supplied wash buffer using a wash bottle, followed by complete removal of fluid at each step. This process was repeated a further three times to a total of four washes. After each step, plates were inverted and blotted against clean paper towels. The

final wash was left on the plate until the detection antibody was ready for use, to avoid the membrane becoming dry.

Detection antibody was prepared as per kit instructions and 100 µl of diluted detection antibody, or appropriate negative control, were added to each well. PBS was used as a negative control for the detection antibody (wells e1 and e2). The plates were incubated overnight at 4^{o}C.

2.8.1.3 Day 3

Streptavidin-AP was prepared as per kit instructions. The detection antibody was discarded and the plates were then washed 4 times as previously. Streptavidin-AP (100 µl) was added to each well and the plate incubated for 2 hours at RT. The streptavidin conjugate was discarded and the plates were washed 4 times. The chromogen (a stabilised mixture of 5-bromo-4-chloro-3'indolylphosphate p-toluidine salt (BCIP) and nitro blue tetrazolium chloride (NBT)) (100 µl) was then added to each well and the plate incubated for 1 hour at RT, in the dark. The chromogen was then discarded and the reaction stopped by rinsing the plate with deionised water. The plate was inverted and tapped against clean paper towels to remove excess water. The aluminium foil and flexible plastic under-drain were removed. The bottom of the plate was wiped thoroughly with clean paper towels and the plate left to dry completely at RT for 60 – 90 min. The plates were then ready for the spots to be counted.

2.8.2 Reading of ELISPOT plates

Spots were counted using an automated ELISPOT plate reader (ViruSpot 4, AID Strasbourg Germany), courtesy of Prof. Brian Willett, Medical Research Council - University of Glasgow Centre for Virus Research. The plate reader is supplied

with integral software. This was used to set parameters for optimal counting (specificity and sensitivity). Parameters were set for "intensity" (darkness of spots; minimum 20 – max 255, no units given), "spot size" (minimum 20 – maximum 5000, no units given) and "gradient" (the angle defined by a tangent from the maximum intensity to the background intensity; minimum 15^{o} – maximum 90^{o}). Uniform settings were used for all experiments to enable comparisons. An example of automated counting in a well is given in Figure 2.7 (overleaf).

Each well was manually reviewed to exclude the possibility of aberrant counting. Clear errors in counting were removed. Causes of such errors include plate movement during incubation or insufficient removal of cellular debris by washing (Figure 2.6). Absolute numbers of spots were counted. The results were exported to Excel (Microsoft corporation) for analysis. A visual record of each plate was recorded as a digital picture file.

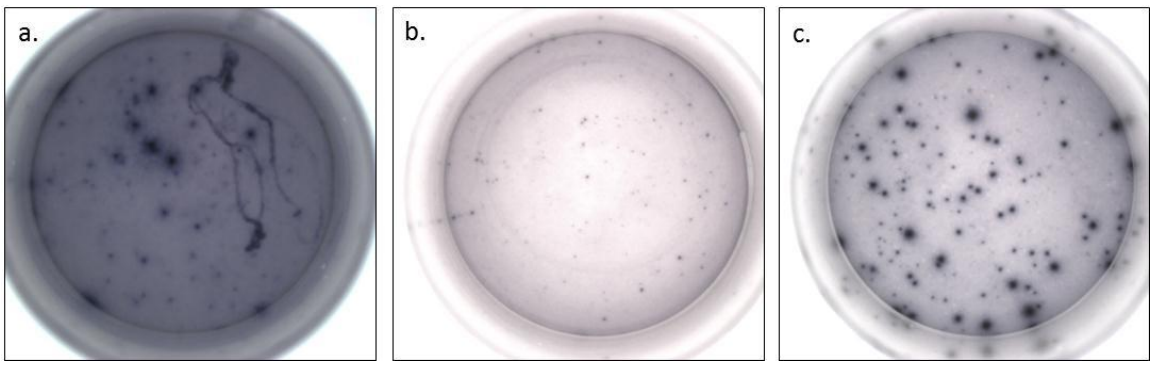


Figure 2-6 Examples of artefact in IFN-γ ELISPOT

a. Artefact seen when a cell secreting IFN-γ moves during the incubation period. b. Small, light spots seen with insufficient removal of cellular debris by washing. c. An example of a "clean" well with low background and good discrimination of well-defined medium-sized spots.

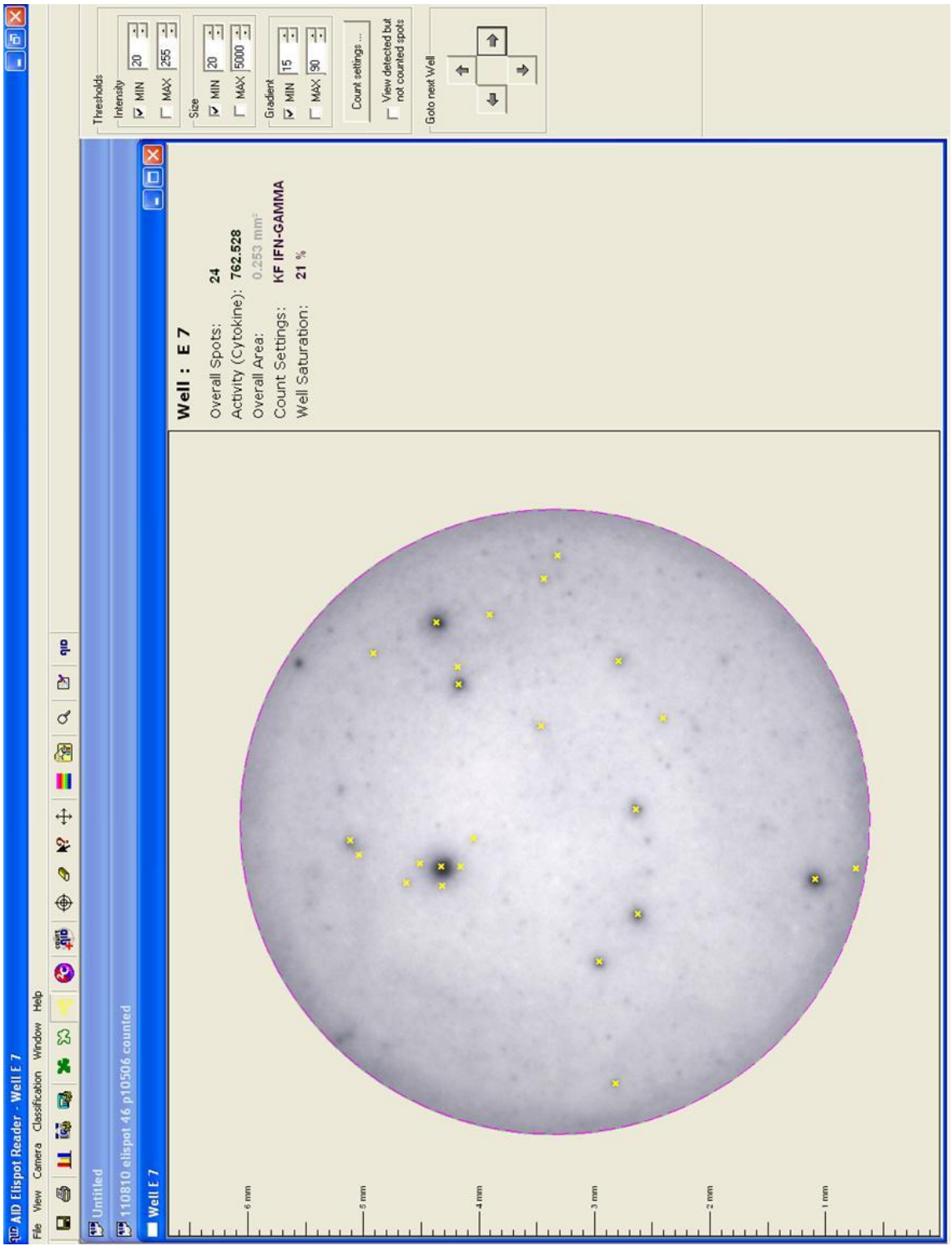


Figure 2-7: Example of an automated counted well

One well from a typical ELISPOT assay as read by the AID automated plate reader. The standard settings used for all assays are given to the right side of the image. The spots counted are seen as yellow “x”s. The tool bars to the top of the frame enable the user to manually review the spots counted, and to add or remove spots counted, or not counted, in error.

2.8.3 Optimisation of ELISPOT experimental conditions

In order to ensure that the results obtained from the study were comparable with other published ELISPOT studies, a number of quality measures were assessed. A number of experiments were undertaken to establish the rationale and validity of the approaches taken in the experiments described in subsequent results Chapters. These results have a direct bearing on the work described in both Chapters 3 and 4.

2.8.3.1 Demonstration of effect of B cell separation procedure on cell types present in PBMCs for ELISPOTs

As the samples were undergoing a cell selection procedure involving the removal of CD20-positive B lymphocytes for use in another study, it was important to ensure that the BD-PBMCs were similar in characteristics to the PBMC prior to the separation. Flow cytometry was performed using CD3/ HLA-DR, CD3/CD20, CD3/CD4/CD8, CD16/56/3, CD15/45/33 antibody combinations in PBMC from 16 donors. The CD16/56/3 antibody was primarily used to identify CD16/56 co-marking NK cells. The CD15 marker identifies monocytes and contaminating neutrophils and eosinophils.

Figure 2.8 demonstrates the pre & post B cell separation levels of CD20, CD3, CD3/ HLA-DR, CD4, CD8, CD16/ CD56 co-marking NK cells, and CD15 in PBMC large bleed samples. The only significant difference in pre & post separation was the expected depletion of CD20-positive B lymphocytes. An increase in the proportion of CD3 and CD4 lymphocytes present was seen, as would be expected given the number of CD20-positive B lymphocytes removed, and the fact that proportions are used in this analysis. No other significant differences between the populations were noted. Once the cellular composition of BD-PBMCs was

demonstrated, the antibody panel that was routinely used on every specimen pre & post B cell separation was limited to CD3/CD20, CD3/HLA-DR and CD4/8/3.

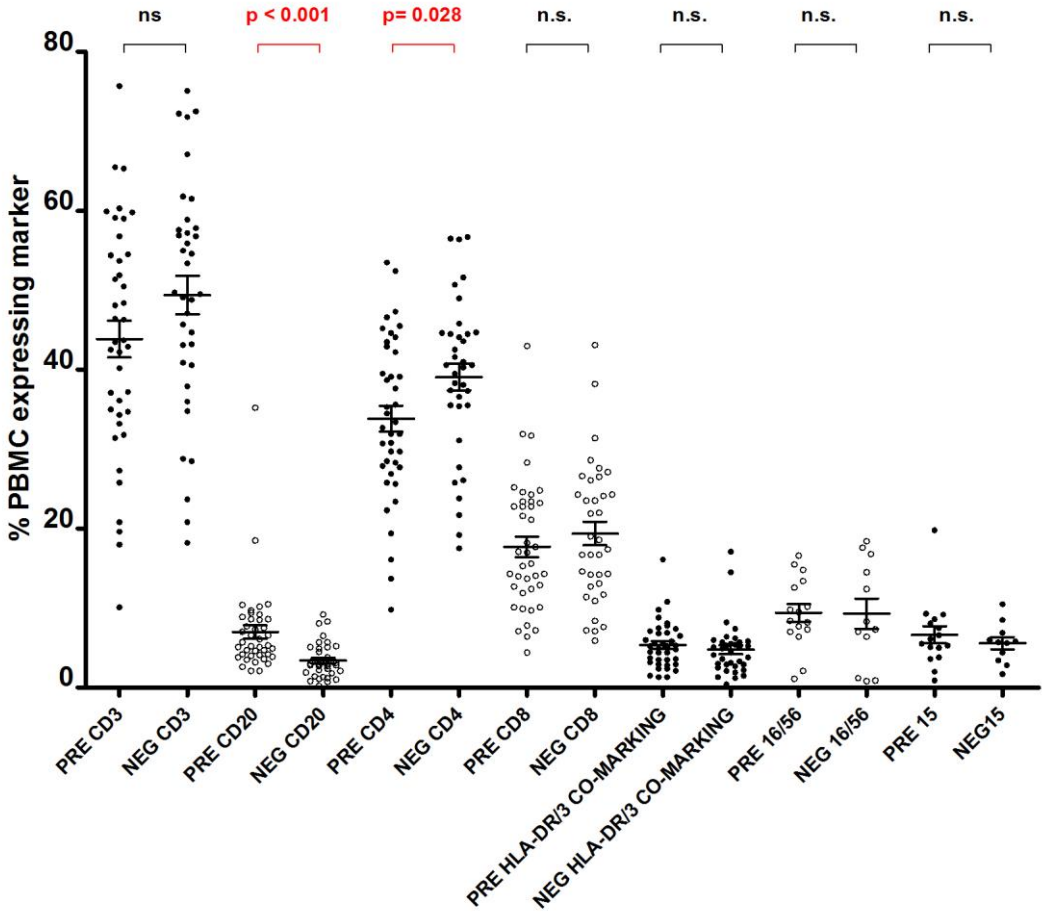


Figure 2-8 Cellular composition of PBMC pre-& post-B cell separation.

PRE, pre-separation sample (PBMC), NEG, CD20 negative fraction (BD-PBMC). Results from 41 donors are presented. Note CD16/CD56 and CD15 staining were performed on only 16 of the 41 donors. Results are reported as percentage of ungated events. The values for pre-separation samples and CD-20 negative fractions (BD-PBMC) were compared by unpaired t-test and the p-values are shown to 3 decimal places. Error bars demonstrate the mean and the standard error of the mean.

2.8.3.2 Optimisation of cell number used in ELISPOTS

The cell concentration or absolute number of cells per well used in ELISPOT assays varies in published series depending on the antigens and cell populations being tested, but is generally between 1 x 10^6 cells / ml and 1 x 10^7 cells / ml. Important features in determining this dose include having sufficient cells to detect a response (sensitivity), not having cells in excess, as this may result in non-specificity and, more pragmatically, the number of cells available for the experiment. In the case of this study, the cell number available for any one experiment was finite as cells were obtained from a single 50 ml blood sample. The volume of cell suspension used in every ELISPOT was 100 µl. Published studies suggest that a cell number of 1-4 x 10^5 cells per well (i.e. 1-4 x 10^6 cells/ ml) yields the highest ratio of spots to cells number (Hobeika *et al*, 2005; Britten *et al*, 2008).

In order to determine the optimal cell number for the ELISPOT experiments in this study, a number of optimisation experiments were carried out. In the first experiment, an HLA-A*02:01 heterozygous donor (p4796) was exposed to three different peptides to which they were known to react. This was performed for cell concentrations of between 2 x10^6/ml and 5 x10^6/ml and the results are presented in Figure 2.9. As can be seen, responses were seen at all cell concentrations. Overall responses were lower when only 2 x 10^6 cells / ml were used, raising concerns regarding loss of sensitivity at this concentration. Sensitivity was higher using the higher cell concentrations.

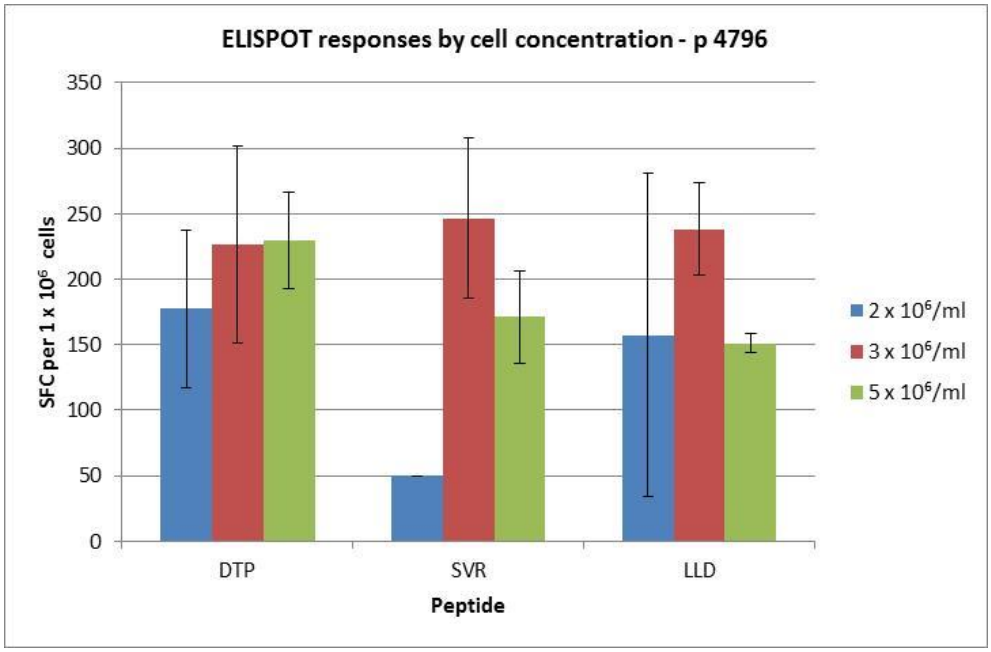


Figure 2-9 Effect of varying cell number on ELISPOT response, donor p4796

Donor p4796 (HLA-A*02:01 heterozygote) exposed to three peptides known to elicit responses in this donor (A*02:01-restricted EBV peptides DTP, SVR, LLD) at 10 µg/ml. Results are expressed as SFC per 1 x 10^6 cells to enable comparison of sensitivity of response by number of cells present. Mean of duplicates shown; error bars demonstrate standard deviation.

Donor p4796 and a further donor who was heterozygous for HLA-A*02:01 (p10158) were exposed to a number of different HLA-A*02:01-restricted peptides. In addition, as a negative control, they were also exposed to an EBV peptide restricted through HLA-B*35:01, which neither donor carried. Figure 2.10 demonstrates the result of this experiment. In both donors, cell concentrations of 5 x 10^5 cells / ml and 1 x 10^6 cells / ml were insufficient to robustly detect a response. In contrast, when the highest cell concentration of 5 x 10^6 cells / ml was used, most experiments showed a drop-off in sensitivity for the number of cells present and, in addition, non-specific responses to the negative control peptide were seen in one donor.

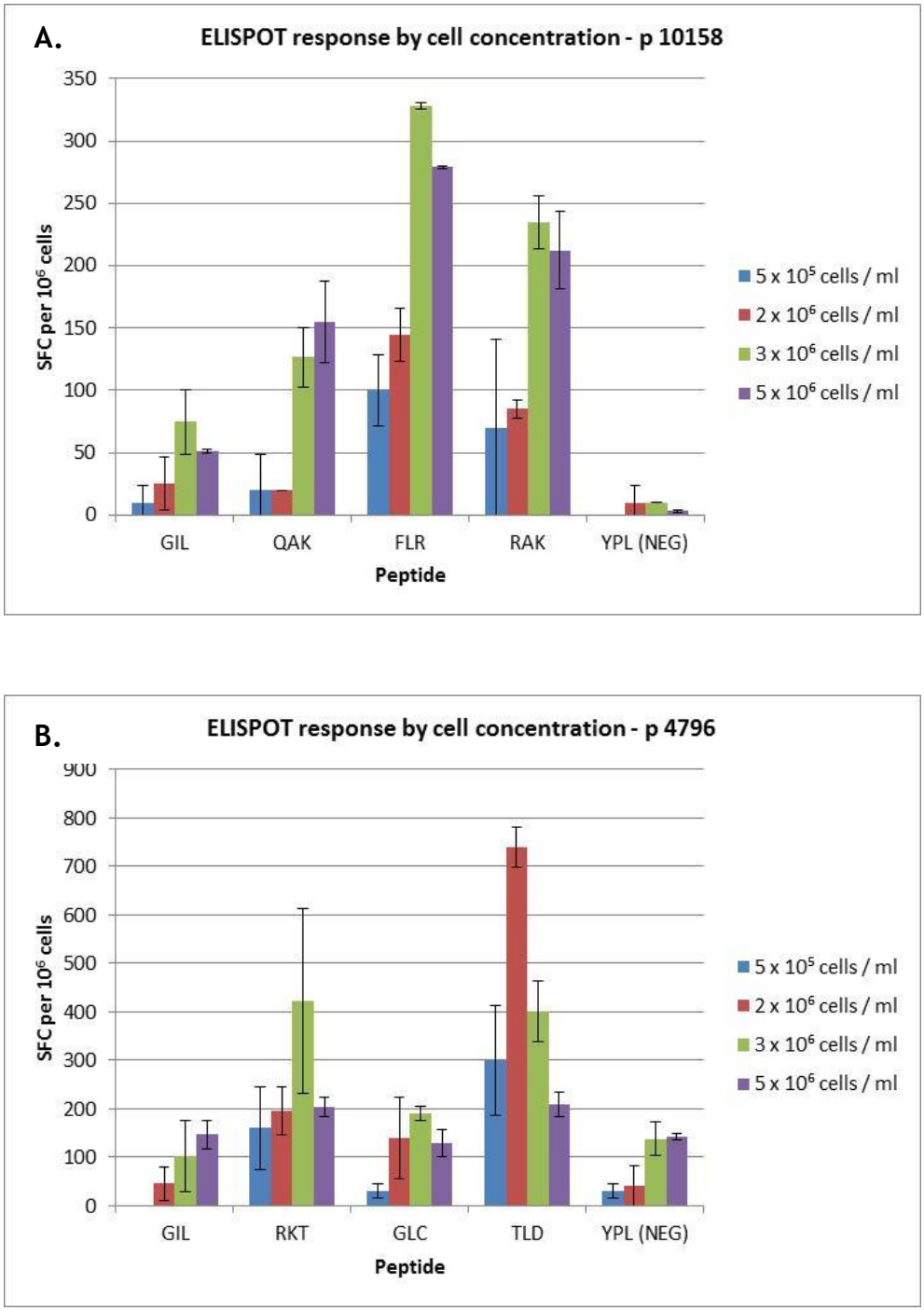


Figure 2-10 Effect of varying cell number on detection of responses

A. Donor p10158. B. Donor p4796. Representative results from two donors. ELISPOT with cells at concentrations between 5 x 10^5 / ml and 5 x 10^6 / ml. Results are expressed as the mean of duplicates in spot-forming cells (SFC) per 1 x 10^6 cells; error bars demonstrate standard deviation. Peptides used are the A*02:01-restricted flu peptide GIL, B*0801 restricted EBV peptide QAK, B*08:01 restricted EBV peptide FLR, B*08:01 restricted EBV peptide RAK, HLA-B*07:01 restricted CMV peptide RKT, A*02:01-restricted EBV peptide GLC and A*02:01-restricted EBV peptide TLD. Note that neither donor has B*35:01; YPL is a B*35:01 restricted EBV peptide to which neither donor would be expected to respond, and is included as a negative control.

The highest sensitivity, with acceptable specificity was a cell concentration of 3 x 10^6 cells / ml (3 x 10^5 cells per well). This concentration also ensured that there were sufficient cells from all donors to perform the planned experiments. For these reasons, a cell concentration of 3 x 10^6 cells / ml was used in all subsequent ELISPOT experiments.

2.8.3.3 Comparison of fresh cells vs. viably-frozen cells

It was important to be able to use the PBMCs at dates subsequent to collection, to maximise the use of the sample donated by subjects and to allow time for the establishment and growth of an LCL where necessary. The literature on use of ELISPOT to monitor CTL responses, e.g. following vaccination, suggests that viably frozen cells, stored at -70^{o}C or in liquid nitrogen can be reliably used in ELISPOTs (Smith *et al*, 2007; Weinberg *et al*, 2009). A cell viability of > 75% following defrosting has been suggested as an "acceptance criterion" for use of cells in functional T cell assays. All cells used in the experiments in the project met this criterion. An additional measure of cell function following freezing and defrosting has been reported to include satisfactory response to PHA or other positive control. It has been demonstrated that, given such checks are in place, the frequency and magnitude of CD8 responses is unaffected by cryopreservation (Kreher *et al*, 2003).

Cells taken fresh from donors were compared with cells from the same donors viably frozen and defrosted up to one year after their initial collection. Donor cells were exposed to a number of peptides and responses assessed by ELISPOT. Examples of results from 2 of 5 donors tested are shown in Figure 2.11.

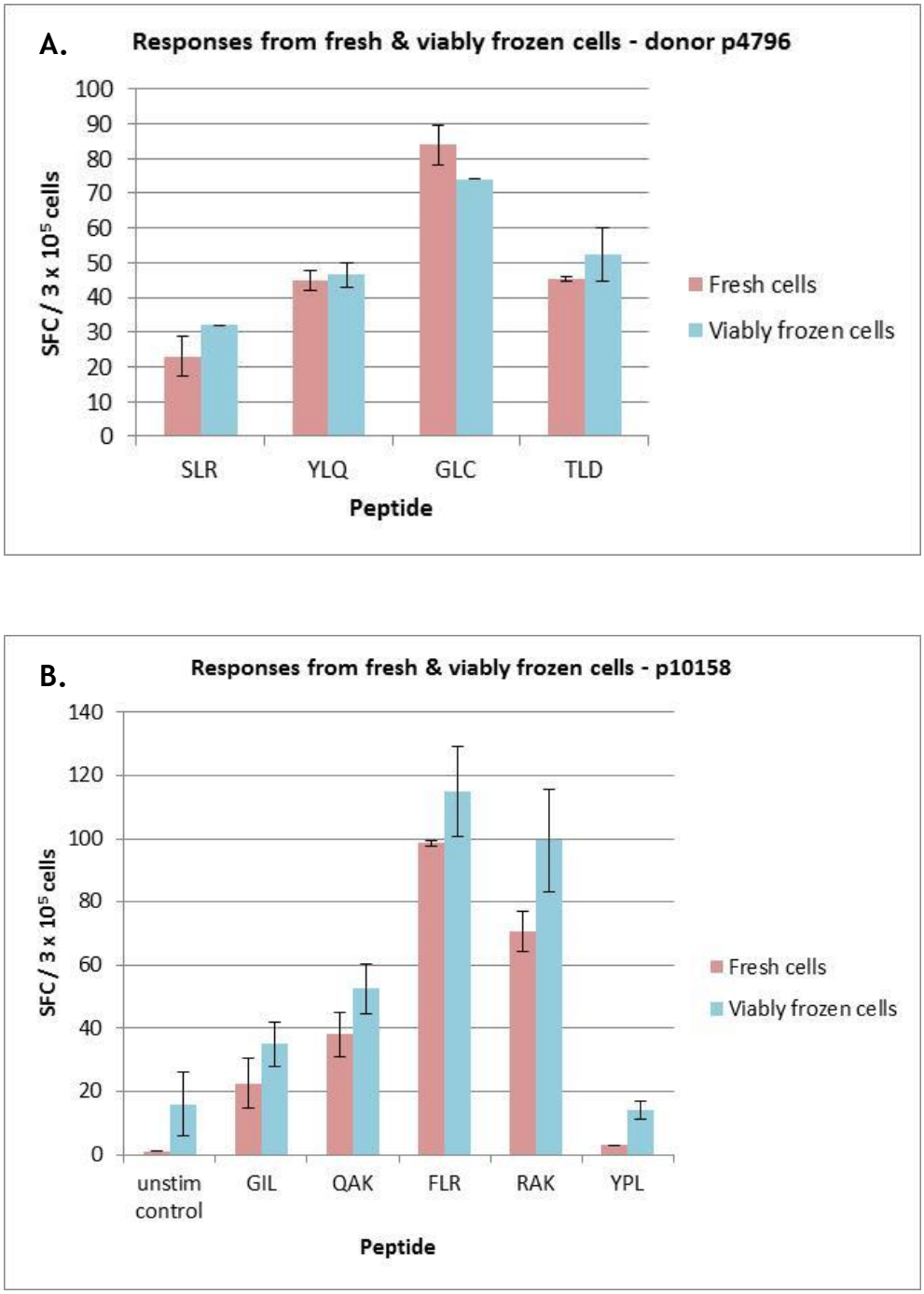


Figure 2-11 Comparison of ELISPOT responses of fresh vs. viably frozen cells

A. Donor p4796. B. Donor p10158. PBMC obtained the same day were compared with PBMC viably frozen for up to one year. Results are expressed as the mean of duplicates in spot-forming cells (SFC) per 3 x 10^5 cells; error bars demonstrate standard deviation. Peptides used are the A*02:01-restricted EBV peptides SLR, YLQ, GLC and TLD, the B*08:01 restricted EBV peptides FLR, QAK and RAK, the A*02:01-restricted flu peptide GIL, and the HLA-B*07:01 restricted CMV peptide RKT. Donors have relevant HLA allotype. Note that neither donor has B*35:01; YPL is a B*35:01 restricted EBV peptide to which neither donor would be expected to respond, and is included as a negative control. The unstimulated control was 10% CCM with DMSO at the same concentration as in the peptide solutions.

These results demonstrate that viably frozen cells give similar results in ELISPOT to fresh cells. However, some non-specific activation was noted in the viably frozen and defrosted cells, and this was more apparent in some donors. Non-specific background reactivity of defrosted cells in ELISPOTs is a well-described phenomenon, and may occur as a result of cellular debris. It should not impair the ability to detect a response, provided the negative controls are dealt with appropriately.

It has also been suggested that the use of foetal bovine serum (FBS) as a freezing medium may contribute to background reactivity (Prof. A. Rickinson and Dr. H. Long, oral communication). Some authors (Britten *et al*, 2008; Janetzki *et al*, 2005; Janetzki *et al*, 2008) have described methods to reduce this non-specific background activation with frozen cells, by optimisation of the defrost and culture conditions for the ELISPOT.

Firstly, it is recognised that resting the cells in CCM for a period of time from 1 – 24 hours following the defrosting and wash steps can be beneficial (Britten *et al*, 2008; Janetzki *et al*, 2008). For this reason, a comparison between unrested cells and cells rested for 1 hour was performed. In addition, some authors have reported that the addition of the exonuclease benzonase, which cleaves extracellular DNA, is helpful in reducing non-specific reactivity caused by cell clumping (Janetzki *et al*, 2005). Other authors have suggested that it is the presence of bovine serum in the CCM which causes non-specific reactivity in ELISPOTs, and that serum-free conditions may be preferable for ELISPOTs (Janetzki *et al*, 2009; Mander *et al*, 2010). Simply omitting the serum from the CCM can lead to poor cell viability. Addressing this, commercial companies have

developed supplemented serum-free media specifically for T cell culture. One such agent, OpTmizer Serum-free medium (Invitrogen), was tested.

The results of comparisons of the above conditions are shown in Figure 2.12. It was found that resting the cells was beneficial, with a reduction in non-specific background reactivity. When cells were rested, it was easier to differentiate between those peptide eliciting a response and those to which no response was detected. In contrast, unrested cells had higher reactivities to the "culture-medium only" negative control and the "irrelevant" negative-control peptide. Again, differences between donors were noted. Donor p10158 demonstrated a reduction in responses when benzonase was added, and both donors demonstrated a reduction in responses with the serum-free media. Donor p4796 demonstrated higher background reactivity with the serum-free media, with both the negative control and YPL negative-control peptide eliciting high responses. On the basis of these comparisons, a rest stage of at least 1 hour following cell defrost was added, but neither exonuclease nor T cell culture supplemented serum-free media were included in the protocol.

Cut-off criteria for the exclusion of background reactivity have been recommended. The Society of Biologic Therapy recommend that the number of spot-forming cells (SFC) more than 2 standard deviations (SD) above the mean value of negative controls tested simultaneously (Keilholz *et al*, 2002) be used as a suitable measure for a responding antigen. This was verified experimentally (Hobeika *et al*, 2005), demonstrating that ELISPOT had a sensitivity of 85.7% and a specificity of 100% when this criterion was used. This criterion was used in this project for the ELISPOTS comparing HLA-A*02:01 responses. This is discussed further in Chapters 4 and 5.

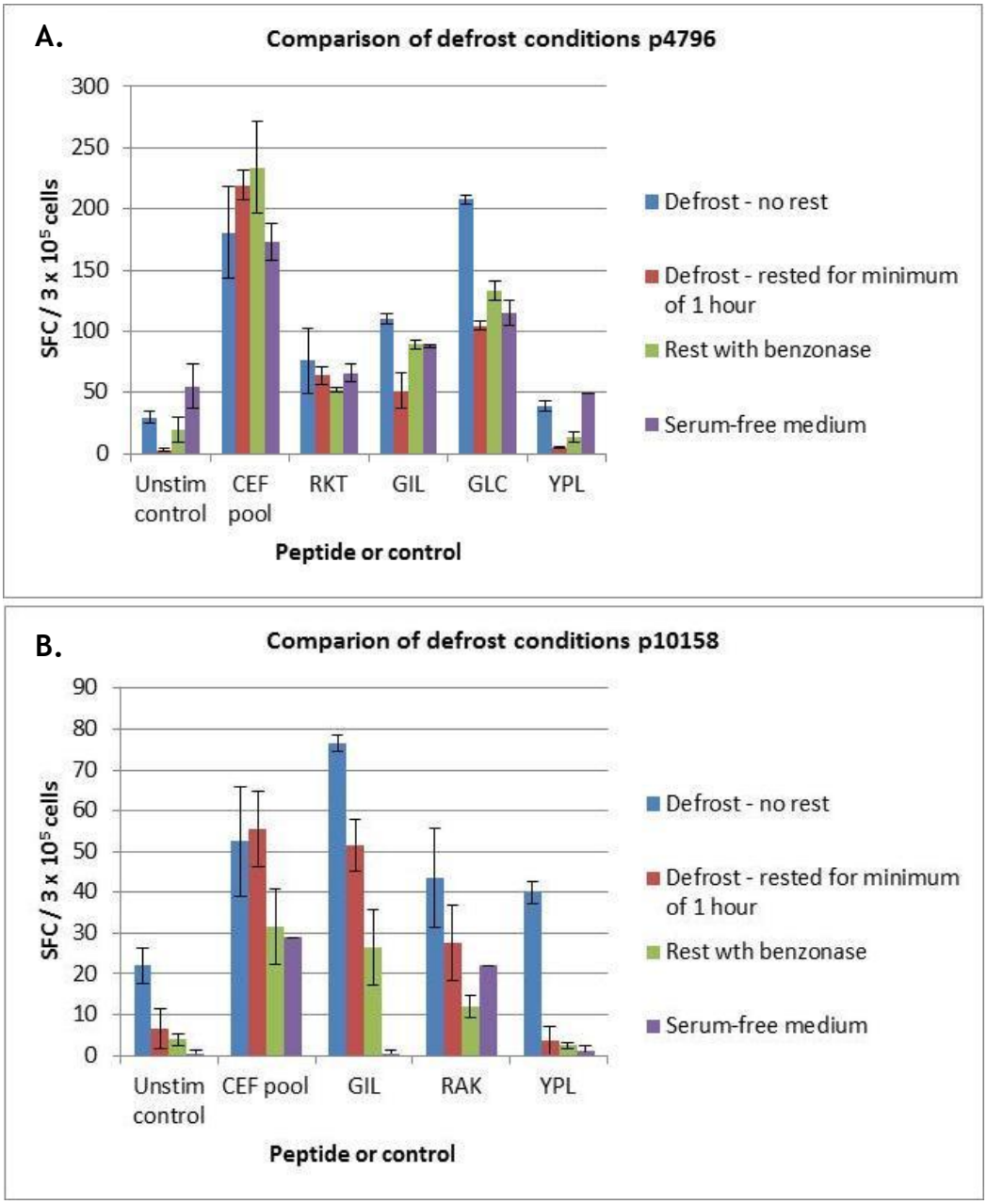


Figure 2-12 ELISPOT responses with differing defrost and culture conditions

A. Donor p4796. B. Donor p10158. Results expressed as the mean of duplicates in spot-forming cells (SFC) per 3 x 10^5 cells; error bars demonstrate standard deviation. Comparisons are shown of viably frozen cells defrosted and used immediately, viably frozen cells defrosted and rested for a minimum of 1 hour in CCM, viably frozen cells defrosted and rested for a minimum of 1 hour in CCM containing benzonase and viably frozen cells defrosted and rested for a minimum of 1 hour in OpTmizer serum-free medium. CEF pool is the pool of 23 CMV, EBV and influenza peptides which is an internationally accepted positive control (see Chapter 2). Peptides used are the A*02:01-restricted EBV peptide GLC, the B*08:01 restricted EBV peptide RAK, the A*02:01-restricted flu peptide GIL, and the HLA-B*07:01 restricted CMV peptide RKT. Note that neither donor has B*35:01; YPL is a B*35:01 restricted EBV peptide to which neither donor would be expected to respond, and is included as a negative control.

2.8.4 Summary of ELISPOT optimisations

Since the first description of IFN-γ ELISPOT to assess CTL responses (Czerkinsky *et al*, 1988; Czerkinsky *et al*, 1983), many published studies have described the optimal conditions for performing ELISPOTs, with the aim of generating meaningful, reproducible and comparable results.

The results of the optimisation experiments described above were generally in line with published results, and remarkably consistent with the guidelines available from the international consensus bodies who advise on the optimal use of ELISPOT, such as the Cancer Vaccine Consortium of the Sabin Vaccine Institute (CVC/SVI) (Janetzki *et al*, 2008) and the International Committee for the Standardisation of ELISPOTS (Janetzki *et al*, 2005). Although peptide concentrations are routinely reported in the published literature, the accurate quantitation of peptides in solution is not possible without HPLC for every specimen. Such an approach is expensive and impractical unless small numbers of peptides are being employed. Consistent with the vast majority of published studies, peptide quantitation in this study assumed full solubility of the dry weight of peptide in DMSO.

The optimisations support the use of cells at 3×10^6 cells / ml in the ELISPOTs (3×10^5 per well) with a final peptide concentration of 10 µg/ml in each well. The use of viably frozen material was acceptable, provided that cells were allowed to rest in CCM for a minimum of one hour prior to use in the ELISPOT, and that appropriate criteria for exclusion of background reactivity were used.

2.9 Cytokine analysis

Supernatants from incubations or stimulations of BD-PBMCs (100 µl) as detailed in Chapters 3 and 4 were aspirated and frozen in 96-well round-bottom plates at -70^{o}C. Analysis of TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and IL-17 was performed using the Human VersaMAP Multiplex Development System 9 Plex kit (R&D Systems) and read using the Bioplex system Luminex 100 plate reader (Bio-Rad). Results are given in pg/ml of each cytokine. Details of the stimulation or controls used in any individual experiment are described in the relevant results Chapters.

2.10 Statistical Analysis

Statistical analyses were performed using SPSS Statistics, version 19 (IBM, UK) and Graphpad (Graphpad Software Inc., La Jolla, California, USA). Details of the statistical analyses performed are given in the relevant results Chapter.

Chapter 3. Does the presence of an HLA-A*01:01 allele modify the magnitude of the CTL response to HLA-A*02:01-restricted epitopes?

3.1 Introduction

Approximately one third of cHL is associated with the EBV. Class I HLA genes are associated with risk of developing EBV+ve cHL, as discussed in the Introduction.

HLA-A*01:01 is associated with increased risk of EBV+ve cHL whereas HLA-A*02:01 is associated with decreased risk, such that there is an almost 10-fold difference in odds of developing EBV+ve cHL between HLA-A*02:01 and HLA-A*01:01 homozygotes with a gradation of risk in intermediate genotypes. This is demonstrated in Figure 3.1.

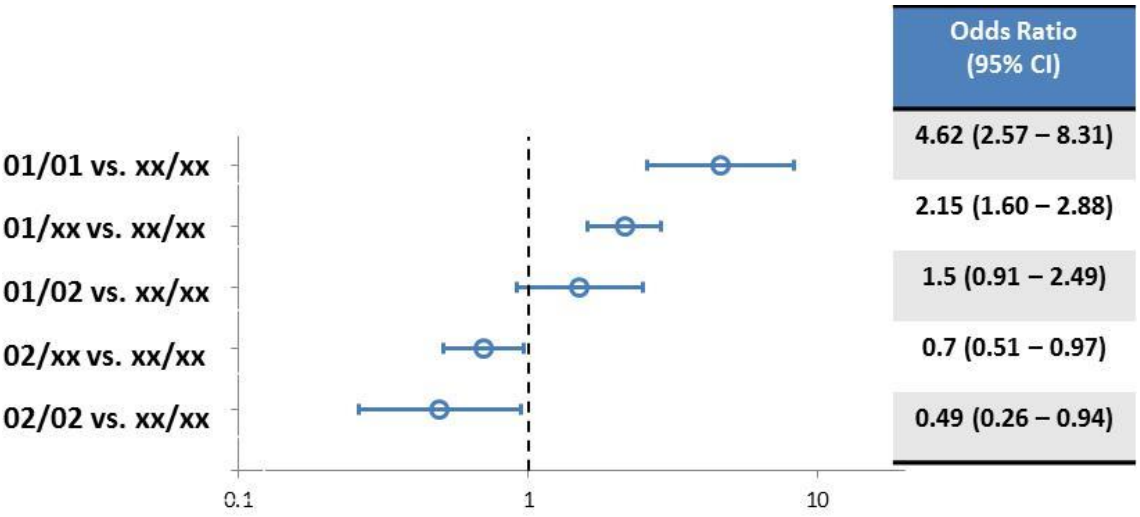


Figure 3-1 Case-series Odds Ratios for EBV+ve cHL by selected combinations of HLA-A*02 genotype.

01, HLA-A*01:01; 02, HLA-A*02:01; x is any allele other than HLA-A*01 or HLA-A*02; CI, confidence interval. Data derived from (Hjalgrim *et al*, 2010).

Class I HLA associations with EBV+ve cHL suggest that EBV-specific CTL responses may be critical in the pathogenesis of EBV+ve cHL. Such responses could be important following initial infection, during persistent latent infection or during oncogenesis. Many EBV peptides are known to be presented by HLA-A*02:01 for recognition by the T cell immune system. Indeed, many of the most

immunodominant EBV peptides presented through any class I allele are restricted through HLA-A*02:01 (Hislop *et al*, 2007). The reason for the elevated risk of disease in HLA-A*02:01/A*01:01 heterozygotes is therefore unclear, since these individuals should be capable of mounting an effective CTL response to EBV.

There is no evidence to date that HLA-A*01:01 is capable of presenting any EBV peptide (examined further in Chapter 5). However, individuals who carry HLA-A*01:01 will also have 4 or 5 additional class I HLA alleles through which they can raise a CTL response (i.e. both B alleles, both C alleles, and the additional HLA-A allele in the case of A*01:01 heterozygotes). EBV peptides can be presented via a wide range of human HLA types, and thus *all* individuals who are EBV seropositive would be expected to have a detectable EBV-directed CTL response. In particular, HLA-B*08:01, which presents a number of immunodominant EBV peptides resulting in a strong CTL response, is in LD with HLA A*01:01; thus, a large proportion of carriers of HLA A*01:01 should be able to effect an efficient anti-EBV CTL response via HLA-B*08:01. That HLA A*01:01 appears to increase risk of disease even in those individuals in whom you would expect to see a good EBV response raises the possibility that HLA-A*01:01 may be able to modify the magnitude of the EBV response restricted through HLA-A*02:01.

This hypothesis led to the primary experimental question: Does the presence of an HLA-A*01:01 allele modify the magnitude of the CTL response to HLA-A*02:01-restricted epitopes? In order to address this, I examined EBV-specific HLA-A*02:01-restricted CTL responses in healthy individuals with the HLA-A genotypes: HLA-A*01:01/ HLA-A*02:01; HLA-A*02:01/ HLA-A*02:01; and HLA-A*02:01/ HLA-A* x (where x is any allele other than A*01:01 or A*02:01).

3.2 Methods

3.2.1 Recruitment

Healthy individuals with the following HLA-A types were recruited (hereafter referred to as the "HLA-A*02 groups"):

- HLA-A*01:01/ HLA-A*02:01 heterozygotes
- HLA-A*02:01/ HLA-A* x heterozygotes (where x is any allele other than A*01:01 or A*02:01)
- HLA-A*02:01/ HLA-A*02:01 homozygotes

These groups with different HLA-A*02:01 genotypes were compared. Power calculations performed prospectively by Dr. Paul Johnson, Robertson Centre for Biostatistics, University of Glasgow determined that 15 individuals of each genotype were required to generate results which would demonstrate statistically significant differences in biological response. With n = 15 per group, any difference in CTL response of 4 percentage units between groups, allowing a within-group SD of 2 percentage units would give 100% power using a two-sample t-test. A difference of 2.81% would give 80% power. Any difference of < 4% between groups is unlikely to be clinically relevant, and therefore all potentially clinically important differences should be detected.

Local population frequencies for class I HLA genotypes were available from the Scotland and Newcastle epidemiological study of Hodgkin's disease (SNEHD) study (Jarrett *et al*, 2003). These data suggested that 150 – 200 adults would need to be HLA-typed to identify ≥ 15 individuals in each of the target groups. The limiting factor in determining this number was the proportion of HLA-

A*02:01 homozygotes in the general population. Supplementing the HLA-unscreened general population with a HLA-targeted approach to donor recruitment via the SNBTS donor population meant that these targets were achieved (see Chapter 2). EBV-seropositive donors with the relevant HLA-type were recalled to provide a "large blood sample" as described in Chapter 2. Thirty-nine of seventy-four recalled donors provided a specimen and their characteristics are described in Table 3.1. They comprised:

- 15 HLA-A*01:01/HLA-A*02:01 heterozygotes ("A*02:01/A*01:01")
- 12 HLA-A*02:01/ HLA-A*02:01 homozygotes ("A*02:01/A*02:01")
- 12 HLA-A*02:01/HLA-A*x heterozygotes ("A*02:01/A*x")

All donors reported that they were well at time of donation of the large specimen. Samples were processed to BD-PBMCs as described in Section 2.3.3.

Table 3-1 Characteristics of individual HLA-A*02:01 donors

LRF patient number	Sex	Age	Self-reported previous IM	HLA-A allele 1	HLA-A allele 2	HLA-B allele 1	HLA-B allele 2	HLA-C allele 1	HLA-C allele 2	Site of donor recruitment	Included in final ELISPOT analysis
4,796	F	51	No	0201	0301	0702	4403	0401	0702	CRF	No
10,040	M	58	Yes	0201	0101	4402	4402	0501	0501	CRF	Yes
10,097	F	57	No	0201	0101	0801	1501	0304	0701	CRF	Yes
10,098	M	44	No	0201	0301	0702	4402	0501	0702	CRF	Yes
10,100	F	24	No	0201	1101	1501	3701	0304	0602	CRF	Yes
10,115	F	28	Yes	0201	0201	4402	4402	0501	0501	CRF	Yes
10,117	F	41	Yes	0201	0201	1501	5101	0303	0401	CRF	Yes
10,121	F	37	No	0201	2902	0702	4403	0702	1601	CRF	No
10,134	F	28	No	0201	0101	1501	5702	0303	0701	CRF	Yes
10,151	F	49	No	0201	2501	1801	3701	0602	1203	CRF	Yes
10,157	M	47	No	0201	0101	0801	4402	0501	0701	CRF	Yes
10,158	M	38	No	0201	0101	0801	4001	0304	0701	CRF	Yes
10,169	F	25	No	0201	0101	0801	1501	0303	0701	CRF	Yes
10,170	M	45	No	0201	0201	1501	5701	0304	0602	CRF	Yes
10,171	F	43	No	0201	0301	0801	5501	0303	0701	CRF	Yes
10,172	F	28	No	0201	0201	1501	5701	0304	0602	CRF	Yes
10,237	F	49	No	0201	0101	0801	4001	0304	0701	CRF	Yes
10,239	F	28	No	0201	6801	1501	4403	0602	0701	CRF	No
10,277	F	43	No	0201	0101	0801	3501	0401	0701	CRF	Yes
10,279	F	24	No	0201	2902	1501	4001	0304	0304	CRF	Yes
10,282	F	53	No	0201	1101	0702	2705	0202	0702	CRF	No
10,284	F	40	No	0201	2301	0702	4403	0401	0702	CRF	No
10,287	F	23	No	0201	0301	1302	2705	0202	0602	CRF	Yes
10,353	F	43	No	0201	2902	4402	4403	0501	1601	CRF	No
10,485	M	50	No	0201	0201	NK	NK	NK	NK	SNBTS	Yes
10,486	M	53	No	0201	0101	NK	NK	NK	NK	SNBTS	No
10,488	NK	23	No	0201	0101	NK	NK	NK	NK	SNBTS	Yes
10,489	F	59	No	0201	0101	NK	NK	NK	NK	SNBTS	Yes
10,490	F	47	No	0201	0101	NK	NK	NK	NK	SNBTS	Yes
10,495	M	51	No	0201	0101	NK	NK	NK	NK	SNBTS	Yes
10,496	M	45	No	0201	0201	NK	NK	NK	NK	SNBTS	Yes
10,503	M	40	Yes	0201	0101	NK	NK	NK	NK	SNBTS	Yes
10,506	M	46	No	0201	0101	NK	NK	NK	NK	SNBTS	Yes
10,541	M	56	No	0201	0201	NK	NK	NK	NK	SNBTS	Yes
10,544	M	56	No	0201	0201	NK	NK	NK	NK	SNBTS	Yes
10,601	M	58	No	0201	0201	NK	NK	NK	NK	SNBTS	Yes
10,605	M	53	No	0201	0201	NK	NK	NK	NK	SNBTS	Yes
10,606	F	35	No	0201	0201	NK	NK	NK	NK	SNBTS	No
10,611	M	44	No	0201	0201	NK	NK	NK	NK	SNBTS	Yes

NK, not known; CRF, Donor recruited through either the CRF or the University of Glasgow; SNBTS, Scottish National Blood Transfusion Service; IM, infectious mononucleosis. Self-reported IM in this case being those donors who stated that they had previous IM which had been confirmed with a blood-test.

3.2.2 Flow cytometry

Flow cytometry using antibodies to CD3, CD4, CD8 and HLA-DR was performed on the BD-PBMCs of all donors (as described in Section 2.5). The proportion of CD3-positive cells that were positive for HLA-DR was measured to assess background T cell activation as this may lead to erroneously high responses in the ELISPOT assay. In addition the CD4/CD8 ratio was assessed in case differences between donors contributed to differences in IFN-γ CTL response measured by ELISPOT.

3.2.3 ELISPOTs

BD-PBMCs from the healthy individuals were subjected to ELISPOT analysis to examine the strength and depth of HLA-A*02:01-restricted EBV CTL responses. All HLA-A*02:01-restricted EBV peptides reported in the literature at time of study initiation (2008) were synthesised (n = 31) (Table 3.2). ELISPOT assays were performed as described in Section 2.8. Responses of 3×10^5 cells to single, un-pooled peptides at a concentration of 10 μg/ml were measured. Each peptide was assayed in duplicate. Positive controls (PHA and the CEF pool) and negative controls (CCM or CCM plus DMSO) were as described in Chapter 2. Plates were read as described in Section 2.8.2 using the same settings and thresholds on the plate reader for all plates.

Table 3-2 HLA-A*02:01-restricted peptides used in the study

Epitope AA sequence	HLA restriction †	Protein	Virus or EBV Latent/ Lytic	Three-letter abbreviation	Peptide pool ‡
VLEETSVML	A2	IE1	CMV	VLA	1
NLVPMVATV	A2	pp65	CMV	NLV	1
GILGFVFTL	A2	Matrix 1	Influenza A	GIL	1
FMVFLQTHI	A2	EBNA1	Latent	FMV	2
DTPLIPLTIF	A2/B51	EBNA2	Latent	DTP	2
SVRDRLARL	A2	EBNA3A	Latent	SVR	2
LLDFVRFMGV	A2.01	EBNA3C	Latent	LLD	2
SLREWLLRI	A2	EBNA-LP	Latent	SLR	2
LLLIALWNL	A2	LMP1	Latent	LLL	2
LLVDLLWLL	A2	LMP1	Latent	LLVD	2
TLLVDLLWL	A2	LMP1	Latent	TLL	2
YLLEMLWRL	A2	LMP1	Latent	YLL	2
YLQQNWWTL	A2	LMP1	Latent	YLQ	2
FLYALALLL	A2	LMP2	Latent	FLY	3
GLGTLGAAI	A2	LMP2	Latent	GLG	3
LIVDAVLQL	A2	LMP2	Latent	LIV	3
LLSAWILTA	A2	LMP2	Latent	LLS	3
LTAGFLIFL	A2	LMP2	Latent	LTA	3
TVCGGIMFL	A2	LMP2	Latent	TVC	3
CLGGLLTMV	A2.01	LMP2	Latent	CLG	3
LLWTLVVLL	A2.01	LMP2	Latent	LLWT	3
LLWAARPRL	A2	BHRF	Lytic	LLWA	4
GLCTLVAML	A2.01	BMLF1	Lytic	GLC	4
TLDYKPLSV	A2.01	BMRF1	Lytic	TLD	4
RALIKTLPRASYSSH	A2	BRLF1	Lytic	RAL	4
YVLDHLIVV	A2.01	BRLF1	Lytic	YVL	4
ILIYNGWYA	A2	GP110	Lytic	ILI	4
LIPETVPYI	A2	GP350	Lytic	LIP	4
QLTPHTKAV	A2	GP350	Lytic	QLT	4
VLQWASLAV	A2	GP350	Lytic	VLQ	4
VLTLLLLLV	A2	GP350	Lytic	VLT	4
LMIIPLINV	A2	GP85	Lytic	LMI	4
SLVIVTTFV	A2	GP85	Lytic	SLV	4
TLFIGSHVV	A2	GP85	Lytic	TLF	4

† HLA-restriction reported is either at low resolution (“A2”) or medium resolution (“HLA-A*02:01”) depending on the method of HLA-typing used in the reporting paper.

‡Note peptides used individually in ELISPOT experiments and pooled only for CD107 degranulation and cytokine secretion experiments.

3.2.3.1 Quality Control of ELISPOT experiments

Plates were read and counted as described in Section 2.8.2. All individual wells were visually inspected at time of plate reading. All plates were reviewed by myself, and then by Prof. Ruth Jarrett and Dr. Karen McAulay. Donors were excluded if positive controls did not elicit a clear detectable CTL response. Plates where the SFC count for the positive controls did no exceed the mean of the highest negative control plus 2 SD were also removed from the analysis. Plates with excessive background reactivity, defined as a response detectable in either of the negative controls, were excluded.

3.2.3.2 Scoring of ELISPOT results

Results for each well were recorded as the number of SFC per 3 x 10^5 cells. The mean of each duplicate was calculated, and the SD obtained. The highest negative control plus 2 SD was taken as the cut-off for a "positive" response to any individual peptide (Figure 3.2). The recorded response to each individual peptide was taken as the mean value of the measured responses minus the value of the highest negative control plus 2 SD.

For each donor the following values were recorded:

1) The total number of HLA-A*02:01-restricted peptides to which a response was detected.

2) The single highest ("maximal") response detected to any peptide.

3) The summative response i.e., the sum of all SFC counts for those peptides to which a response was detected.

The first of these measures gives an idea of breadth of the HLA-A*02:01-restricted CTL response to EBV. The maximal and summative responses give measures of the depth of EBV response, and are probably the most physiologically relevant as they represent the highest overall (maximal) and cumulative (summative) strength of the CTL immune response to EBV restricted through HLA-A*02:01.

The analyses performed looked at responses to (a) all known HLA-A*02:01-restricted EBV peptides, (b) peptides derived from EBV latent proteins, (c) peptides derived from EBV lytic proteins, and (d) peptides derived from those EBV proteins expressed by HRS cells (latency II).

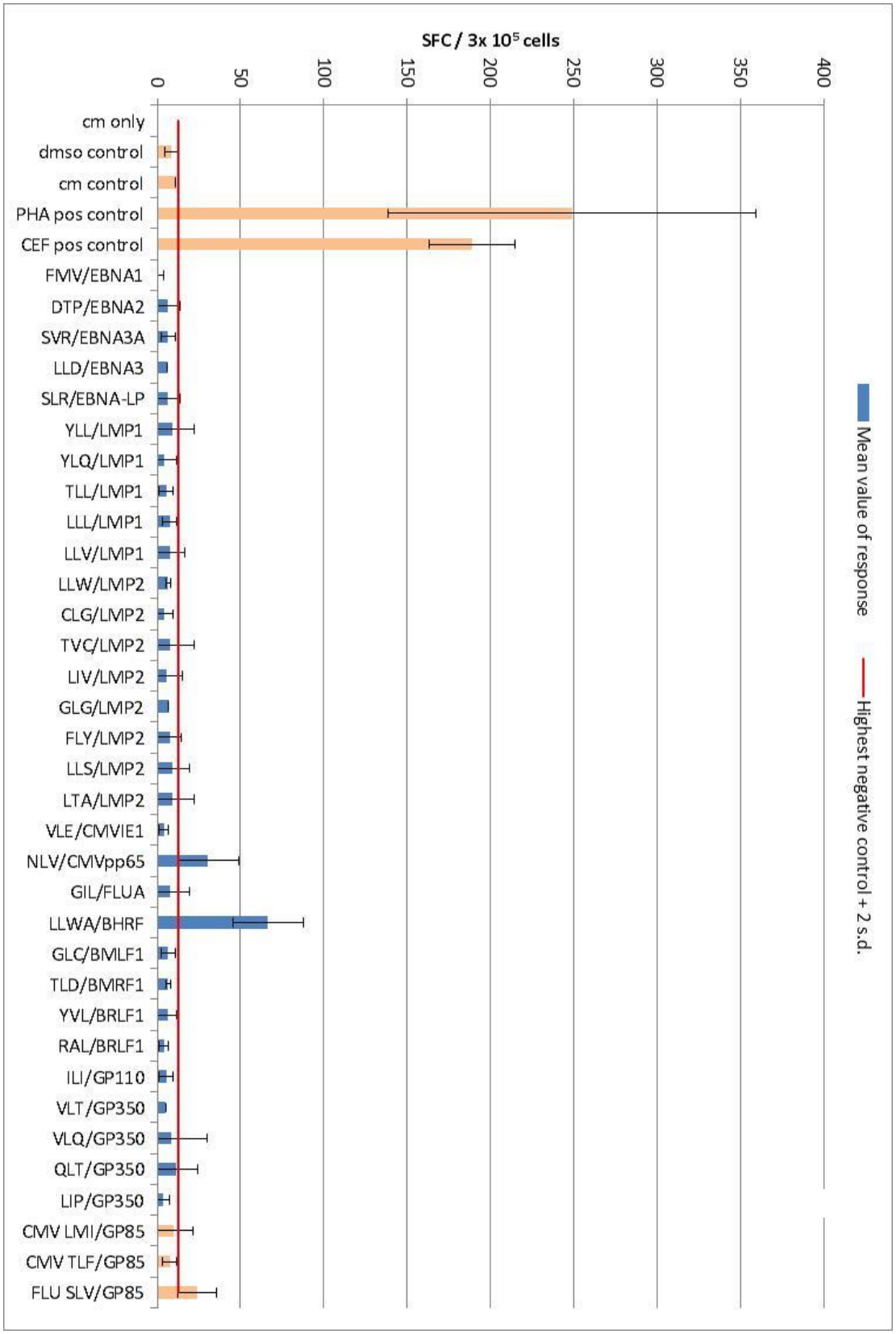


Figure 3-2 Visual representation of ELISPOT scoring

Responses to peptides or controls (x-axis) in spot-forming cells (SFC) per 3 x 10^5 cells. Controls are coloured pale orange. Error bars represent 2 SD. The red-line represents the cut off for a "positive response" i.e. the highest negative control plus 2 SD. As can be seen in this analysis, one EBV peptide (LLWA) and one CMV peptide (NLV) gave positive results.

3.2.4 CTL degranulation by assessment of CD107 expression

Assessment of cytotoxicity by degranulation was performed by flow cytometric analysis of CD107 expression after exposure to peptide pools. CD107 is expressed on the surface of CTLs when degranulation has occurred (Kannan *et al*, 1996) and it is possible to measure this using flow cytometry (Betts *et al*, 2003; Rubio *et al*, 2003).

Cells from the same donors as used in the ELISPOTs (Table 3.1) were stimulated with peptide pools for 4 hours in the presence of anti-CD107a conjugated to PE. BD-PBMCs from donors were prepared as described and suspended in CCM at 3 x 10^6 cells / ml in individual 5 ml tubes. Peptide pools (Table 3.2) were added to give a final concentration of each peptide of 10 µg/ml. PHA and the CEF peptide pool were used as positive controls. Cells in CCM plus DMSO at the same concentration as in the peptide pools were used as the negative control. Anti-CD107a conjugated to phycoerythrin (PE) (BD BioSciences) (20 µl) was added to each tube and the cells incubated for 4 hours. One tube with cells plus CCM was used as an isotype control for each donor, incubated for 4 hours with the isotype control antibody instead of the anti-CD107a. Flow cytometry was performed using the Beckman FC500 flow cytometer. Five thousand events were counted. The percentage of CD107 positive events in the ungated population was recorded and the change in percentage expression (ΔCD107, the percentage CD107 in stimulated cells minus the percentage CD107 in unstimulated cells) used to assess CTL degranulation.

3.2.5 Cytokine analysis

Cells from the same donors as used in the ELISPOTs (Table 3.1) were also used to assess cytokine secretion in response to EBV peptides. Peptide pools (10 µl at a final concentration of each peptide of 10 µg/ml) were added to 3 x 10^5 BD-PBMCs in 100 µl CCM, prepared as described before. Due to limitations in available cell number, peptide pools 2 and 3 (latent peptides) were combined. Cells were incubated for 24 hours at 37°C in humidified 5% CO_2, to replicate the conditions of the ELISPOTs. Supernatants (100 µl) were aspirated and frozen in 96-well round-bottom plates at -70°C. Due to limitation of resource, stimulation controls (DSMO, PHA and CEF pool) were not used in this experiment, and baseline cytokine levels were not assessed. Analysis of TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and IL-17 was performed using the Human VersaMAP Multiplex Development System 9 Plex kit (R&D Systems) and read using the Bioplex system Luminex 100 plate reader (Bio-Rad). Calibration controls were used to create the standard curve, and positive controls for beads and analytes were supplied with the kit. Results for each cytokine are given in pg/ml.

3.2.6 Statistical Analysis

3.2.6.1 ELISPOTS

Data were analysed for the normality of distribution of results. The summary results obtained for each donor were grouped according to HLA-A*02 genotype; results from each group were then compared using a Kruskal-Wallis test for analysis of variance across groups or a Mann-Whitney test for comparison between pairs of groups (HLA-A*02:01/A*01:01 heterozygotes versus HLA*02:01/x heterozygotes, HLA-A*02:01/A*01:01 heterozygotes versus HLA-A*02:01 homozygotes and HLA*02:01/x heterozygotes versus A*02:01 homozygotes).

The primary analysis was for response to all HLA-A*02:01-restricted EBV peptides. To correct for multiple testing in the primary analysis, a p-value of < 0.025 is considered significant. This is based on the assumption that the 3 measures of response (number of peptides, maximal response and summative response) are not independent.

Further exploratory analysis was performed where responses were broken down by protein expression group (lytic, latent and latency II). In the exploratory analyses p-values of < 0.05 are reported.

3.2.6.2 CTL Degranulation

Data were analysed for the normality of distribution of results. The summary results obtained for each donor were grouped according to HLA-A*02 genotype; results from each group were then compared using Mann-Whitney test for comparison between pairs of groups (HLA-A*02:01/A*01:01 heterozygotes versus HLA*02:01/x heterozygotes, HLA-A*02:01/A*01:01 heterozygotes versus HLA-A*02:01 homozygotes and HLA*02:01/x heterozygotes versus A*02:01 homozygotes). As responses to the different pools of peptides are independent, to correct for multiple testing, a p-value of < 0.025 is considered significant.

3.2.6.3 Cytokine analysis

Median cytokine levels (pg/ml) were compared between groups. The primary question in this exploratory analysis was whether HLA-A*01:01 resulted in a change in cytokine profile, so the primary comparison was HLA-A*02:01/A*01:01 heterozygotes versus the other HLA-A*02:01 groups considered together, using a Mann-Whitney test. In a secondary analysis, secretion was compared between all groups using a Kruskal-Wallis test for analysis of variance. Correction for

multiple testing was not performed in this exploratory analysis; p-values of < 0.05 are reported.

3.2.6.4 Analysis for confounding variables

In order to identify variables that could potentially confound the above analyses, the distribution of selected demographic and laboratory variables was analysed. These included: age; sex; prior IM confirmed by blood test; whether the subject had ever smoked; time to processing of blood sample; percentage of CD3 cells positive for HLA-DR; and CD4/CD8 ratio. Analysis of confounders was performed using simple regression for continuous variables (age, HLA-DR level, time to processing and CD4/CD8 ratio) and by Pearson chi-square analysis for categorical variables (sex, previous IM, ever smoker). For the analyses of confounding, a p value of < 0.10 was considered significant.

3.3 Results

3.3.1 Donor Characteristics

Characteristics of the 39 donors included in the experiments are given in Table 3.3. The three HLA-A genotype groups were well-matched for age, deprivation score, previous IM and smoking status. Females were significantly over-represented in the HLA-A*02:01/x heterozygote group (Table 3.3, p = 0.01). In addition, due to the recruitment strategy, no HLA-A*02:01/x heterozygotes were recruited from SNBTS platelet donors. As the latter samples were more likely to have been processed the following day, this meant that "same-day processed" samples were over-represented in HLA-A*02:01/x heterozygotes (p = 0.006).

Table 3-3 Summary of donor characteristics by HLA-A genotype

	HLA-A* 02:01 Group			p-value for differences between groups
	HLA-A* 02:01 / A*01:01 heterozygotes	HLA-A*02:01 homozygotes	HLA-A*02:01 / other (x) heterozygotes	
Total Number	15	12	12	N/A
Female, n (%)	7 (46.7)	4 (33.3)	11 (91.7)	p = 0.01 ‡
Age (median, range)	47 (23 -59)	45 (28-58)	41.5 (23-53)	p = 0.45 †
DEPCAT 5 (median, range)	1 (1-5)	4 (1-5)	3 (1-4)	p = 0.43 †
Monospot-confirmed previous IM §, n (%)	2 (13.3)	2 (16.7)	0 (0)	p = 0.36 ‡
Suspected previous IM §, n (%)	2 (13.3)	2 (16.7)	1 (8.3)	p = 0.79 ‡
Current smoker, n (%)	1 (6.7)	1 (8.3)	2 (16.7)	p = 0.39 ‡
Ever smoked? n (%)	3 (20.0)	5 (41.7)	3 (25.0)	p = 0.67 ‡
Processed within 24 hours, n (%)	11 (73.3)	5 (41.7)	12 (100)	p = 0.01 ‡

† regression (2-tailed t-test) ‡ Pearson Chi-square analysis

§ Note all IM is self-reported. As defined in this table, suspected previous IM is where an individual reported previous IM which was not confirmed by a blood test, and Monospot-confirmed IM is where the individual told us that a doctor confirmed the IM diagnosis using a blood test.

A p-value of < 0.05 is considered significant (highlighted in blue).

3.3.2 IFN-γ ELISPOT results by HLA-A*02:01 genotype

Results presented are of IFN-γ ELISPOT experiments conducted using BD-PBMCs from healthy volunteers. In total, 8 of the 39 donors were excluded from the analysis as a result of the QC described in Section 3.2.3.1; one on the basis of lack of response to the positive controls and seven on the basis of high background reactivity in the negative controls. The excluded donors are indicated in Table 3.1. There was no common pattern in HLA-B or C alleles, or in the second HLA-A allele in HLA-A*02:01/x heterozygotes in the excluded donors (in case of systematic bias in cross-presentation causing background reactivity). The HLA type of the donor who failed to respond to the positive controls was HLA-A*02:01/HLA-A*03:01.

Results from 31 donors were included in the analysis of ELISPOT results: 14 HLA-A*01:01/ HLA-A*02:01 heterozygotes; 11 HLA-A*02:01/ HLA-A*02:01 homozygotes; and 6 HLA-A*02:01/ A* x heterozygotes.

Responses to EBV-derived peptides were observed in 30 of the 31 donors. One donor, an A*02:01 homozygote, demonstrated no detectable IFN-γ CTL response to any of the peptides tested. The median number of peptides recognised was 8 (range 2 – 30 peptides). The mean maximal response to any peptide in all donors was 38 SFC / 3 x 10^5 cells (range 5-150 SFC / 3 x 10^5 cells). The mean summative response in all donors was 256 SFC / 3 x 10^5 cells (range 11 – 2059 SFC / 3 x 10^5 cells).

In the primary analysis of response (number of peptides, maximal response or summative response) to all EBV peptides tested, no significant differences were observed between the groups. No significant difference in response to A*02:01-

restricted EBV peptides was observed between A*01:01/A*02:01 heterozygotes and A*02:01 homozygotes in any of the comparisons (number of peptides, maximal response or summative response).

HLA-A*02:01/ A*x heterozygotes recognised more EBV peptides than either A*01:01/A*02:01 heterozygotes ($p = 0.03$) or A*02:01 homozygotes ($p = 0.06$), and this effect was greatest for lytic peptides ($p = 0.01$) (Figure 3.3). Where HLA-A*02:01/ A*x heterozygotes also demonstrated recognition of greater number of EBV lytic peptides than HLA-A*02:01 homozygotes ($p = 0.01$). No difference was observed in the number of HLA-A*02:01-restricted EBV peptides to which a response was detected between A*01:01/A*02:01 heterozygotes and A*02:01 homozygotes.

Maximal responses to any EBV peptide (Figure 3.4) were highest in A*02:01/ A*x heterozygotes. Differences reached significance when responses to lytic peptide were compared between HLA-A*02:01/ A*x heterozygotes and HLA-A*02:01/A*01:01 heterozygotes ($p = 0.02$) and when responses to latent peptide ($p = 0.05$) or latency II peptides ($p = 0.04$) were compared between A*02:01/x heterozygotes and HLA-A*02:01 homozygotes. No difference in single maximal response to A*02:01-restricted EBV peptides was observed between A*01:01/A*02:01 heterozygotes and A*02:01 homozygotes.

The summative response to all HLA-A*02:01-restricted EBV peptides tested was higher in A*02:01/ A*x heterozygotes compared with HLA-A*02:01/A*01:01 heterozygotes ($p = 0.05$). For lytic peptides, significant differences were observed between A*02:01/ A*x heterozygotes and HLA-A*02:01/A*01:01 heterozygotes ($p = 0.02$) and between A*02:01/ A*x heterozygotes and HLA-A*02:01 homozygotes ($p = 0.01$).

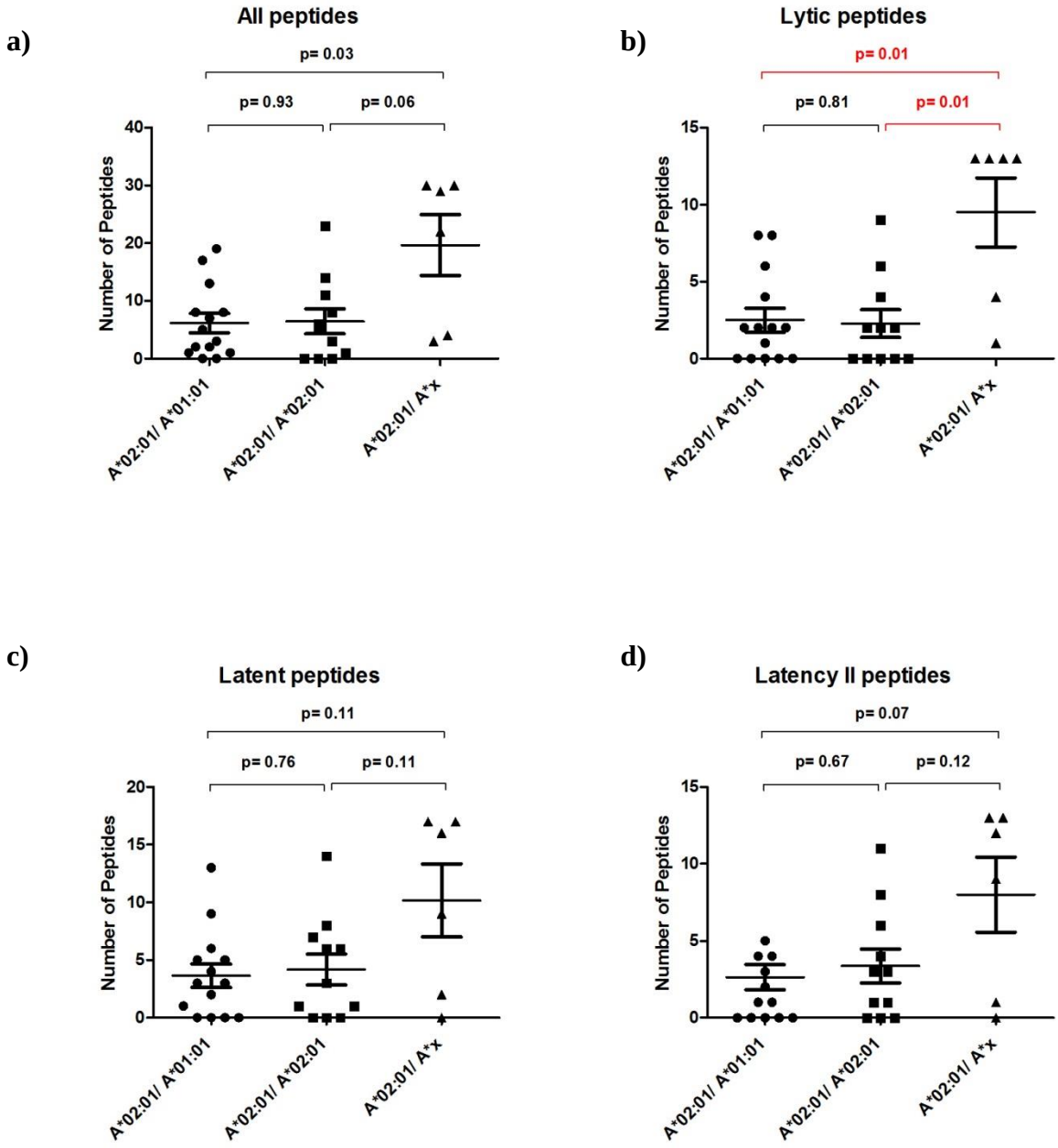


Figure 3-3 Number of HLA-A*02:01-restricted EBV peptides eliciting responses

Results are presented by a) total number of peptides recognised, b) number of lytic peptides recognised, c) number of latent peptides recognised and d) number of latency II peptides recognised, all by HLA-A genotype. Error bars demonstrate the mean and the standard error of the mean. Lytic peptides, peptides derived from proteins expressed during lytic cycle replication; Latent peptides, peptides derived from proteins expressed during viral latency; Latency II peptides, peptides derived from peptides derived from those EBV proteins expressed in the latency II expression programme, as in HRS cells. For the primary analysis (all peptides), p values of < 0.025 are considered significant. For the exploratory analyses of lytic, latent and latency II peptides, p-values of < 0.05 are considered significant, and are shown in red.

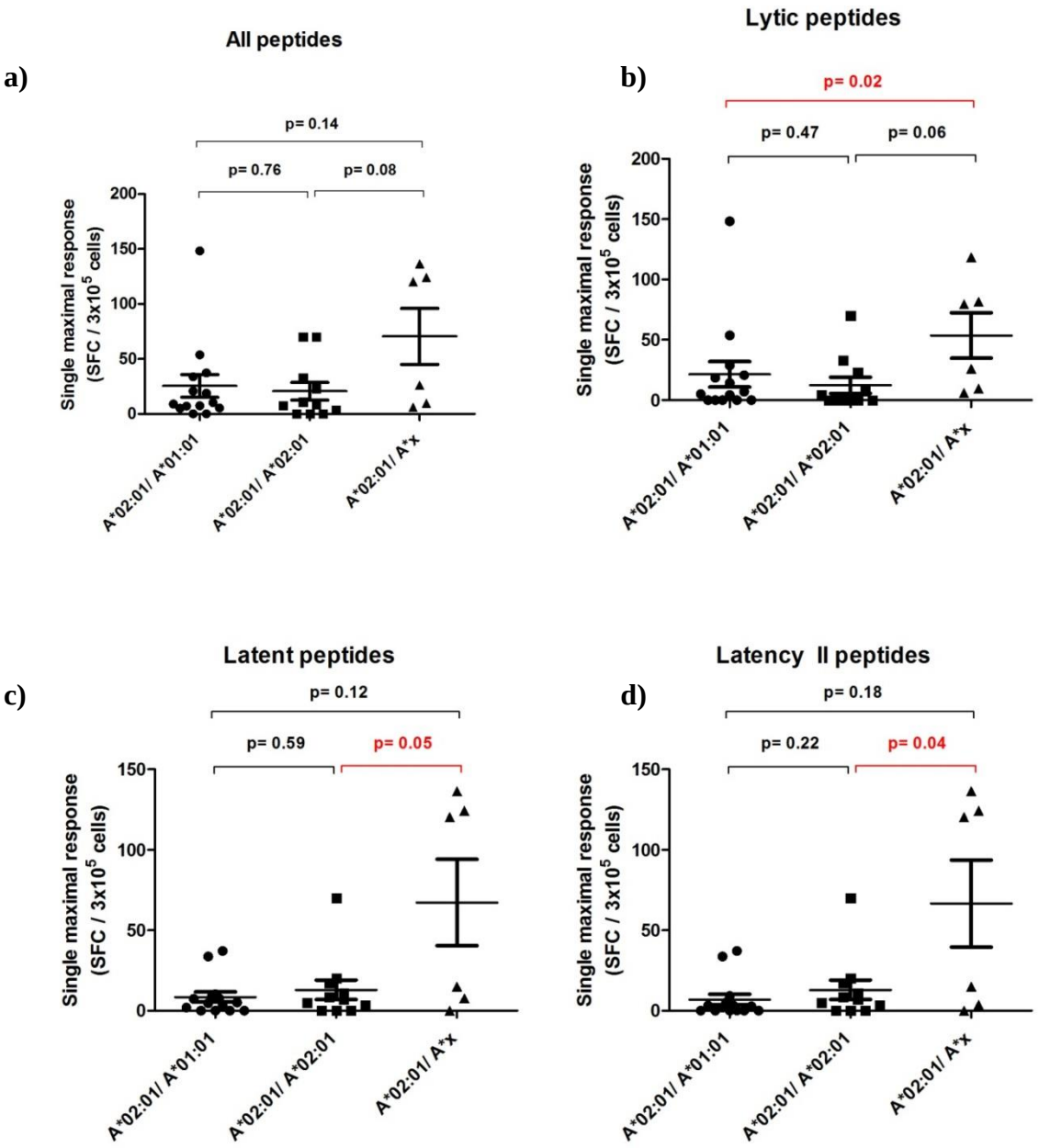


Figure 3-4 Single maximal response to HLA-A*02:01-restricted EBV peptides

Results are presented by a) maximal response to any HLA-A*02:01-restricted EBV peptide, b) maximal response to any HLA-A*02:01-restricted EBV lytic peptide, c) maximal response to any HLA-A*02:01-restricted EBV latent peptide and d) maximal response to any HLA-A*02:01-restricted EBV latency II peptide, all by HLA-A genotype. Error bars demonstrate the mean and the standard error of the mean. Latent peptides, peptides derived from proteins expressed during viral latency; Latency II peptides, peptides derived from peptides derived from those EBV proteins expressed in the latency II expression programme, as in HRS cells. For the primary analysis (all peptides), p values of < 0.025 are considered significant. For the exploratory analyses of lytic, latent and latency II peptides, p-values of < 0.05 are considered significant, and are shown in red.

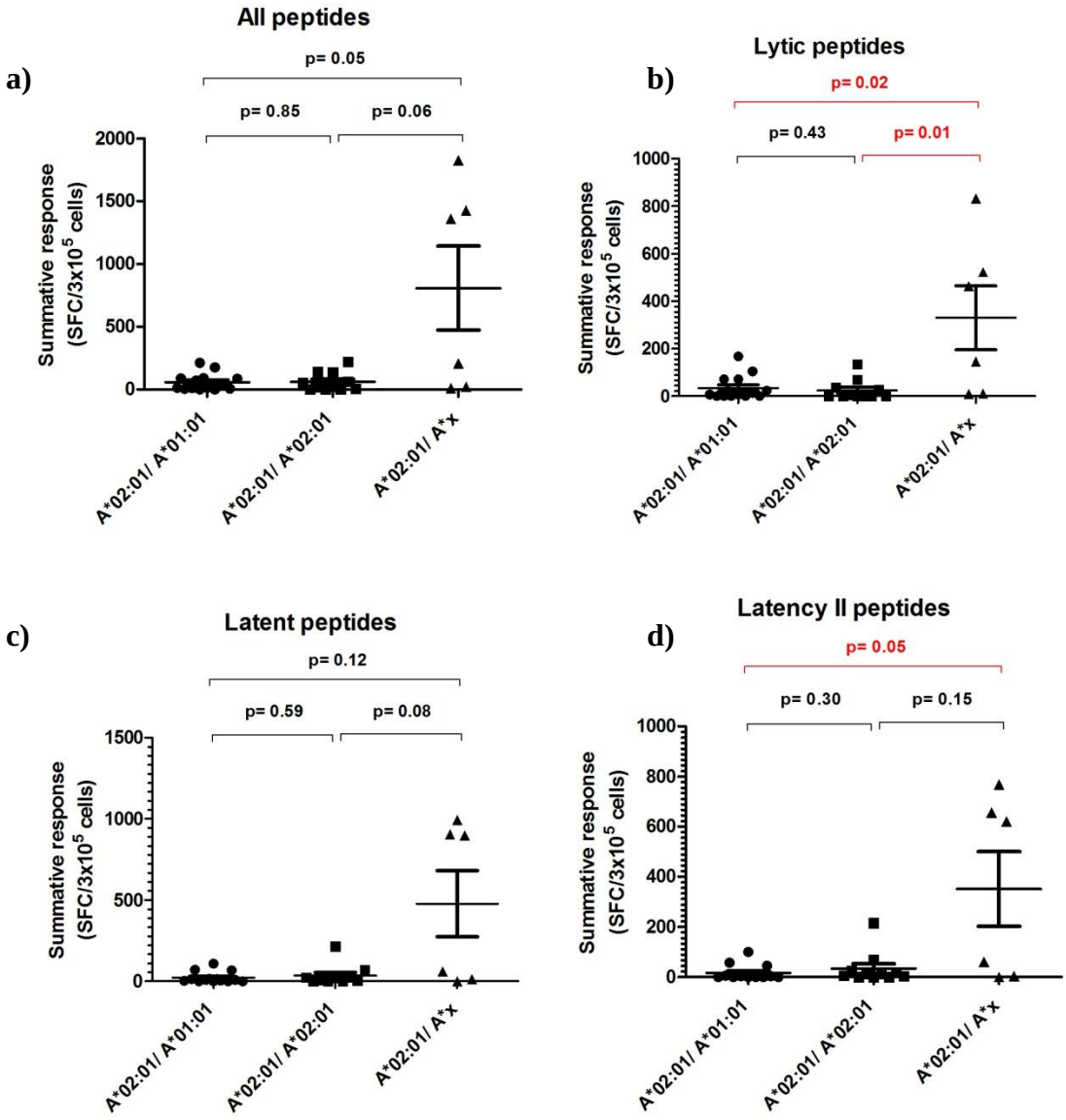


Figure 3-5 Summative (total) responses to HLA-A*02:01-restricted EBV peptides

Results are presented by a) summative response to any HLA-A*02:01-restricted EBV peptide, b) summative response to any HLA-A*02:01-restricted EBV lytic peptide, c) summative response to any HLA-A*02:01-restricted EBV latent peptide and d) summative response to any HLA-A*02:01-restricted EBV latency II peptide, all by HLA-A genotype. Error bars demonstrate the mean and the standard error of the mean. Latent peptides, peptides derived from proteins expressed during viral latency; Latency II peptides, peptides derived from peptides derived from those EBV proteins expressed in the latency II expression, as in HRS cells. For the primary analysis (all peptides), p values of < 0.025 are considered significant. For the exploratory analyses of lytic, latent and latency II peptides, p-values of < 0.05 are considered significant, and are shown in red.

3.3.3 Confounding variables in ELISPOT analysis

Given the surprising finding that the responses measured in the ELISPOT analysis were of greater breadth and depth in HLA-A*02:01/ A*x heterozygotes, compared to either HLA-A*02:01 homozygotes or A*02:01/A*01:01 heterozygotes, the data were examined to look for potential confounding variables.

First, in order to exclude cross-presentation by another allele as cause of the results, particularly in the high responders, the other class I alleles present in donors were examined manually for any trends. There was no systematic difference between higher and lower responders in terms of the other class I alleles present. The high-responding outlying donors in the responses were different for number of peptides recognised (donors 10170, 10237, 10279 and 10287), maximal response (donors 10170, 10237, 10279 and 10503) and summative responses (donors 10151, 10171 and 10279.)

Second, the data were analysed for potential confounding variables. The variables analysed included time to processing of specimen, sex, donor self-reported blood test-confirmed IM, if ever-smoked, percentage of CD3 cells positive for HLA-DR and CD4/CD8 ratio.

Potential confounding variables were first compared by genotype group. Given that 7 of the 8 donors excluded from the analysis were female and 6 of the 8 processed the same day, the comparison of donors described in Section 3.3.1 was repeated for those donors included in the final ELISPOT analysis (n = 31, Table 3.1). A summary of the characteristics of these 31 donors and the potentially confounding variables is given in Table 3.4.

Significant differences between the groups are noted in regard to sex, time to processing and the percentage of CD3 cells positive for HLA-DR. There were significantly more female donors and samples that were processed on the same day in the HLA-A*02:01/ A*x heterozygote group compared to the other groups. Fewer CD3 cells were HLA-DR-positive in the HLA-A*02:01/ A*x heterozygote group compared to the other groups, although all were within the expected normal range of < 20% (source; personal communication, Gartnavel General Hospital Haematology Laboratory, Glasgow).

Where differences between the groups were noted, analysis for potential confounding was performed. A summary of the analysis of potentially confounding variables by HLA-group is shown in Table 3.5.

Table 3-4 Characteristics of the 31 donors included in the ELISPOT analysis

	HLA-A* 02:01 Group			p-value for differences between groups
	HLA-A* 02:01 / A*01:01 heterozygotes n (%)	HLA-A*02:01 homozygotes n (%)	HLA-A*02:01 / other (x) heterozygotes n (%)	
Total Number	14	11	6	N/A
Female	7 (53.8)	3 (27.3)	5 (83.3)	p = 0.08 ‡
Age *	47 (23 -59)	45 (28-58)	41.5 (23-53)	p = 0.21 †
DEPCAT 5 *	2.23(1-5)	3.37 (1-5)	3 (1-4)	p = 0.10 †
Monospot-confirmed previous IM §	2 (14.3)	2 (18.2)	0 (0)	p = 0.55 ‡
Suspected previous IM §	2 (14.3)	2 (18.2)	1 (16.7)	p = 0.68 ‡
Current smoker	1 (7.1)	2 (18.2)	1 (16.7)	p = 0.68 ‡
Ever smoked?	1 (7.1)	1 (9.1)	1 (16.7)	p = 0.80 ‡
Processed within 24 hours	11 (78.6)	5 (45.5)	6 (100)	p = 0.04 †
% of CD3 cells positive for HLA-DR *	5.5 (2.1-10.8)	7.10 (4.4-16.1)	3.95 (1.5-5.5)	p = 0.030 †
CD4/CD8 ratio *	2.29 (1.42-5.00)	2.53 (1.43-4.80)	1.80 (0.93-3.20)	p = 0.277 ‡

* Age, DEPCAT 5, CD3/HLA-DR and CD4/CD8 ratio are presented as median and range.

† regression (2-tailed t-test)

‡ Pearson Chi-square analysis

§ Note all IM is self-reported. As defined in this table, suspected previous IM is where an individual reported previous IM which was not confirmed by a blood test, and Monospot-confirmed IM is where the individual has told us that a doctor confirmed their IM using a blood test.

Results presented by HLA-group. A p-value of < 0.05 is considered significant (highlighted in blue).

Table 3-5 Confounding by ELISPOT responses

Measure of ELISPOT response	p-value for associations with confounders †		
	Time to processing	Sex	% of CD3 cells positive for HLA-DR
Total number of peptides	0.198	0.519	0.158
Maximum of all peptides	0.015	0.033	0.230
Sum of all peptides	0.030	0.008	0.170

† Equal variances not assumed, data assumed to be not normally distributed Mann Whitney analysis performed for categorical variables and Kruskal-Wallis analysis performed for continuous variables. A p-value of < 0.10 is considered significant (highlighted in blue).

In the analysis of potential confounding variables by ELISPOT response, only sex and time to processing were associated with significant differences in response.

Female donors demonstrated higher maximal (p = 0.001) and summative responses (p = 0.03) (Figure 3.6). Samples processed on the same day as collection also demonstrated significantly higher maximal (p = 0.02) and summative (p = 0.03) responses (Figure 3.7).

Separating the effects of sex and time to processing on the ELISPOT outcome measures is difficult, as there was tight association between the two variables; 14 of 15 female donor specimens were processed the same day, and 8 of 9 samples processed after more than 24 hours came from male donors (Table 3.6).

Table 3-6 Association of time to processing with sex of the donor

		Time to sample processing (number of donors)	
		Same Day	**Next Day**
Sex (number of donors)	**Male**	7	8
	Female	14	1

Chi square analysis p = 0.014.

In this table, n = 30; datum on sex was missing for 1 donor.

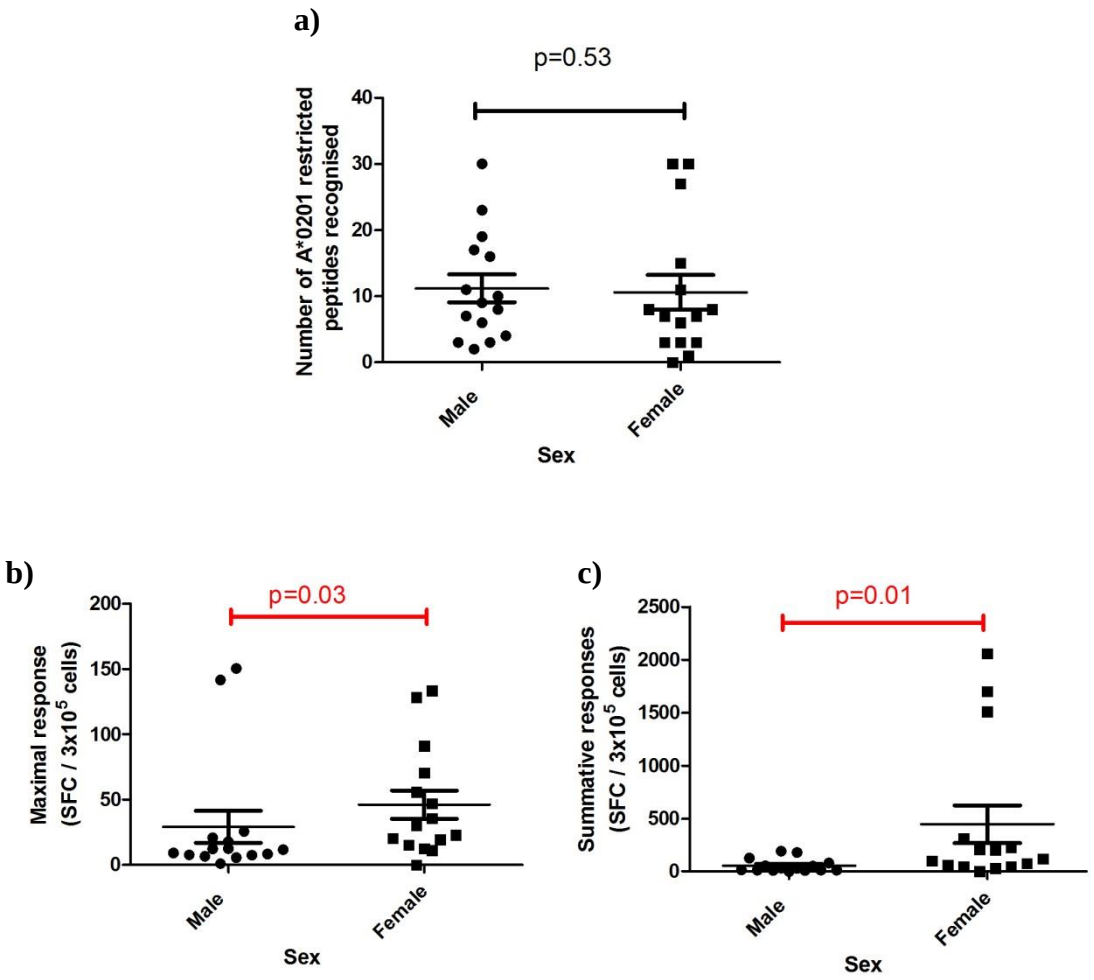


Figure 3-6 Effect of sex on ELISPOT responses to HLA-A*02:01-restricted peptides

a) Number of peptides recognised, b) maximal response to any peptide and c) summative response to any peptide by sex. Maximal and summative responses to HLA-A*02:01-restricted EBV peptides, although not number of peptides recognised, are significantly higher in the CTLs from female donors. p-values of < 0.05 are considered significant, and are shown in red.

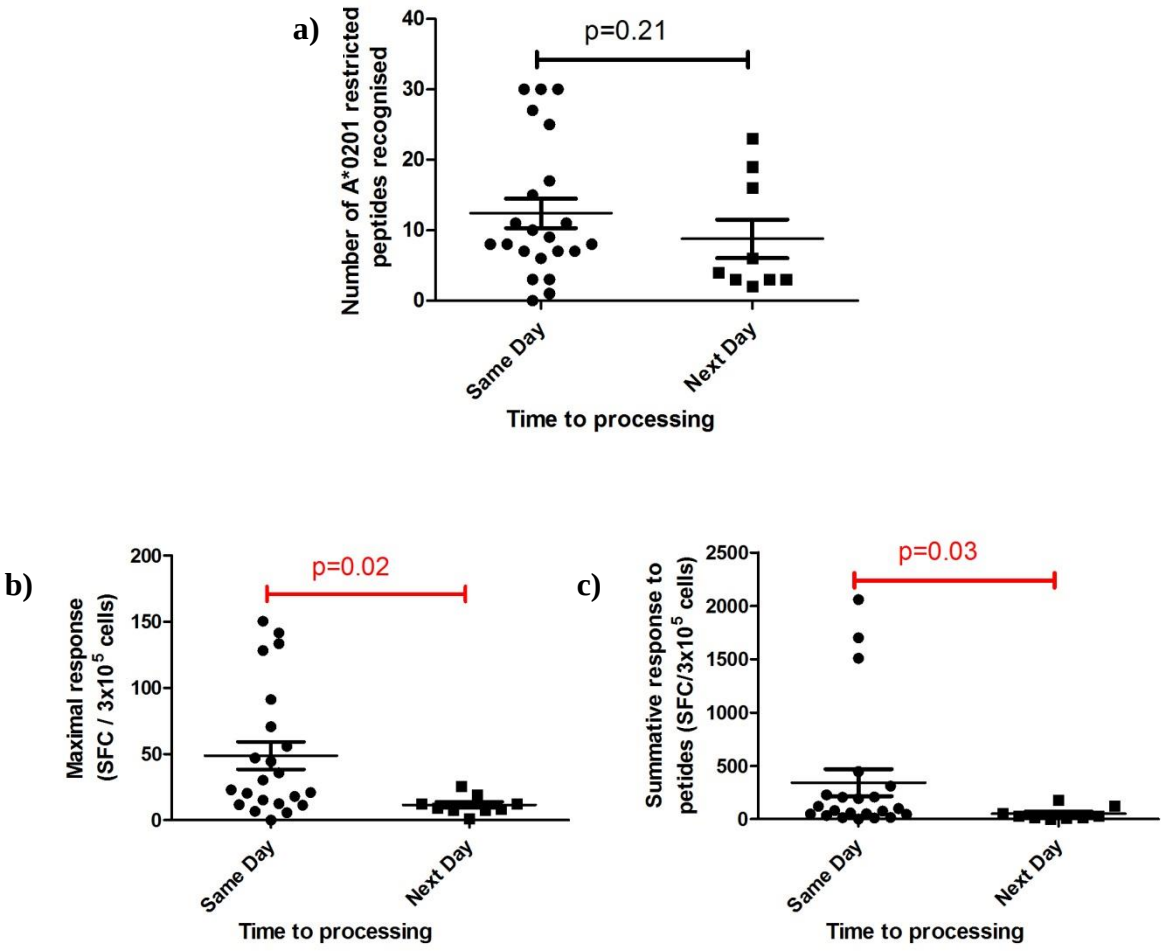


Figure 3-7 Effect of time-to-processing on ELISPOT responses to HLA-A*02:01-restricted peptides

a) Number of peptides recognised, b) maximal response to any peptide and c) summative response to any peptide time to processing. Maximal and summative responses to HLA-A*02:01-restricted EBV peptides, although not number of peptides recognised, are significantly higher in samples processed on the same day as collection, versus those samples processed the next day. p-values of < 0.05 are considered significant, and are shown in red.

3.3.3.1 Sub-group analyses to assess effect of confounding variables

On the basis of the differences observed in CTL response by sex and time-to-processing, these variables were studied in further detail, to assess if differences by HLA group would continue to be observed. Sub-group analyses of CTL response by HLA genotype were performed including samples only from female donors (n = 15, Figure 3.8), or with only samples processed the same day (n = 22, Figure 3.9). In the analysis of female donors only, significant differences were no longer observed when the number of peptides eliciting a response and the single maximal response were compared between A*02:01/x heterozygotes and the other two groups. HLA-A*02:01/x heterozygotes were still observed to have higher summative responses than either HLA-A*02:01 homozygotes or HLA-A*02:01/A*01:01 heterozygotes (p = 0.01). In the analysis of samples processed on the same day, significant differences in number of peptides eliciting a response and the single maximal response by HLA-A*02:01 genotype were no longer observed. Higher summative responses continued to be observed in HLA-A*02:01/x heterozygotes compared with HLA-A*02:01/A*01:01 heterozygotes, and this approached significance (p = 0.08).

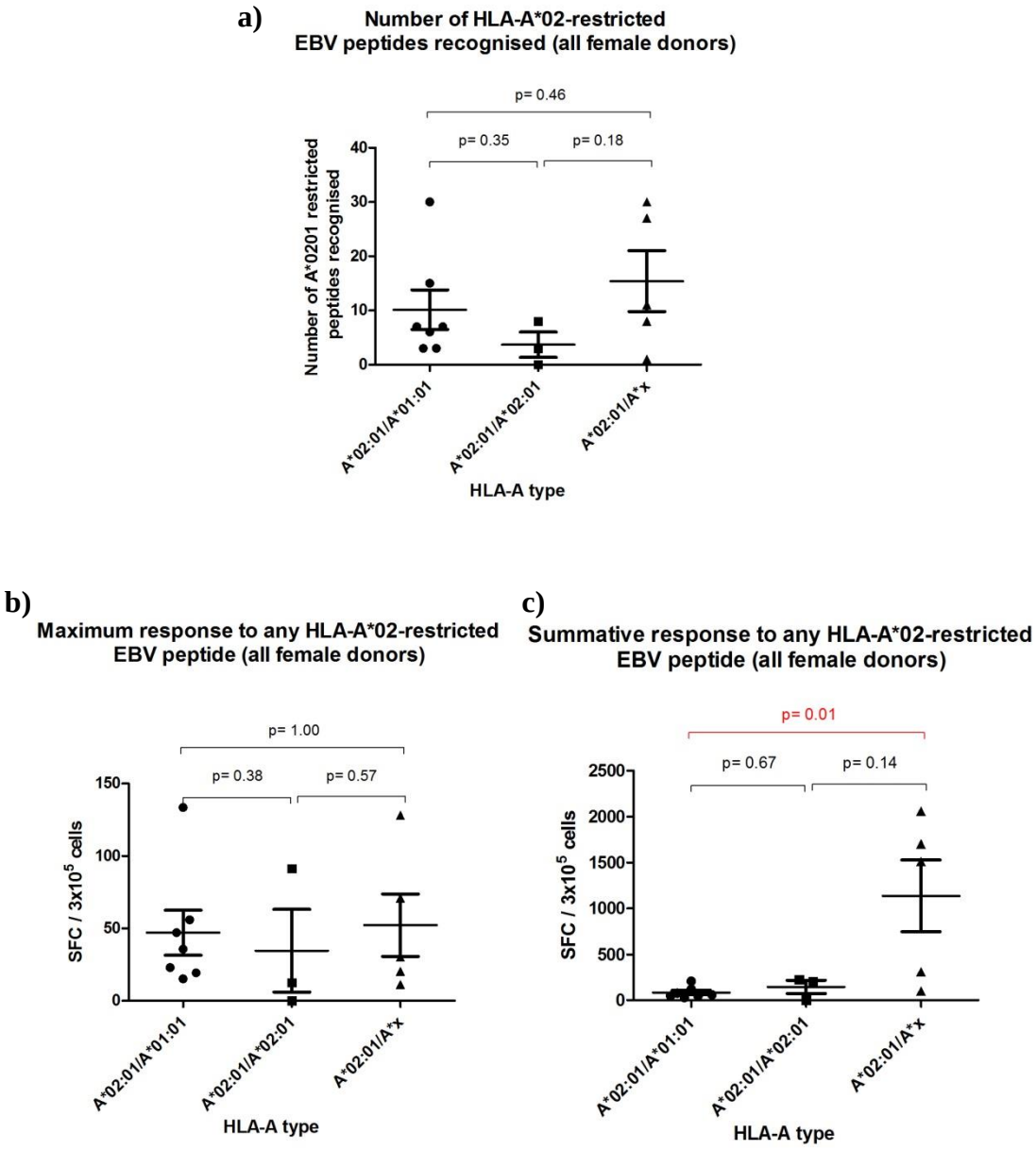


Figure 3-8 Sub-group analysis of responses in female donors only

Responses to all peptides in samples from female donors by HLA-A genotype group. Results are presented by a) number of HLA-A*02:01-restricted EBV peptides recognised, b) maximal response to any HLA-A*02:01-restricted EBV peptide, c) summative response to any HLA-A*02:01-restricted EBV peptide. Error bars demonstrate the mean and the standard error of the mean. p-values of < 0.05 are considered significant, and are shown in red.

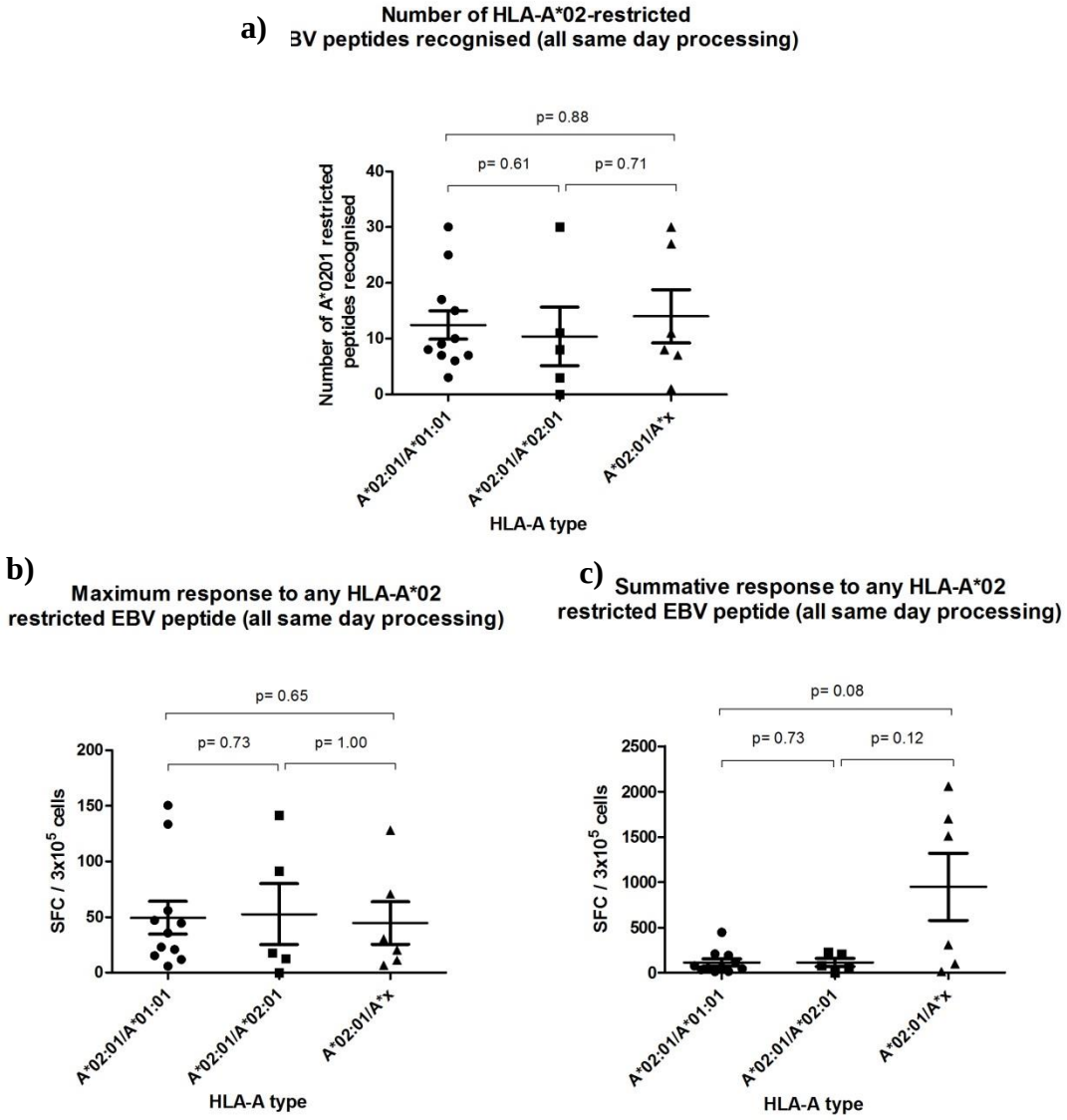


Figure 3-9 Sub-group analysis in samples processed on the same day as collection

Responses to all peptides in samples processed on the same day as collection by HLA-A genotype group. Results are presented by a) number of HLA-A*02:01-restricted EBV peptides recognised, b) maximal response to any HLA-A*02:01-restricted EBV peptide, c) summative response to any HLA-A*02:01-restricted EBV peptide. Error bars demonstrate the mean and the standard error of the mean. p-values of < 0.05 are considered significant, and are shown in red.

*3.3.3.2 Analysis of difference between HLA-A*02:01/A*01:01 heterozygotes and HLA-A*02:01 homozygotes if HLA-A*02:01/A*x heterozygotes are excluded*

It was apparent from the analyses of confounding variables that sex and time to sample processing were associated both with HLA genotype and ELISPOT response. It was also apparent that the HLA-A*02:01/x heterozygotes were the group with most excluded donors (7 of the 8 excluded donors), the highest proportion of females (5/6), and in which all of the samples were processed on the same day. It is probably not valid, therefore, to draw any conclusions for ELISPOT responses in this group.

The other two groups (HLA-A*02:01/A*02:01 homozygotes and HLA-A*02:01/A*01:01 heterozygotes) were well matched (Table 3.7), and therefore any differences in ELISPOT responses observed between these groups would be likely to be real. As mentioned above, no significant difference in any measure of ELISPOT response was noted between these two groups.

Table 3-7 Comparison of donor characteristics between HLA-A*02:01 homozygotes and HLA-A*02:01/A*01:01 heterozygotes only

	HLA-A* 02:01 Group		p-value for differences between groups
	HLA-A* 02:01 / A*01:01 heterozygotes	HLA-A*02:01 homozygotes	
Total Number	14	11	N/A
Female, n (%)	7 (53.8)	3 (27.3)	p = 0.24 ‡
Age (median, range)	47 (23 -59)	45 (28-58)	p = 0.63 †
DEPCAT 5 (mean, range)	2.23 (1-5)	3.37 (1-5)	p = 0.10 †
Monospot-confirmed previous IM §, n (%)	2 (14.3)	2 (18.2)	p = 1.00 ‡
Suspected previous IM §, n (%)	2 (14.3)	2 (18.2)	p = 1.00 ‡
Current smoker, n (%)	1 (7.1)	2 (18.2)	p = 0.57 ‡
Ever smoked? n (%)	1 (7.1)	1 (9.1)	p = 1.00 ‡
Processed within 24 hours, n (%)	11 (78.6)	5 (45.5)	p =0.12 ‡

† Regression (2-tailed t-test)

‡ Fisher's exact test

§ Note all IM is self-reported. As defined in this table, suspected previous IM is where an individual reported previous IM which was not confirmed by a blood test, and Monospot-confirmed IM is where the individual has told us that a doctor confirmed their IM using a blood test. A p-value of < 0.05 is considered significant.

Linear logistic regression modelling, to adjust for the effects of confounding variables, was not performed as a) ELISPOT response results were not normally distributed, b) donor numbers were small, and c) the inclusion of only one male donor in the HLA-A*02:01/x group precluded adjustment for the effect of sex.

3.3.4 CTL Degranulation by donor HLA-A*02:01 genotype

Assessment of T cell cytotoxicity by measuring cellular degranulation was performed by flow cytometric analysis of CD107 expression after exposure to peptide pools. PBMCs from the same 39 donors described in Section 3.3.1 were exposed to the pools of EBV peptides described in Section 2.7.1 (two pools of latent peptides and one pool of lytic peptides). Two cases were excluded, one because of unacceptably high levels of staining in the isotype negative control (LRF patient number 10495) and the other for high baseline and negative control CD107 levels rendering the result uninterpretable (LRF patient number 10485). Results are presented in Figure 3.10

Four individual donors (three HLA-A*02:01/A*x heterozygotes and one A*02:01 homozygote, Figure 3.10) demonstrated higher levels of CD107 expression following exposure to the EBV peptide pools (latent and lytic). These donors, p10098, p10100, p10115 and p10279, were not the same donors in whom particularly high ELISPOT responses were observed. HLA-A*02:01/x heterozygotes demonstrated greater cytotoxicity assessed by CD107 expression than either A*01:01/A*02:01 heterozygotes or A*02:01 homozygotes. No difference was observed between HLA-A*02:01 homozygotes and HLA-A*02:01/A*01:01 heterozygotes, thus echoing the pattern in the IFN-γ ELISPOTS.

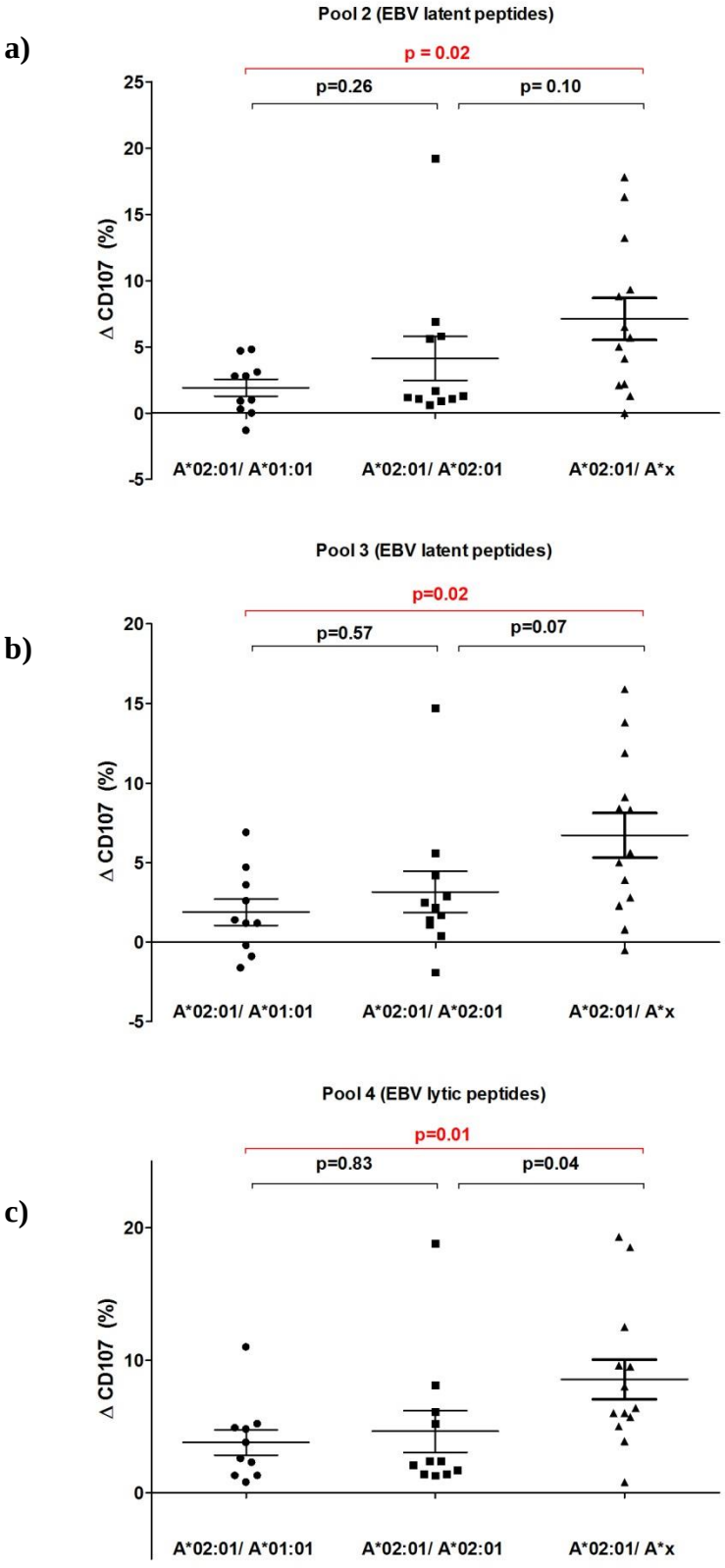


Figure 3-10 CTL degranulation, by HLA-A*02:01 genotype

As assessed by CD107 expression following stimulation. ΔCD107, Difference in CD107 expression from baseline (in percent) genotype group. Responses shown are to a) Pool 2 (a pool of HLA-A*02:01-restricted EBV latent peptides), b) Pool 3 (a pool of HLA-A*02:01-restricted EBV latent peptides), and c) Pool 4 (the pool of HLA-A*02:01-restricted EBV lytic peptides). Error bars demonstrate the mean and the standard error of the mean. p-values of < 0.025 are considered significant, and are shown in red.

Analysis of potential confounding variables was performed (Table 3.8). Unlike in the ELISPOT analyses, sex of the donor had no effect on the level of CD107 expression following stimulation by EBV peptide pools. However, same day processing was associated with higher ΔCD107 values; thus, as with the ELISPOT responses, any differences observed in the HLA-A*02:01/A*x heterozygote group cannot be considered valid.

Table 3-8 Potential confounding variables and CD107 responses

ΔCD107 response	p-value for associations with confounders (2-tailed t test)					
	Time to process sample	Sex	Donor reported blood test confirmed IM	Ever smoked	% of CD3 cells positive for HLA-DR	CD4/CD8 ratio
Pool 2	**0.03**	0.71	0.09	0.18	0.16	0.54
Pool 3	**0.05**	0.17	0.17	0.64	0.23	0.99
Pool 4	**0.07**	0.53	0.55	0.97	0.17	0.89

† Equal variances not assumed, data not normally distributed, Mann Whitney analysis performed for categorical variables and Kruskal-Wallis analysis performed for continuous variables. p-values of < 0.10 are considered significant (highlighted in blue).

3.3.5 Cytokine production following stimulation of BD-PBMCs with EBV peptides by donor HLA-A*02:01 genotype

Cells from the same 39 donors used for the CD107 flow cytometry experiments were also used to assess cytokine secretion by HLA-A*02:01 genotype in response to EBV peptide. The same pools of peptides derived from EBV proteins expressed during latent infection ("latent peptides") and EBV proteins expressed during the lytic replication phase ("lytic peptides") as described in Section 2.7.1 were used in an overnight stimulation (described in Section 3.2.5).

Results are given as level of a given cytokine in pg/ml following the stimulation. Results of responses to lytic peptides are given in Figure 3.11, and those in response to latent peptides in Figure 3.12.

The mean level of each cytokine as secreted in response to overnight stimulation with lytic peptides is given in Table 3.9 and to latent peptides in Table 3.10. The statistical analyses of the cytokine secretion levels are presented in Table 3.11.

In response to lytic peptides, HLA-A*02:01/A*01:01 heterozygotes produced significantly higher levels of IL-10 (mean 1427 vs. 155 pg/ml $p = 0.03$), IL-17 (84 vs. 70 pg/ml $p = 0.02$) and IL-5 (35 vs. 29 pg/ml $p = 0.04$) compared to both other HLA-A groups. In response to latent peptides, IFN-γ secretion was higher in A*02:01 /x heterozygotes compared to other groups (2552 vs. 552 pg/ml, $p = 0.007$).

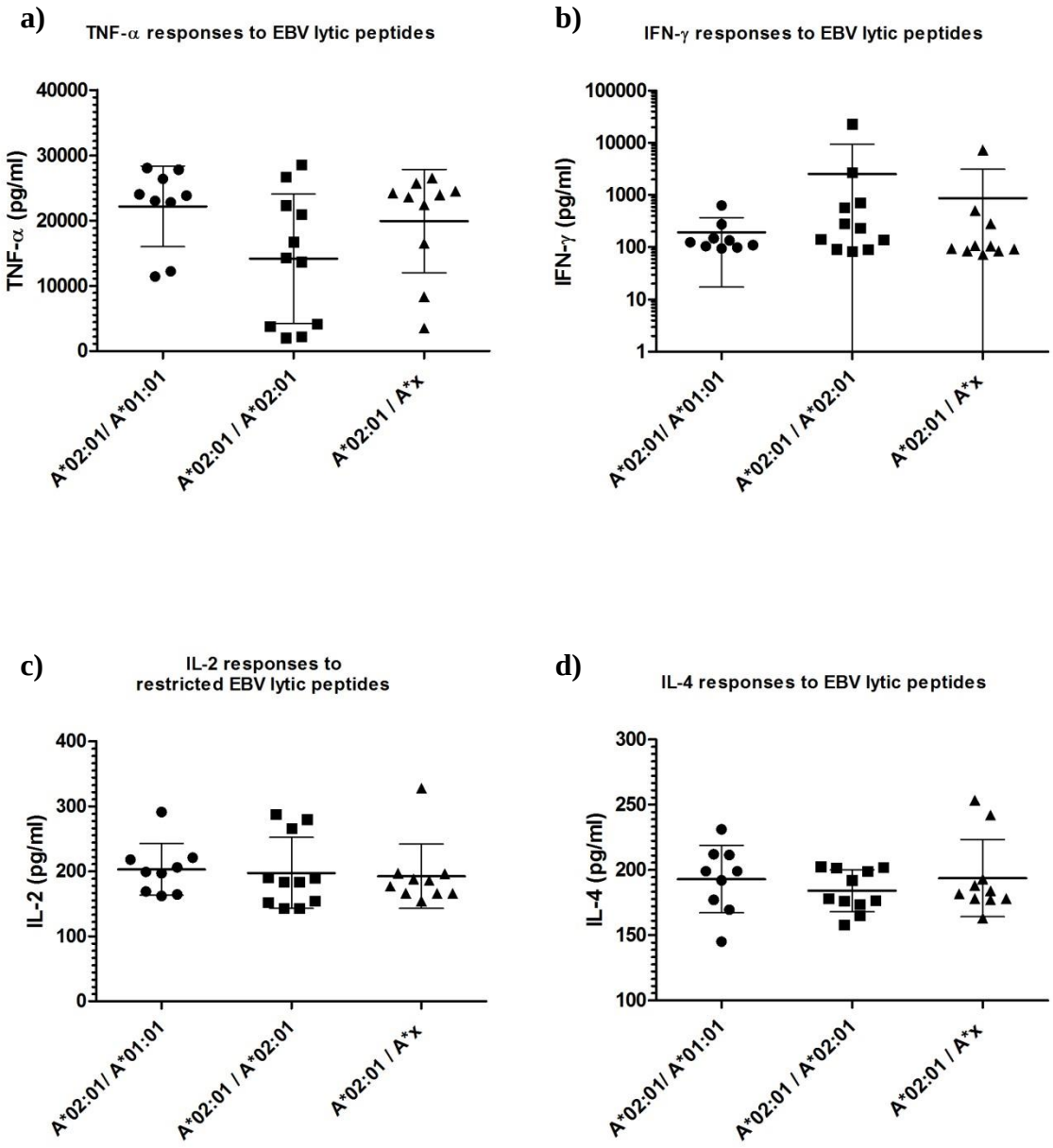


Figure 3-11 Cytokine secretion in response to EBV lytic peptides, by HLA group

a) TNF-α, b) IFN-γ, c) IL-2, d) IL-4, e) IL-5, f) IL-6, g) IL-10, h) IL-12 and i) IL-17. Figure over two pages (this page and overleaf). Concentrations are in pg/ml. Note different scales on y-axis used due to differences in levels of cytokines secreted. Bars represent mean and standard deviation. For statistical analysis of these data see Table 3.11.

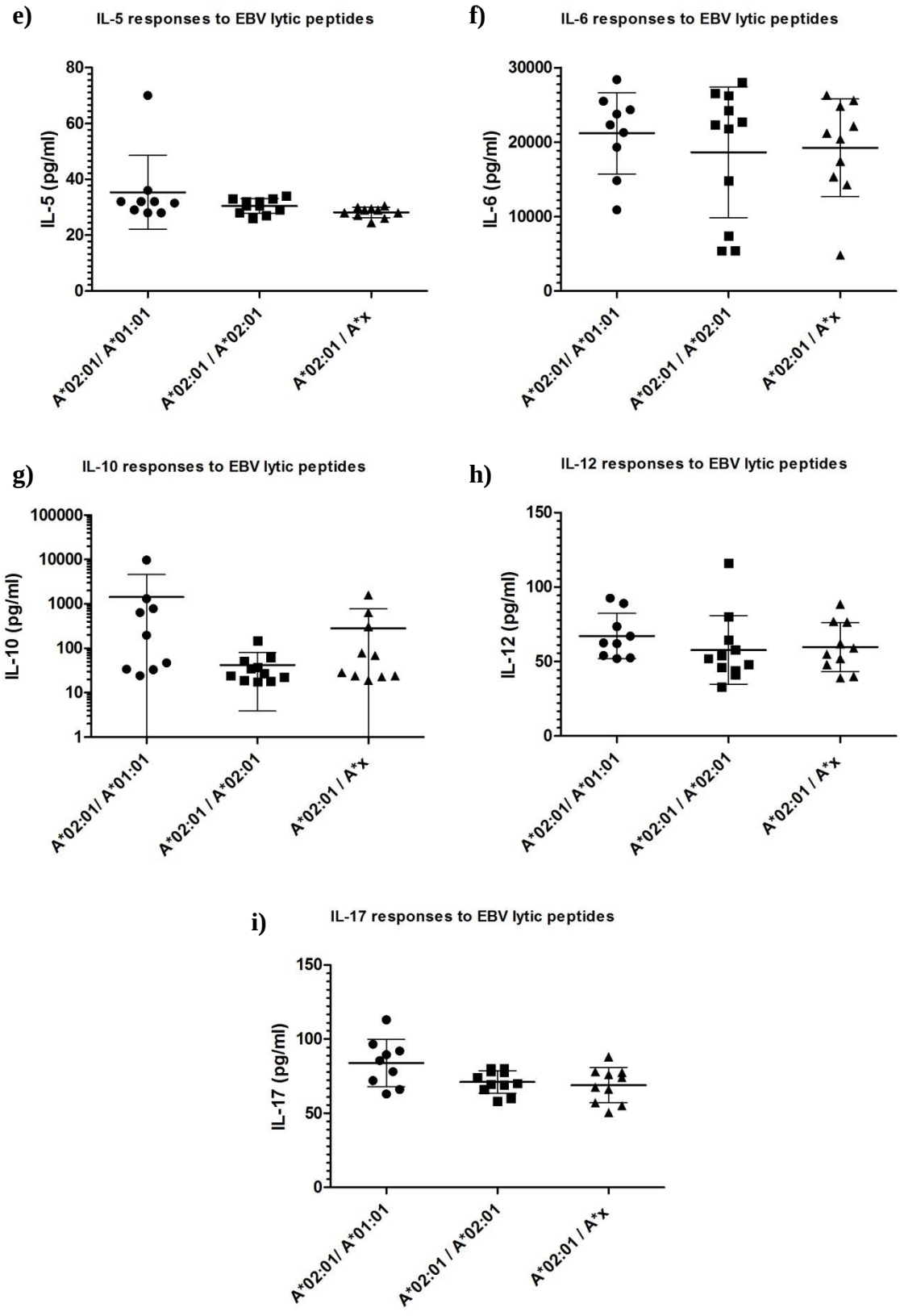


Figure 3-11 (Continued) Cytokine secretion in response to EBV lytic peptides, by HLA group

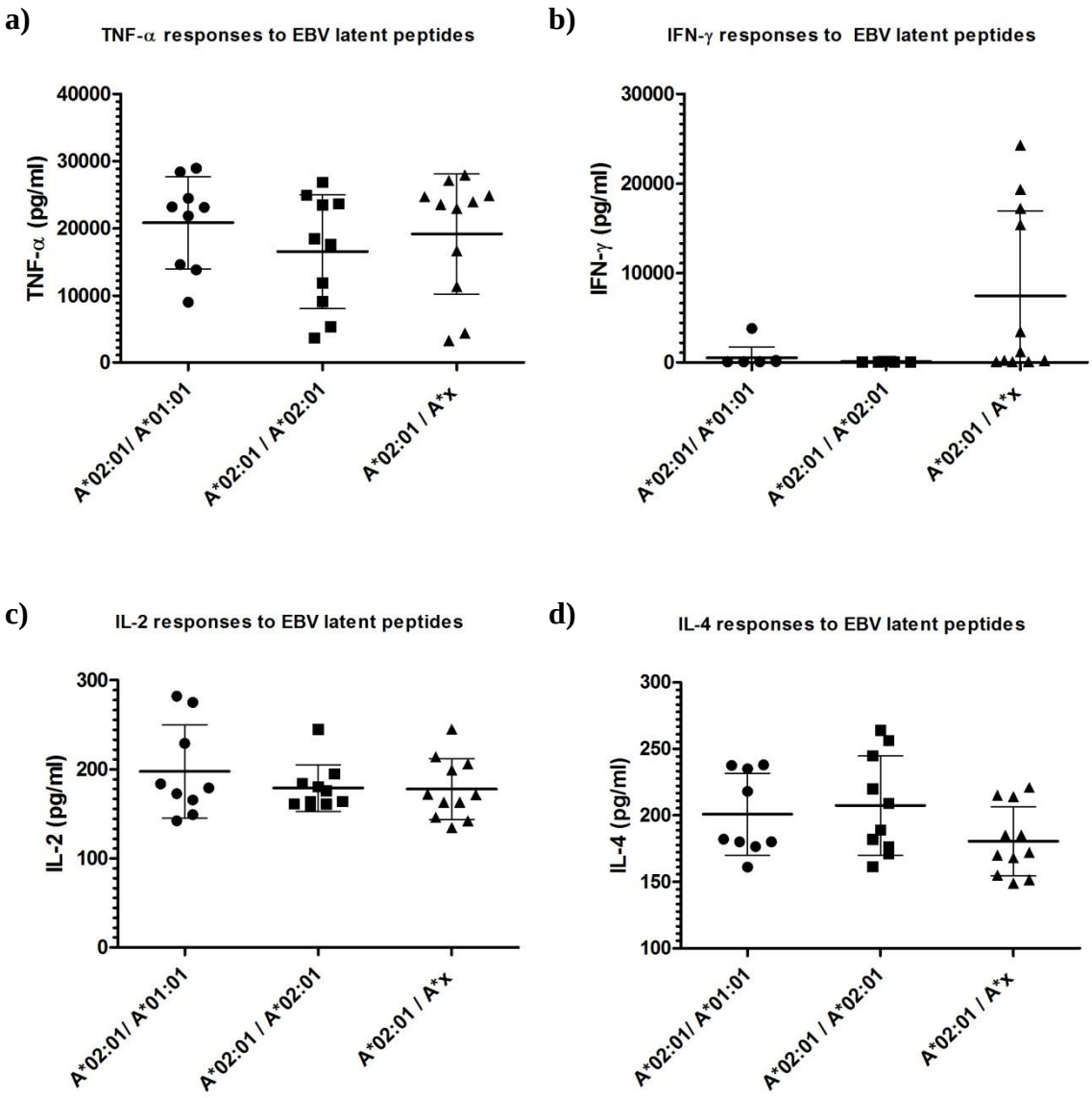


Figure 3-12 Cytokine secretion in response to EBV latent peptides, by HLA group

a) TNF-α, b) IFN-γ, c) IL-2, d) IL-4, e) IL-5, f) IL-6, g) IL-10, h) IL-12 and i) IL-17. Figure over two pages (this page and overleaf). Concentrations are in pg/ml. Note different scales on y-axis used due to differences in levels of cytokines secreted. Bars represent mean and standard deviation. For statistical analysis of these data see Table 3.11.

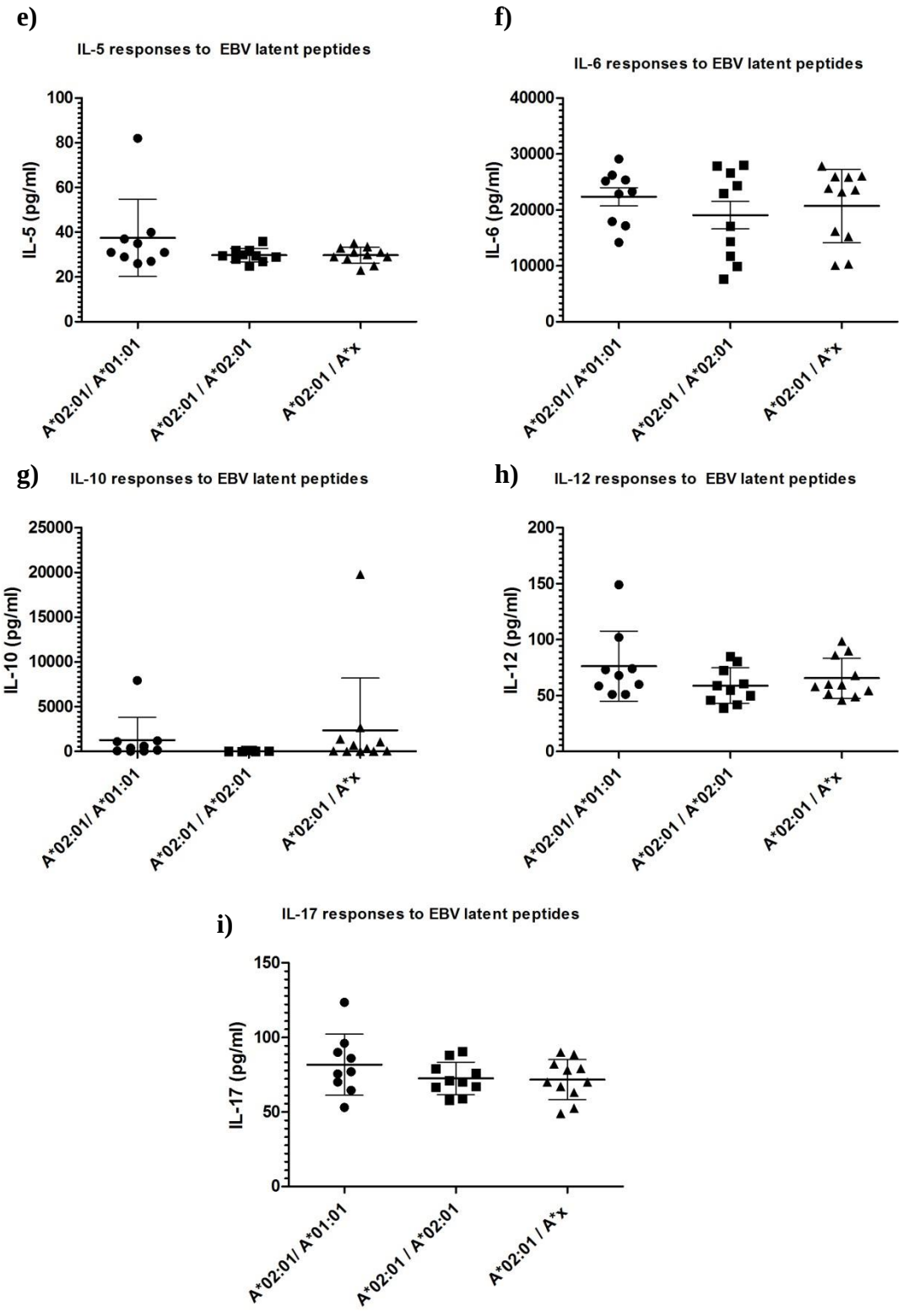


Figure 3-12 (Continued) Cytokine secretion in response to EBV latent peptides, by HLA group

Table 3-9 Cytokine secretion in response to lytic HLA-A*02:01-restricted EBV peptides

Cytokine	HLA Group					
	HLA-A*02:01/ A*01:01 heterozygotes		HLA-A*02:01 / A*02:01 homozygotes		HLA-A*02:01 / other heterozygotes	
	Mean (pg/ml)	SD	Mean (pg/ml)	SD	Mean (pg/ml)	SD
IL-10	1427.33	3161.29	42.14	38.28	279.50	498.61
IL-4	192.89	25.82	184.00	16.02	193.80	29.63
IL-2	203.11	39.94	197.59	54.89	192.50	49.72
IL-12 p70	67.22	15.11	57.86	23.01	59.70	16.50
IFN-γ	192.06	174.68	2552.27	6816.35	876.90	2272.62
IL-17	83.94	15.96	71.09	7.64	68.95	11.93
IL-5	35.39	13.22	30.45	2.65	28.15	1.87
TNF-α	22164.05	6182.87	14135.09	9903.47	19914.20	7939.74
IL-6	21183.33	5447.03	18637.00	8773.70	19240.60	6548.13

Lytic EBV peptides, peptides derived from proteins expressed during EBV replicative cycle. Raw data shown as mean (pg/ml) and standard deviation. Results presented by HLA group.

Table 3-10 Cytokine secretion in response to HLA-A*02:01-restricted latent EBV peptides

Cytokine	HLA Group					
	HLA-A*02:01/ A*01:01 heterozygotes		HLA-A*02:01 / A*02:01 homozygotes		HLA-A*02:01 / other heterozygotes	
	Mean (pg/ml)	SD	Mean (pg/ml)	SD	Mean (pg/ml)	SD
IL-10	1282.50	2539.99	50.45	44.93	2377.18	5837.97
IL-4	200.89	30.80	207.45	37.36	180.50	26.11
IL-2	197.50	52.15	179.00	26.09	177.82	34.28
IL-12 p70	76.28	31.41	58.95	15.91	65.55	17.91
IFN-γ	531.17	1235.08	104.50	30.39	7430.50	9509.47
IL-17	81.72	20.48	72.50	10.98	71.73	13.43
IL-5	37.56	17.29	29.80	3.04	29.77	3.57
TNF-α	20816.55	6862.47	16528.00	8486.19	19157.91	8931.80
IL-6	22355.89	4898.52	19060.85	7831.87	20721.96	6564.74

Latent EBV peptides, peptides derived from proteins expressed in EBV latent phase. Raw data shown as mean (pg/ml) and standard deviation (SD). Results presented by HLA group.

Table 3-11 Statistical analysis of cytokine secretion in response to EBV peptides

	Response to Lytic Peptides		Response to Latent Peptides	
	Primary analysis HLA - A*02:01/A*01:01 vs. other groups	Secondary analysis - between all three groups	Primary analysis HLA - A*02:01/A*01:01 vs. other groups	Secondary analysis - between all three groups
Cytokine	Mann-Whitney (p-value)	Kruskal-Wallis (p-value)	Mann-Whitney (p-value)	Kruskal-Wallis (p-value)
IL-10	0.03	0.04	0.06	0.10
IL-4	0.46	0.63	0.51	0.15
IL-2	0.21	0.44	0.37	0.64
IL12 p70	0.10	0.22	0.20	0.32
IFN-γ	0.98	0.31	0.59	< 0.01
IL-17	0.03	0.08	0.25	0.51
IL-5	0.09	0.04	0.17	0.39
TNF-α	0.17	0.15	0.57	0.65
IL-6	0.60	0.81	0.51	0.76

Latent EBV peptides, peptides derived from proteins expressed in EBV latent phase; lytic EBV peptides, peptides derived from proteins expressed during EBV replicative cycle. P-values are given to 2 decimal places. A p-value of < 0.05 is considered significant (highlighted in blue). Results presented by HLA group.

3.3.5.1 Analysis of cytokine secretion for potential confounding variables

As confounding had been detected in the analysis of ELISPOT and CD107 results, analysis for confounding on the cytokine secretion results was performed. As can be seen, the distribution of levels of some cytokines was different by time to processing, sex, smoking status and CD4/CD8 ratio; however smoking status and CD4/CD8 ratio were not significantly different in the three HLA groups and therefore would not have affected the results. Echoing the result of the ELISPOT experiments, secretion of cytokines could plausibly be confounded by time to processing and sex, and therefore any differences in cytokine secretion observed in A*02:01 /x heterozygotes in response to EBV peptides cannot be regarded as valid.

Table 3-12 Cytokine secretion by potentially confounding variables

Peptide pool	Cytokine	p-value for associations with confounders					
		Time to process sample	Sex	Donor reported blood test confirmed IM	Ever smoked	% of CD3 cells positive for HLA-DR	CD4/CD8 ratio
Latent	TNF-α	0.23	0.82	0.80	0.18	0.24	0.32
	IL-10	***0.05***	0.53	0.33	0.79	0.11	0.29
	IL-4	***0.06***	***0.00***	0.70	0.81	0.12	0.38
	IL-2	0.82	0.46	0.18	0.25	0.13	0.37
	IL-12	0.35	0.59	0.77	0.53	0.17	0.35
	IL-6	***0.03***	0.53	0.90	0.50	0.73	0.48
	IFN-γ	***0.08***	0.16	0.73	0.54	0.86	0.20
	IL-17	0.31	***0.02***	0.26	0.37	0.28	0.32
	IL-5	0.15	0.88	0.76	0.38	0.88	***0.02***
Lytic	TNF-α	0.26	***0.05***	0.61	0.18	0.35	0.28
	IL-10	0.81	0.19	0.42	0.97	0.13	0.37
	IL-4	0.12	***0.05***	0.82	***0.05***	0.79	0.13
	IL-2	0.30	0.65	0.92	0.40	0.84	0.44
	IL-12	0.30	***0.07***	0.72	0.12	**0.09**	0.33
	IL-6	0.24	0.86	0.38	0.16	0.69	0.65
	IFN-γ	***0.00***	***0.10***	0.42	***0.01***	0.27	0.42
	IL-17	0.47	0.43	0.68	0.99	0.97	0.47
	IL-5	0.25	0.50	0.32	***0.04***	0.25	0.16

† Equal variances not assumed, data assumed to be not normally distributed, Mann Whitney analysis performed for categorical variables and Kruskal-Wallis analysis performed for continuous variables. A p-value of < 0.10 is considered significant (highlighted in bold).

As in the ELISPOT experiments, further analysis for confounding was performed if the most confounded group, the HLA-A*02:01/A*x heterozygotes, were removed. This analysis is given in Table 3.12. Of those cytokines where significant differences in secretion by HLA group were noted (IL-10, IL-17 and IL-5) no confounding was found, and, as discussed, HLA-A*02:01 homozygotes and HLA-A*02:01/A*01:01 heterozygotes were well matched such that any comparisons between these two groups remain valid.

3.3.6 Summary of Results

HLA-A*02:01-restricted EBV-specific CTL responses in healthy individuals were examined to assess if the presence of an HLA-A*01:01 allele would modify the magnitude of the CTL response elicited.

Significant differences by HLA-A*02:01 genotype in CTL responses to HLA-A*02:01-restricted EBV peptides as assessed by IFN-γ ELISPOT were observed. In these experiments, HLA-A*02:01/x heterozygotes demonstrated responses of greater magnitude (summative and maximum) and greater breadth (number of peptides recognised), as compared with both HLA-A*02:01 homozygotes and HLA-A*01:01/A*01:01 heterozygotes. This effect was observed for lytic and latent peptides. These data therefore disproved the null hypothesis that responses should be the same in all HLA-A*02:01 genotype groups; however there were no differences between HLA-A*02:01 homozygotes and HLA-A*02:01/A*01:01 heterozygotes and therefore the magnitude of responses did not reflect the risk of developing EBV+ve cHL.

The results were unexpected and analysis of potentially confounding variables shed some light on the possible reasons for this, demonstrating higher ELISPOT responses in females and in samples collected and processed on the same day. The HLA-A*02:01/A*x heterozygote group was found to be highly confounded by these variables, with 100% of specimens being processed on the same day and 5 of the 6 donors being female. The effect of the confounding variables also affected the CTL degranulation experiments, and probably also accounted for the elevated secretion of IFN-γ observed HLA-A*02:01/A*x heterozygote group in the cytokine analysis. Thus, whilst this group did demonstrate greater CTL toxicity assessed by several outcome measures used, this cannot be concluded to

be due to HLA phenotype. As discussed in Section 3.2.6, linear regression modelling with adjustment for these confounding variables was not possible due to non-normally distributed data, small numbers and the inclusion of one male subject in the HLA-A*02:01/A*x group.

Only 31 ELISPOTs passed the QC criteria, falling short of the 45 required by the power calculation, and with the majority of excluded donors falling in to the HLA-A*02:01/A*x heterozygote group. Recruitment to the study reached required targets, but incomplete recall and exclusion of results on the basis of QC criteria led to incomplete attainment of the numbers of required donors. Further studies are being carried out in the laboratory at present to increase numbers in the different HLA groups.

The power calculation was performed prospectively based on assumptions of the nature of the ELISPOT responses which would be seen. However, in retrospect, the data generated could not justify the type of analysis used in the power calculation, as data were not normally distributed and the variability seen was greater than expected.

In response to lytic peptides, HLA-A*02:01/A*01:01 heterozygotes produced significantly higher levels of IL-10 (mean 1427 vs. 155 pg/ml $p = 0.03$), IL-17 (84 vs. 70 pg/ml $p = 0.02$) and IL-5 (35 vs. 29 pg/ml $p = 0.04$) compared to the other HLA-A*02 groups. As all lytic peptides were in the same pool, it was not possible to determine if this effect was principally due to differences in responses to particular group of lytic peptides i.e. those derived from IE vs. late proteins.

3.4 Discussion

That female sex and time-to-processing led to greater IFN-γ CTL responses is perhaps not surprising, although has not previously been reported with regard to EBV responses. Greater magnitude of immune responses in females is a well-recognised phenomenon (Klein, 2012) and is thought, in part, to account for the higher prevalence of autoimmune disease in women. More specifically, the CTL immune response has been shown to be of greater magnitude in women (Hewagama *et al*, 2009), an effect which may be seen in stronger responses to viruses and vaccines (Peacock *et al*, 2004; Villacres *et al*, 2004). It is possible that the higher EBV-specific CTL responses from females observed in this study may go some way to explaining the higher prevalence of EBV-associated malignancies such as cHL (Shenoy *et al*, 2011; Glaser *et al*, 1997), Burkitt's lymphomas (Boerma *et al*, 2004) and NPC (Yu & Yuan, 2002) in men as compared with women.

Samples processed on the same day as donation gave higher responses in the ELISPOTs, again perhaps not surprising, as instinctively one would feel that T cells freshly removed from the body would be capable of greater responses. This was not addressed in the optimisation stage, as ELISPOTS have been demonstrated to be possible and reliable in samples processed up to 30 hours from venesection (Matijevic & Urban, 2005) and in samples shipped at RT e.g. in studies of CTL responses to vaccines (Schultes & Whiteside, 2003; Ferrari *et al*, 1997). The magnitude of difference this made to the results was therefore surprising. In studies where qualitative responses are analysed, in situations where all samples are processed after the same delay or in situations where comparisons are between groups that are well matched this may not matter. However, because of the recruitment strategies adopted in this study, most HLA-

A*02:01/A*x heterozygotes (as the commonest group) were recruited via the CRF (University and NHS staff). The majority of these donors were female and their samples were processed on the same day, principally because of the timing and location of collection. In contrast, HLA-A*02:01/A*01:01 heterozygotes and HLA-A*02:01 homozygotes were more evenly distributed between the CRF donors and the SNBTS donors; SNBTS donors having a more equal sex distribution and more likely to have samples processed the following day.

If this study were to be repeated or extended, groups would have to be matched for sex, or restricted to one sex, and to include only samples processed on the same day as collection.

Delays in processing resulting from the B cell selection (Section 2.3.3) should also be avoided, given these results. Pragmatically adopted to maximise the use of a donation from a given donor, the separation step did not lead to any difference in ELISPOT responses, but all comparisons were performed on samples processed the same day as collection.

Analysis of a panel of inflammatory cytokines measured in response to stimulation of PBMC by EBV peptides demonstrated significant differences between the HLA-phenotype groups. The elevated secretion of IFN-γ by HLA-A*02:01/A*x heterozygotes in response to EBV peptides is likely to be confounded by the same factors discussed above, and cannot be accepted as valid.

However, this analysis did demonstrate a possible effect of HLA-A*01:01 in HLA-A*02:01/A*01:01 heterozygotes, as this group demonstrated significantly higher secretion of IL-10 (with a nearly 10-fold difference), IL-17 and IL-5 in response to

stimulation with EBV peptides. Analysis of potential confounders suggests not only that comparisons of these cytokines were not affected by confounding, but also that comparisons between HLA-A*02:01/A*01:01 heterozygotes and HLA-A*02:01 homozygotes are valid. Whilst a criticism of any multiplexed approach is detection of significant differences through multiple analysis (Bonferroni correction was not performed due to sample size), the magnitude of the difference observed for IL-10 is provocative and should be investigated further.

The null hypothesis of this study, that all HLA-A*02:01 carriers should have the same response to HLA-A*02:01-restricted EBV peptides was not disproved. Responses in the HLA A*02:01 / A*x heterozygote group were greater than in the other groups but had to be disregarded for reasons of confounding, and adjusted analysis was not possible.

Comparisons of CTL responses in HLA-A*02:01/A*01:01 heterozygotes and HLA-A*02:01 homozygotes were valid, but differences by ELISPOT, CTL degranulation as assessed by CD107 expression and by IFN-γ secretion were not demonstrated. I did not find evidence that HLA-A*01:01 specifically inhibited CTL responses to A*02:01-restricted EBV peptides.

The primary experimental question "*Does the presence of an HLA-A*01:01 allele modify the magnitude of the CTL response to HLA-A*02:01-restricted epitopes?*" was partly answered. Exploratory analysis of cytokine levels in response to stimulation with EBV peptides did demonstrate differences in HLA-A*02:01/A*01:01 heterozygotes, compared to other groups. In response to EBV lytic peptides, A*02:01/*01:01 heterozygotes demonstrated significantly higher levels of cytokines associated with a T-helper (Th) cell response. A Th response is typically generated by CD4 cells via a HLA class II (not class I) restricted

response. A number of possibilities may explain this; first the existence of the Ts population, a population of inhibitory, regulatory CD8+ve, CD28-ve T cells (Chess & Jiang, 2004; Cortesini *et al*, 2001), which initiate a secondary immune response which includes secretion of cytokines associated with suppression of a CTL response. Second, as discussed in the Introduction, the class I HLA genes sit in a large cluster of genes on chromosome 6, the function of many of which are immune in nature. This includes many genes directly or indirectly involved in cytokine expression. There are high levels of LD in this area of the chromosome, therefore the level of secretion of any given cytokine may not be directly causal, but instead could be as a result of association with a class I HLA gene. Therefore, it is biologically plausible that different levels of "Th2" cytokines may be seen in response to viral stimuli by class I HLA genotype.

IL-5 is produced by Th2 cells and is important in the antibody mediated immune response and in recruitment of eosinophils. IL-5 has also been shown to be important as an autocrine growth factor in EBV-transformed LCLs (Baumann & Paul, 1992). In addition, a Th2 profile which includes IL-5 as part of the process driving eosinophilia, is known to be implicated in the pathogenesis of cHL (Di Biagio *et al*, 1996).

IL-17 is a pro-inflammatory cytokine and is the archetypal cytokine of a group of cytokines which conventionally determine the response of the subset of CD4+ Th cells known as Th17 cells. Physiologically, the IL-17 response is thought to be important in mucosal immunity. IL-17 producing T-cell subsets have been implicated in the pathogenesis of autoimmune diseases such as MS and type 1 diabetes mellitus (Mills, 2008), and also chronic active EBV infection (Ohta et al, 2013) where seemingly immune competent individuals can succumb to severe,

potentially fatal illness in the face of EBV infection. In addition, in recent years, it has been recognised that IL-17 can also be produced by novel CD8+, CD161+ T lymphocytes, the so-called Tc17 cells (Tajima *et al*, 2011; Sundrud & Trivigno, 2013). Such Tc17 cells have been observed in solid tumours such as gastric cancer where they influence the tumour microenvironment by promoting migration of myeloid derived suppressor cells and suppressing CD8+ CTL function (Zhuang *et al*, 2012). Tumour associated macrophages are known to be important in cHL where they are associated with poor outcome (see Section 1.3.7) and it is notable that these can stimulate differentiation of Tc17 cells (Zhuang *et al*, 2012). It is possible that the differential IL-17 secretion is observed by class I HLA type in this study may be due to antigen specific Tc17 responses. Whilst not assessed in this study, this hypothesis could be evaluated, e.g. by flow cytometry, in future studies of HLA-A*01:01 restricted responses to EBV.

IL-10 was originally thought to be produced only by Th2 cells. It is now known that IL-10–secreting CD8 T cells represent a phenotypically distinct Ts cell lineage (Noble, Blood, 2006) and that the IL-10 produced by effector Th1 cells normally help to limit the collateral damage caused by exaggerated inflammation (Trinchieri, JEM, 2007). There is strong evidence that IL-10 may play an important role in the pathogenesis of cHL. In addition to the described immune suppressive roles, there is also some evidence that IL-10 can enhance B cell proliferation (Guedez *et al*, 2001). Levels of IL-10 have been found to be elevated in adults with cHL, where they are associated with poor prognosis (Sarris *et al*, 1999; Rautert *et al*, 2008). IL-10 has been particularly implicated in the pathogenesis of EBV+ve cHL (Ohshima *et al*, 1995), and in a recent study, a particular IL-10 gene polymorphism (rs 1800890) was associated with lower risk

of HL in adults over 40, whose disease is more commonly EBV-associated (Yri *et al*, 2012). Differential secretion of IL-10 by HLA-A*02:01 genotype in response rubella vaccination has been reported (Ovsyannikova *et al*, 2007), although has not yet been described in response to EBV.

These data suggest that overall HLA-A phenotype does not significantly affect the EBV-specific CTL response restricted through HLA-A*02:01. However, the cytokine profiles observed following stimulation with EBV peptides might begin to explain some of the HLA-associated differences in risk of developing EBV+ve cHL, and merit further exploration.

Chapter 4. Can HLA-A*01:01-restricted responses to EBV be detected?

4.1 Introduction

Class I HLA genes determine CTL responses, keeping viral infection under tight control in healthy hosts. As discussed in the Introduction and in Chapter 3, HLA-A*01:01 is associated with increased risk of EBV+ve cHL whereas HLA-A*02:01 is associated with decreased risk (Hjalgrim *et al*, 2010). In the model described in this paper, the OR for EBV-related cHL conveyed by each additional HLA-A*01 allele was 2.15 (95% CI, 1.60–2.88).

The association between HLA-A*01:01 and EBV+ve cHL suggests that such EBV-specific immune responses might be important in the pathogenesis of this disease. Many immunodominant EBV peptides are known to be presented by class I alleles other than HLA-A*01:01. To date, no HLA-A*01:01-restricted response to any EBV-derived peptide has been described. However, individuals who carry HLA-A*01:01 will also have other class I HLA alleles through which they may be able to raise an EBV-specific CTL response. It is not clear whether the increased risk associated with HLA-A*01:01 is simply because there are no EBV-specific responses restricted through this allele, or whether HLA-A*01:01 exerts qualitative or inhibitory changes to the EBV-specific immune response.

EBV is an intensively studied virus. Over the last 20 years, a number of groups have interrogated the EBV-specific CTL responses. Generally, these studies were concerned with mapping T cell epitopes and HLA-restrictions, and did not specifically seek or examine the HLA-A*01:01-restricted response. It is notable, however, that in spite of intensive study, no EBV peptide epitopes have been described that present through HLA-A*01:01 (Hislop *et al*, 2007; Long *et al*, 2011b; Moss *et al*, 2001). HLA-A*01:01 is able to efficiently present peptides from viruses other than EBV. For instance, immunodominant peptides from CMV

and Influenza raise very efficient CTL responses via this allele (Currier *et al*, 2002; DiBrino *et al*, 1993; Elkington *et al*, 2003; Wills *et al*, 1996). Thus, there is no suggestion that HLA-A*01:01 is in any way deficient in terms of antigen presentation capability.

The only study to specifically examine the HLA-A*0:01 response (Brennan & Burrows, 2008) used a strategy of stimulating responses with A*01:01 expressing 721.221 LCLs, assessing responses with intracellular cytokine staining for IFN-γ. Negligible responses were detected; however, good EBV-specific responses were noted to 721.221 LCLs transduced with other class I alleles, and to HLA-A*01:01-restricted peptides from other viruses e.g. CMV. Potential weaknesses of this study are that weak responses might represent allogeneic responses to minor histocompatibility antigens presented by the 721.221 cells, and that intra-cellular cytokine staining assay is less sensitive than other techniques, such as ELISPOT, for detecting CTL responses. However, in general, this study supported the hypothesis that there are no HLA-A*01:01-restricted CTL responses to EBV.

In the present study we sought to determine whether HLA-A*01:01-restricted EBV-specific CTL responses could be detected. In addressing this question, a sensitive methodology (ELISPOT) was adopted, and a strategy of using synthesised EBV-sequence derived peptides to avoid erroneous detection of allogeneic responses to minor histocompatibility antigens. A stimulation step using EBV-infected LCLs was employed to boost any low-level memory-CD8 CTL responses present. As a secondary exploratory question, I examined whether any inhibitory cytokine responses could be detected following stimulation of PBMCs with LCLs expressing HLA-A*01:01.

4.2 Methods

4.2.1 Power calculation

To determine whether HLA-A*01:01-restricted EBV-specific CTL responses can be detected by ELISPOT analysis, healthy individuals homozygous for HLA-A*01:01 were studied.

The sample size was determined prospectively using power calculations performed by Dr. Paul Johnson, Robertson Centre for Biostatistics, University of Glasgow. It was determined that 10 individuals homozygous for HLA-A*01:01 were required to detect statistically significant EBV-specific CTL responses. With n = 10, assuming a background negative control SFC of 3% as compared to the positive controls, a SD of 2 percentage units and a cut-off for a positive response of 5% of the positive control, n = 10 will give 85% power to detect any response using a paired t-test. As most published ELISPOT studies report positive responses at a level of at least 8% of the positive control, I should be able to detect any HLA-A*01:01-restricted EBV-specific CTL responses should they exist.

Local population frequencies for class I HLA genotypes were available from the in-house SNEHD study controls (Jarrett *et al*, 2003). HLA-A*01:01 homozygotes are relatively uncommon in the population (approximately 5%) and thus, these data suggested that approximately 200 adults would need to be HLA-typed to identify 10 individuals homozygous for HLA-A*01:01. Supplementing the HLA-unscreened general population with a HLA-targeted approach to donor recruitment via the SNBTS donor population meant that these targets were achieved (see Chapter 2). EBV-seropositive individuals homozygous for HLA-A*01:01 (n = 11) were selected from a pool of healthy adults who agreed to take part in this study. The donors used are listed in Table 4.1. None of these HLA-

A*01:01 homozygous donors reported previous IM or were smokers. Additional donors heterozygous for HLA-A*01:01 and HLA-A*02:01 (n = 2) were used in comparisons – these were donors p10158 and p10134 as detailed in Table 3.1 (Chapter 3). Donors were recalled to provide a "large blood sample" (Section 2.3.2). All donors reported that they were well at time collection of this specimen. Samples were processed to BD-PBMCs (Section 2.3.3). Autologous LCLs were established from donors as described in Section 2.4.6.

4.2.2 Peptide prediction

As discussed above, HLA-A*01:01-restricted responses to EBV have never been described and therefore sequences of HLA-A*01:01-restricted peptides are not available in the published literature. In order to predict those peptides derived from EBV proteins which might be suitable for presentation in the binding groove of HLA-A*01:01, peptide prediction software was used. Prediction strategies have been successfully used previously to identify such epitopes, e.g. in the case of CMV (Elkington *et al*, 2003).

A number of different software packages are freely available on-line. In order to determine the optimal prediction package, at time of study initiation in 2008, four of the leading packages (described in the Introduction) were compared. They were tested on their ability to identify previously characterised epitopes of known HLA-specificity from two viral proteins, namely HLA-A*01:01-restricted CMV IE-1 and HLA-A*02:01-restricted EBV LMP2 epitopes. The proportion of previously characterised peptides identified by the software was recorded. The software packages require the full AA sequence of the protein to be entered, and a HLA restriction selected. The reference sequence of these

Table 4-1 HLA-A*01:01 homozygous donors

LRF Patient Reference Number	Sex	Age	Carstairs 5-point deprivation score †	HLA-A Allele 1	HLA-A Allele 2	HLA-B Allele 1	HLA-B Allele 2	HLA-C Allele 1	HLA-C Allele 2	Site of donor recruitment	Used in ELISPOTs with no prior stimulation	Used in stimulated ELISPOTs	Used in cytokine analysis
10,116	F	68	NK	0101	0101	0801	0801	0701	0701	CRF	Yes	Yes	Yes
10,288	M	25	5	0101	0101	0801	4402	0501	0701	CRF	No	Yes	Yes
10,494	M	51	0	0101	0101	NK	NK	NK	NK	SNBTS	Yes	No	Yes
10,498	M	41	3	0101	0101	NK	NK	NK	NK	SNBTS	Yes	No	Yes
10,504	F	48	4	0101	0101	NK	NK	NK	NK	SNBTS	Yes	No	No
10,534	M	52	0	0101	0101	NK	NK	NK	NK	SNBTS	Yes	Yes	Yes
10,553	F	65	1	0101	0101	NK	NK	NK	NK	SNBTS	Yes	No	Yes
10,568	F	38	5	0101	0101	NK	NK	NK	NK	SNBTS	Yes	No	Yes
10,569	M	46	2	0101	0101	NK	NK	NK	NK	SNBTS	Yes	No	Yes
10,613	M	40	1	0101	0101	NK	NK	NK	NK	SNBTS	Yes	No	Yes
10,616	M	49	1	0101	0101	NK	NK	NK	NK	SNBTS	Yes	No	Yes

† Carstairs deprivation scores described in Section 2.3.1.4.

NK, not known; for SNBTS donors, only HLA-A* genotype information was available; CRF, Donor recruited through either the CRF or the University of Glasgow; SNBTS, Scottish National Blood Transfusion Service.

proteins was derived from UniProt KB (Uniprot Consortium, 2008), (http://www.uniprot.org/, accessed 18/11/08). As shown in Table 4.2, SYFPEITHI predicted the highest proportion of actual epitopes, with the other three packages performing poorly. On the basis of the above comparisons, SYFPEITHI was used to predict EBV- derived epitopes restricted through HLA-A*01:01.

Table 4-2 Comparison of peptide prediction programmes

Software	Proportion of HLA-A*02:01-restricted EBV LMP2 epitopes correctly predicted (%)	Number of HLA-A*01:01-restricted CMV IE-1 epitopes correctly predicted (%)
SYFPEITHI	7/8 (87.5%)	2/2 (100%)
RANKPEP	0/8 (0%)	1/2 (50%) – but not predicted to bind with sufficient strength to stimulate immune response
ProPred	1/8 (12.5%)	1/2 (50%)
BIMAS (NIH)	2/8 (25%)	2/2 (100%) – but only 1 predicted to bind with sufficient strength

4.2.3 HLA-A*01:01 predicted peptides

The AA sequences of the following EBV proteins were obtained from UniProt (Uniprot Consortium, 2008), (http://www.uniprot.org/, accessed 18/11/08): the latency II proteins EBNA1, LMP1, LMP2A, LMP2B; additional proteins expressed in the latency III pattern namely EBNA-LP, EBNA2, EBNA3A, EBNA3B, EBNA3C; and lastly, proteins expressed in the early lytic phase including BZLF1, BRLF1, BMLF1, BMRF1 (EA protein D), BALF2 (major DNA binding protein) and BALF5 (DNA polymerase).

Peptides with a score of > 23% (which would usually predict binding sufficient to elicit a T cell response) were selected for synthesis. If no peptides from an individual protein met this criteria, the top five candidate epitopes per protein

were selected, unless a binding strength of < 16% was predicted, in which case fewer than five peptides were synthesised. The total number of peptides synthesised was 61. Nonamers (epitopes of 9 AAs length) were selected as this is the optimal binding length for the class I HLA molecule.

The AA sequences of these peptides and the protein from which they are derived are listed in Table 4.4 (overleaf). Peptides were synthesised and reconstituted as described in Sections 2.7.3 and 2.7.4. Pools of no greater than 6 peptides were used (Table 4.4) and each peptide was used at a final concentration of 10 µg/ml.

*4.2.3.1 HLA-A*01:01 Control peptides*

HLA-A*01:01-restricted peptides derived from either CMV or influenza A (n = 5) were used as controls (Table 4.3). Used in combination in the HLA-A*01:01 experiments, they are referred to as “peptide pool 1”.

Table 4-3 HLA-A*01:01-restricted CMV and Influenza A derived peptides used as positive controls

Epitope AA sequence	HLA restriction	Protein	Virus	Three-letter abbreviation	Peptide pool
YSEHPTFTSQY	A0101	pp65	CMV	YSE	1
CTELKLSDY	A1	NP	Influenza A	CTE	1
CVETMCNEY	A1	IE1	CMV	CVE	1
DEEEAIVAY	A1	IE1	CMV	DEE	1
VSDGGPNLY	A1	PB1	Influenza A	VSD	1

Table 4-4 Peptides predicted to be presented through HLA-A*01:01 and used in the study

AA sequence	HLA restriction	Protein	Latent/ Lytic	Abbreviation	Peptide pool
ATSYHRDIY	A1 PRED	BALF2	LY	ATS	2
PDDEPRYTY	A1 PRED	BALF2	LY	PDD	2
PPEWLKGHY	A1 PRED	BALF2	LY	PPE	2
QSQPGPCGY	A1 PRED	BALF2	LY	QSQ	2
YKDLVKSCY	A1 PRED	BALF2	LY	YKD	2
ILDRARHIY	A1 PRED	BALF5	LY	ILD	3
REDSSWPSY	A1 PRED	BALF5	LY	RED	3
STGEEAGRY	A1 PRED	BALF5	LY	STG	3
VIDILNQAY	A1 PRED	BALF5	LY	VID	3
VYDILETVY	A1 PRED	BALF5	LY	VYD	3
CTDESYGKR	A1 PRED	BMLF1	LY	CTD	4
KEETGNSSY	A1 PRED	BMLF1	LY	KEE	4
STDGEISSS	A1 PRED	BMLF1	LY	STD	4
VVETLSSSY	A1 PRED	BMLF1	LY	VVE	4
YNFVKQLFY	A1 PRED	BMLF1	LY	YNF	4
YNFVKQLFY	A1 PRED	BMLF2	LY	YNF	4
ALAVLSKCY	A1 PRED	BMRF1	LY	ALA	5
AVSFRNLAY	A1 PRED	BMRF1	LY	AVS	5
GEACLTLDY	A1 PRED	BMRF1	LY	GEA	5
NPDLYVTTY	A1 PRED	BMRF1	LY	NPD	5
VSVPILRFY	A1 PRED	BMRF1	LY	VSV	5
HSRVRAYTY	A1 PRED	BRLF1	LY	HSR	6
QLGSLVSDY	A1 PRED	BRLF1	LY	QLG	6
SSHAGQRSY	A1 PRED	BRLF1	LY	SSH	6
SVEITLRSY	A1 PRED	BRLF1	LY	SVE	6
VTDRFFIQA	A1 PRED	BRLF1	LY	VTDR	6
AFDQATRVY	A1 PRED	BZLF1	LY	AFD	7
APENAYQAY	A1 PRED	BZLF1	LY	APE	7
DSELEIKRY	A1 PRED	BZLF1	LY	DSE	7
LPQGQLTAY	A1 PRED	BZLF1	LY	LPQ	7
SSENDRLRL	A1 PRED	BZLF1	LY	SSE	7
TTDEGTWVA	A1 PRED	EBNA1	LA	TTD	8
TWVAGVFVY	A1 PRED	EBNA1	LA	TWV	8
VGEADYFEY	A1 PRED	EBNA1	LA	VGE	8
YGGSKTSLY	A1 PRED	EBNA1	LA	YGG	8
DTDSLGNPS	A1 PRED	EBNA2	LA	DTD	9
ESPSSDEDY	A1 PRED	EBNA2	LA	ESP	9
LALHGGQTY	A1 PRED	EBNA2	LA	LAL	9
LSVIPSNPY	A1 PRED	EBNA2	LA	LSV	9
PLDRDPLGY	A1 PRED	EBNA2	LA	PLD	9
ATEVLDLSI	A1 PRED	EBNA3A	LA	ATE	10
FLQRTDLSY	A1 PRED	EBNA3A	LA	FLQ	10
RSEGPGPTR	A1 PRED	EBNA-LP	LA	RSE	10
VTTQRQSVY	A1 PRED	EBNA-LP	LA	VTT	10
AVTQAPTEY	A1 PRED	EBNA3B	LA	AVT	11
KNIPQTLPY	A1 PRED	EBNA3B	LA	KNI	11
LVSSGNTLY	A1 PRED	EBNA3B	LA	LVSS	11
TNEEIDLAY	A1 PRED	EBNA3B	LA	TNE	11
VTDFSVIKA	A1 PRED	EBNA3B	LA	VTDF	11
ESSDDELPY	A1 PRED	EBNA3C	LA	ESS	12
ISHEEQPRY	A1 PRED	EBNA3C	LA	ISH	12
KTIGNFKPY	A1 PRED	EBNA3C	LA	KTI	12
PKDAKQTDY	A1 PRED	EBNA3C	LA	PKD	12
PSMPFASDY	A1 PRED	EBNA3C	LA	PSM	12
ATDDSGHES	A1 PRED	LMP1	LA	ATD	13
LLALLFWLY	A1 PRED	LMP1	LA	LLAL	13
LLVLGIWIY	A1 PRED	LMP1	LA	LLVL	13
PHGPVQLSY	A1 PRED	LMP1	LA	PHG	13
CLPVIVAPY	A1 PRED	LMP2	LA	CLP	14
LLAAVASSY	A1 PRED	LMP2	LA	LLAA	14
MLVLLILAY	A1 PRED	LMP2	LA	MLV	14
SEERPPTPY	A1 PRED	LMP2	LA	SEE	14

4.2.4 ELISPOTs

EBV-specific CTL response were investigated in BD-PBMCs with and without stimulation with LCLs using IFN-γ ELISPOT analysis (Figure 4.1).

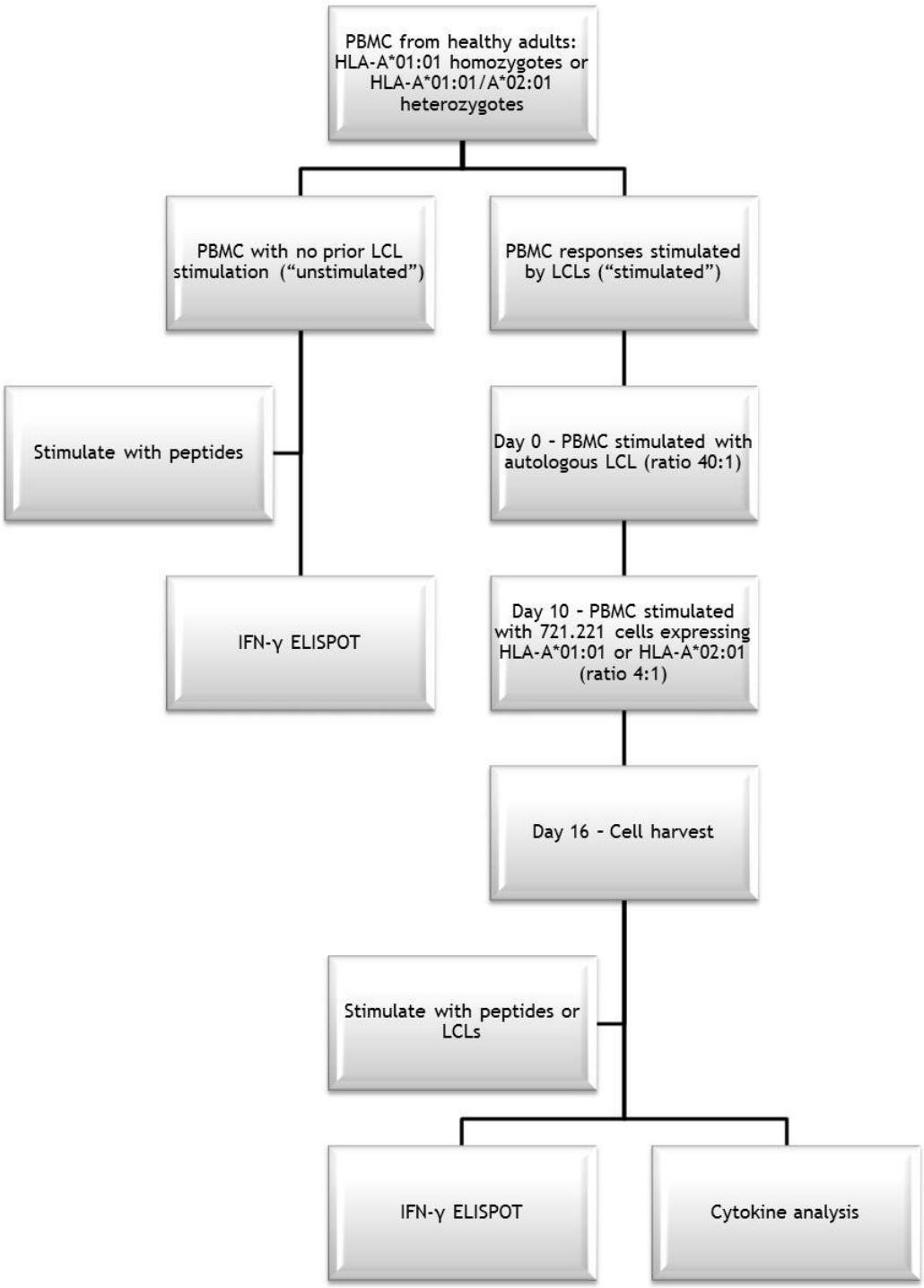


Figure 4-1 Flowchart of experiments performed in Chapter 4

CTL responses in HLA-A*01:01 homozygotes and HLA-A*01:01/A*02:01 heterozygotes, either with prior stimulation by LCLs or with no prior stimulation; full details given in text.

In the first set of experiments, donor BD-PBMCs, prepared as in Section 2.3.3.2, were exposed to either test peptides (in pools) or positive and negative controls. BD-PBMCs (100 µl at 3 x 10^6 cells / ml) were exposed to peptides at a final concentration in the well of 10 µg/ml. All assays were performed in duplicate. ELISPOTS were performed as described in Section 2.8; in these experiments, overnight exposure to peptide or control was the only stimulation.

Plates were read and counted as described (Section 2.8.2). All individual wells were visually inspected at time of plate reading. All plates were reviewed by myself, and then by Prof. Ruth Jarrett and Dr. Karen McAulay. Any donor where the positive controls did not elicit a clear detectable CTL response was excluded. Plates where the SFC for the positive controls did not exceed the mean of the highest negative control plus one SD were also removed from the analysis. Plates with excessive background reactivity, defined as a response detectable in either of the negative controls, were excluded. ELISPOTs satisfying these QC criteria were included in the analysis. To maximise sensitivity in detecting any response, the highest mean negative control plus 1 SD was taken as the cut-off for a "positive" response.

In the second set of experiments, responses were examined following stimulation of donor BD-PBMCs with irradiated autologous LCLs at day 0 and irradiated 721.221 cells, a HLA class I-deficient LCL transduced to express only a single class I HLA allele (courtesy Prof Trowsdale, University of Cambridge, Section 2.4.4), at day 10. In the case of HLA-A*01:01 homozygotes, day 10 stimulation was with HLA-A*01:01 expressing 721.221 cells and, in the case of HLA-A*02:01/A*01:01 heterozygotes, the culture was split at day 10 and

stimulated with either HLA-A*01:01 expressing or A*02:01 expressing 721.221 cells. At day 16, the stimulated BD-PBMCs were subjected to ELISPOT analysis.

4.2.4.1 PBMC Stimulation - Day 0

Autologous LCLs (Section 2.4.6) were counted, washed and re-suspended in 20% CCM at a concentration of 1 x 10^6 cells / ml. These were placed in a 25 cm^3 flask, and the lid was replaced and sealed with Parafilm.

The cell in these flasks were irradiated at 4000 rad (40Gy) using the RS225 high-voltage irradiator (Xstrahl Ltd, UK), courtesy of Prof. Anthony Chalmers, Beatson Institute of Cancer Research, Glasgow.

BD-PBMCs (1 – 2 x 10^7 cells) were thawed as described in Section 2.4.3. Viable cells were counted (Section 2.3.4), washed and re-suspended in 20% CCM at a density of 4 x 10^6 cells / ml. Aliquots of 2 x 10^6 cells (500 µl) were dispensed into each well of a 48-well plate. Irradiated LCLs (5 x 10^4 cells in 50 µl) were added to each well giving an effector: stimulator ratio of 40:1. The cells were incubated at 37°C in 5% CO_2 for 10 days.

4.2.4.2 PBMC Stimulation - Day 10

721.221 cells expressing HLA-A*01:01 or HLA-A*02:01 were prepared as described in Section 2.4.4. Cells were counted, washed and re-suspended in 20% CCM at a concentration of 1 x 10^6 cells / ml in 25 cm^3 flasks. The cells in these flasks were irradiated at 4000 rad (40Gy) as above.

Supernatant (50 µl) was removed from each of the wells of the 48-well plate described in Section 2.9. These were frozen at -70°C in screw-cap tubes for later use. The stimulated cultures from the wells in the 48-well plate were pooled and

counted. A volume containing at least 1 x 10^5 cells was removed for flow cytometry. The remainder was re-suspended and combined with the irradiated 721.221 LCLs expressing HLA-A*01:01 or HLA-A*02:01 at a ratio of 4:1 (e.g. 4 x 10^6 cells from the stimulated cultures: 1 x 10^6 irradiated 721.221 A01 cells). IL-2 (Gibco, Invitrogen Ltd., UK) was then added to a final concentration of 20 IU/ ml. The combined cell suspension was aliquoted into wells of a 48-well plate (500 µl per well, number of wells determined by number of cells retrieved from the stimulated cultures) and incubated at 37°C in 5% CO_2 for a further 6 days.

4.2.4.3 Day 16: Cell harvest

Supernatant (50 µl) was removed from each of the wells of the 48-well plate. These were frozen in screw-cap tubes at -70°C for later use. The cell cultures in the wells were pooled and a viable cell count performed. A volume containing at least 1 x 10^5 cells was removed for flow cytometry. The cells were washed and re-suspended in CCM at a concentration of 1.5 x 10^6 cells / ml for use in ELISPOTs.

4.2.4.4 ELISPOTS using stimulated BD-PBMCs

ELISPOTs were set up as in Section 2.8. In addition to the pools of A*01:01 predicted peptides and the appropriate positive and negative controls, wells were set up with either HLA-A*01:01 or HLA-A*02:01 expressing 721.221 cells at a ratio of 8:1 (PBMC:LCL), and with autologous LCLs at ratio of 8:1 (PBMC:LCL). The LCLs did not require to be irradiated for this short incubation. The ELISPOTs were then performed as previously described in Section 2.8.1, with the difference that due to limitation in available cell number, PBMCs were plated at 1.5 x 10^5 cells / well. At the end of the 24 hour incubation (prior to development

of the ELISPOT), supernatants (50 µl) were collected from each well and stored at -70^{o}C for later use. QC of the ELISPOTS was as described.

4.2.4.5 Flow cytometry on Day 10 and Day 16 stimulated cell cultures

In order to determine the characteristics of the stimulated cell cultures, flow cytometry (Section 2.5) for CD3/CD20, CD16/CD56/CD3, CD3/HLA-DR and CD4/CD8/CD3 was performed on the samples from the day 10 and day 16 stimulated cell cultures.

4.2.5 Cytokine analysis

In exploratory analyses, supernatants from the cultures were sampled at day 10 and day 16 to assess cytokine secretion in response to stimulation with EBV-infected LCLs. Analysis of TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and IL-17 was performed using a multiplex assay as described in Chapter 3 using the Human VersaMAP Multiplex Development System 9 Plex kit (R&D Systems) and read using the Bioplex system Luminex 100 plate reader (Bio-Rad). Calibration controls used to create the standard curve, and positive controls for beads and analytes were supplied with the kit. Cytokine levels are given in pg/ml.

TGF-ß cannot be measured in the multiplex assay as the cytokine requires ex-vivo acid activation before it can be measured. TGF-ß was assayed using an enzyme-linked immune assay (ELISA) kit (E-bioscience, UK). The supernatants from the same donors and stimulations as used in the multiplex assay were analysed. Samples were diluted 1:10 with the provided assay buffer as per kit instructions (180 µl assay buffer (1x) and 20 µl sample). Samples were then acid activated by the addition of 20 µl 1N hydrochloric acid (HCl) to the 200 µl of

diluted sample, and incubated for 1 hour at RT. Neutralisation was achieved by the addition of 20 µl 1N NaOH (sodium hydroxide). The result of the dilution and acid-activation steps was a 1:12 dilution of the samples. Samples were then added in duplicate to the microwell plate and the ELISA performed as per kit instructions. The plates were read on a colourimetric plate reader at 450 nm. Standards for creation of a standard curve were included with the kit.

4.2.5.1 Statistical Analysis

Cytokine levels were compared using an independent samples 2-tailed t-test analysis; Mann-Whitney analysis was not possible due to the presence of < 3 samples in some of the groups. Equal variances were not assumed. A p-value of < 0.05 is taken as significant. Correction for multiple testing was not performed.

Cytokine levels in the following samples were compared:

- Day 10 culture supernatants from HLA-A*02:01/A*01:01 heterozygotes and HLA-A*01:01 homozygotes
- Day 16 culture supernatants from HLA-A*01:01 homozygotes stimulated with A*01:01 expressing 721.221 LCLs and HLA-A*01:01/A*02:01 heterozygotes stimulated with A*01:01 expressing 721.221 LCLs
- Day 16 culture supernatants from HLA-A*01:01 homozygotes stimulated with A*01:01 expressing 721.221 LCLs and HLA-A*01:01/A*02:01 heterozygotes stimulated with A*02:01 expressing 721.221 LCLs
- Day 16 culture supernatants from all those stimulations performed with A*01:01 expressing 721.221 LCLs and those performed with A*02:01 expressing 721.221 LCLs (remembering that all donors used in this experiment carried at least 1 copy of HLA-A*01:01).

4.3 Results

4.3.1 Flow Cytometry

The characteristics of the cells present in the stimulated cell cultures at day 10 and day 16 were analysed by flow cytometry. CD16 and CD56 expression was analysed to assess presence of NK cells. Events positive for CD16 and CD56 were counted and given as a percentage of forward scatter/side scatter-gated events. The gate was set to include viable cells and exclude the cellular debris seen following stimulation which renders interpretation of ungated events difficult. The gate was the same for all analyses performed. In all donors, a CD16/CD56 co-marking population was present at day 10 (median 2.05%, range 0.9% - 16.5%) and at day 16 (median 6.35%, range 1.9% - 12.5%).

T cell activation, as demonstrated by the proportion of CD3-positive cells also positive for HLA-DR, increased following stimulation with LCLs from a median of 4.8% at day 0 to a median of 65.3% at day 16. This is demonstrated in Figure 4.2. There was an increase in the proportion of CD8 T cells present in the cultures following stimulation. This is demonstrated in Figure 4.3.

In all cases, the stimulated cell cultures contained populations of cells associated with antigen presentation (e.g. NK cells, monocytes) and cell-mediated immune response (CD4 and CD8 T cells) (data not shown).

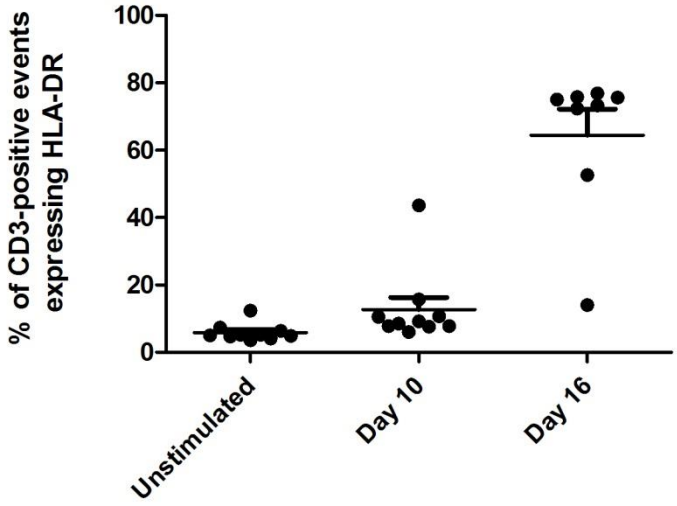


Figure 4-2 Activation of CD3 positive T cells following stimulation.

Activation demonstrated percentage of CD3-positive events also positive for HLA-DR. Events in the forward-scatter/side-scatter gate defined in Section 4.3.1 were counted. Error bars demonstrate mean and S.E.M.

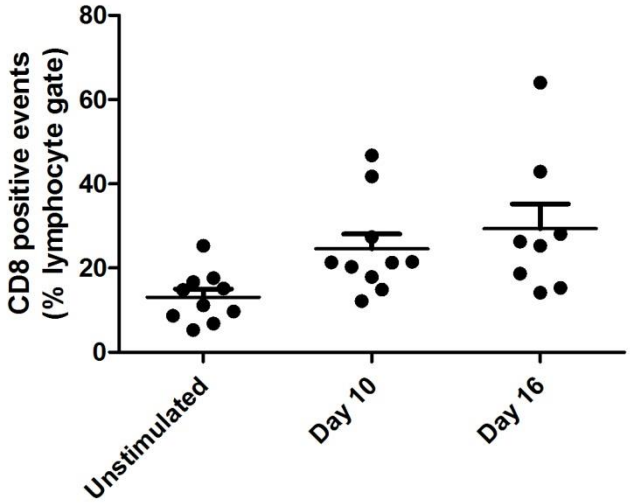


Figure 4-3 Proportion of CD8-positive T cells following stimulation.

The percentage of CD8-positive events is given. Events in the lymphocyte gate counted only. Error bars demonstrate mean and S.E.M.

4.3.2 ELISPOTS with no prior stimulation

In the ELISPOTs using unstimulated PBMC, plates from 10 donors passed QC criteria, as detailed in Table 4.1. The ELISPOT from donor p10288 was rejected on the basis of high background counts in the negative control wells.

Responses to positive controls or HLA-A*01:01-restricted CMV and influenza peptides were present in all of the donors. No response to any of the predicted HLA-A*01:01-restricted EBV peptides was detected in any of the 10 donors.

4.3.3 Stimulated ELISPOTS

ELISPOTs using stimulated cells were analysable for 3 of 11 HLA-A*01:01 homozygous donors (p10534, p10116 and p10288). Two ELISPOTS were excluded on the basis of lack of response in the positive control wells (p10494 and p10498) and five because of high background counts in the negative control wells (p10553, p10568, p10569, p10613 and p10616). For one donor (p10504), an LCL could not be raised, and thus it was not possible to perform stimulation.

ELISPOTs from five donors were included in the analysis. In the three analysable ELISPOTS from homozygous donors, responses to positive controls or HLA-A*01:01-restricted CMV and influenza peptides were detected. No response to any of the HLA-A*01:01-restricted EBV peptides was detected. The two ELISPOTS from HLA-A*01:01/ A*02:01 heterozygotes demonstrated positive responses to positive controls or HLA-A*01:01-restricted CMV and influenza peptides. In the heterozygotes, responses to HLA-A*02:01-restricted EBV peptides, but not HLA-A*01:01-restricted EBV peptides, were detected.

The three homozygous donors mounted strong responses to their autologous EBV-infected LCL. This is a well-recognised phenomenon. Only one of the three donors generated a response to the A*01:01-restricted 721.221 cell line. The lack of a response in two of the three donors suggests lack of EBV antigen presentation via HLA-A*01:01. The response seen in the third donor could be an HLA-A*01:01-restricted EBV response, or an allogeneic response to minor histocompatibility antigens on the 721.221 cells. The strong responses to the autologous LCL but weaker responses to the HLA-A*01:01 expressing 722.221 LCLs support the hypothesis that class I HLA alleles other than HLA-A*01:01 are driving the EBV-specific CTL response.

The HLA-A*02:01/A*01:01 heterozygotes demonstrated strong ELISPOT responses to their autologous EBV-infected LCL and to the HLA-A*02:01 expressing 721.221 LCL, but only weak responses to the HLA-A*01:01 expressing 722.221 LCLs.

4.3.4 Cytokine responses

As described in Section 4.2.5, in an exploratory analysis, supernatants from the stimulations were collected at day 10 and day 16, and analysis of TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and IL-17 performed using a multiplex assay.

Results of the stimulations at day 10 are presented in Figure 4.4 and Table 4.5. Results of the stimulations at day 16 are presented in Figure 4.5 and Tables 4.6 and 4.7.

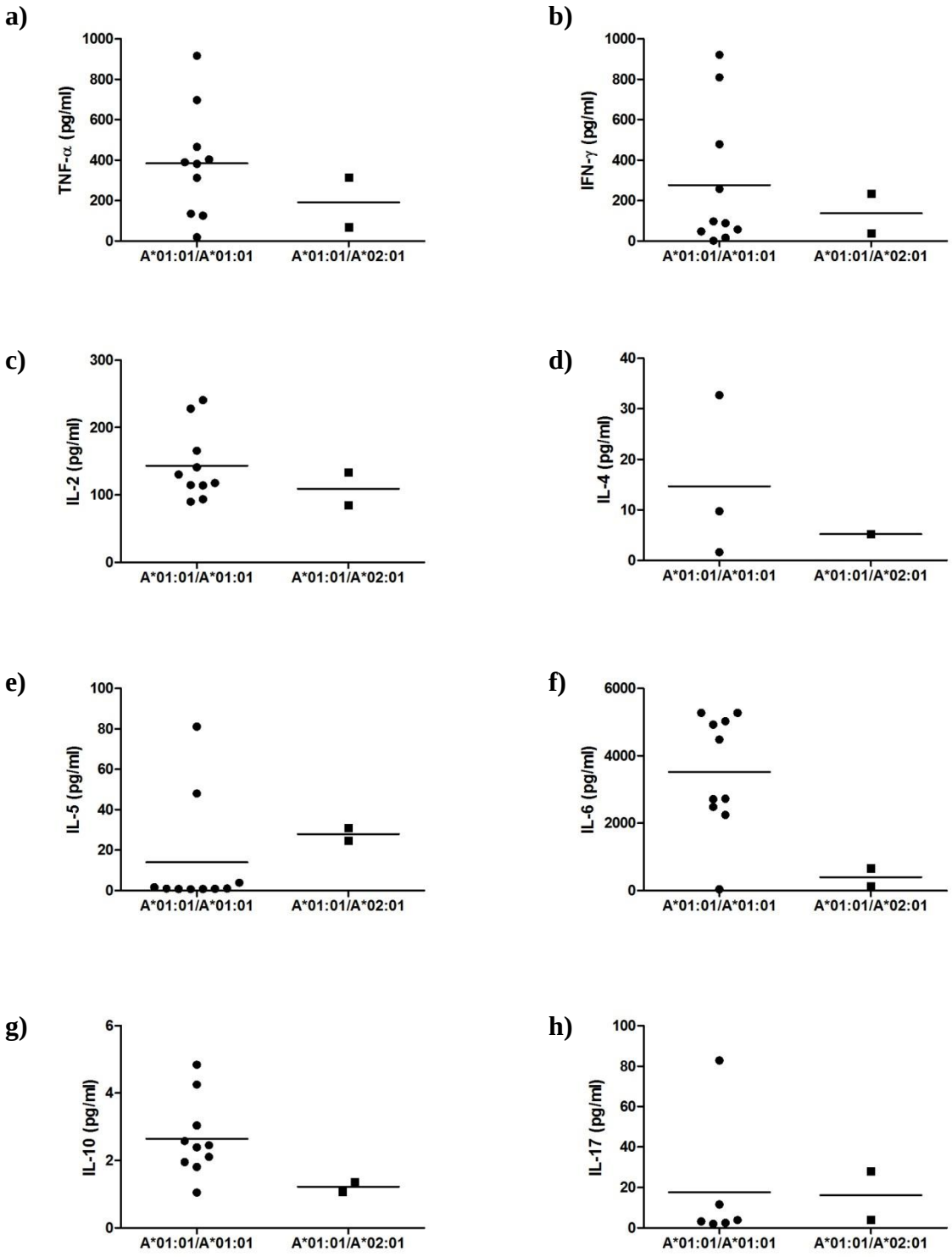


Figure 4-4 Levels of cytokine at day 10 of stimulation

a) TNF-α, b) IFN-γ, c) IL-2, d) IL-4, e) IL-5, f) IL-6, g) IL-10 and h) IL17. Concentrations are in pg/ml. Bars represent the mean. Statistical analyses of these data are presented in Table 4.5. Note IL-12 is omitted as this cytokine was not detected in any sample.

Table 4-5 Levels of cytokine at day 10 of stimulation

Cytokine	HLA Group				p-value of comparison between groups *
	HLA-A*01:01/ A*01:01 homozygotes		HLA-A*02:01/ A*01:01 heterozygotes		
	Mean (pg/ml)	SD	Mean (pg/ml)	SD	
TNF-α	384.48	270.04	191.66	174.36	0.320
IFN-γ	277.38	342.07	136.78	139.83	0.390
IL-2	143.59	52.70	109.23	34.41	0.359
IL-4 †	14.67	16.11	5.22	NA*	NR
IL-5	13.99	27.80	27.84	4.55	0.170
IL-6	3519.88	1747.82	395.01	368.83	0.001
IL-10	2.65	1.14	1.22	0.20	0.004
IL-17 ‡	17.64	32.11	16.11	16.90	0.737
IL-12 p70 §	ND	ND	ND	ND	ND

Mean cytokine levels are shown along with SD

NR, not detectable

† IL-4 7/12 samples had levels below the dynamic range of the assay

‡ IL-17 3/12 samples had levels below the dynamic range of the assay

§ IL-12 12/12 samples had levels below the dynamic range of the assay

* Independent samples t-test, equal variances not assumed, comparisons not performed for IL-4 and IL-12 as invalid given missing results. p-values given to 3 decimal places, a p-value of < 0.05 is considered significant (highlighted in blue)

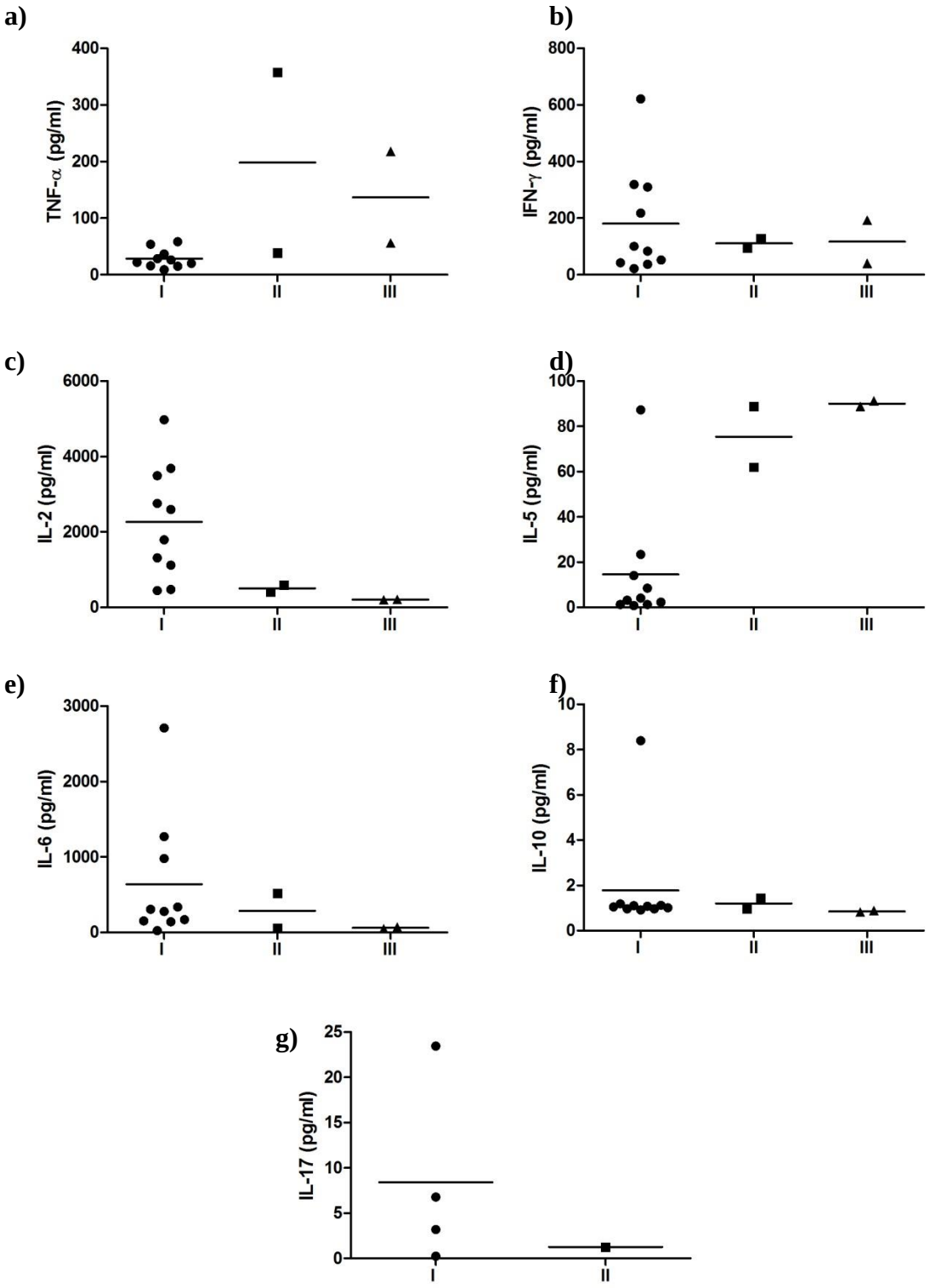


Figure 4-5 Levels of cytokine at day 16 of stimulation

a) TNF-α, b) IFN-γ, c) IL-2, d) IL-5, e) IL-6, f) IL-10 and g) IL-17. Groups are I) HLA-A*01:01 homozygotes vs. A*01:01 expressing 721.221 LCLs, II) HLA-A*01:01/A*02:01 heterozygotes vs. A*01:01 expressing 721.221 LCLs and III) HLA-A*01:01/A*02:01 heterozygotes vs. A*02:01 expressing 721.221 LCLs. Concentrations are in pg/ml. Bars represent the mean. Statistical analyses of these data are presented in Table 4.7. Note IL-4 and IL-12 are omitted due to levels below the dynamic range of the assay.

Table 4-6 Levels of cytokine at day 16 of stimulation

Cytokine	HLA Group					
	HLA-A*01:01/ A*01:01 homozygotes		HLA-A*02:01/ A*01:01 heterozygotes, A*01:01 stimulation		HLA-A*02:01/ A*01:01 heterozygotes, A*02:01 stimulation	
	Mean (pg/ml)	SD	Mean (pg/ml)	SD	Mean (pg/ml)	SD
TNF-α	28.46	16.43	198.21	225.77	136.98	114.13
IFN-γ	180.49	191.30	110.84	23.30	116.68	108.38
IL-2	2266.05	1496.52	502.88	137.12	206.18	7.89
IL-5	14.64	26.52	75.38	18.99	90.03	1.74
IL-6	636.95	830.52	286.98	324.95	62.17	17.78
IL-10	1.78	2.33	1.20	0.34	0.86	0.05
IL-17†	8.42	10.38	1.25	ND	ND	ND
IL-4‡	23.86	10.99	ND	ND	ND	ND
IL-12 p70§	ND	ND	ND	ND	ND	ND

Mean cytokine levels are shown along with SD

ND, not detectable

† IL-17 9/14 samples had levels below the dynamic range of the assay

‡ IL-4 5/10 HLA-*01:01 homozygote and 4/4 HLA-A*02:01/A*01:01 heterozygote samples had levels below the dynamic range of the assay

§ IL-12 14/14 samples had levels below the dynamic range of the assay

Table 4-7 Statistical analysis of cytokine levels at day 16 of stimulation

Cytokine	HLA-A*01:01/ A*01:01 homozygotes vs. HLA-A*02:01/ A*01:01 heterozygotes stimulated with A*01 expressing 721.221 LCLs	HLA-A*01:01/ A*01:01 homozygotes vs. HLA-A*02:01/ A*01:01 heterozygotes stimulated with A*02 expressing 721.221 LCLs	All stimulations with A*01 expressing 721.221 LCLs vs. All stimulations with A*02 expressing 721.221 LCLs
	p-value	p-value	p-value
IL-10	0.472	0.241	0.204
IL-2	0.367	0.056	0.040
IFN-γ	0.284	0.184	0.165
IL-5	0.002	0.005	0.009
TNF-α	0.480	0.406	0.493
IL-6	0.293	0.568	0.626

* Independent samples t-test, equal variances not assumed, comparisons not performed for IL-4, IL-12 or IL-17 as invalid given missing results. P-values given to 3 decimal places, a p-value of < 0.05 is considered significant (highlighted in blue).

4.3.4.1 Summary of cytokine responses to stimulation

At day 10, HLA A*01:01 homozygotes produced almost 10-fold higher levels of IL-6 than HLA-A*01:01/A*02:01 heterozygotes (mean 3519 vs. 395 pg/ml p = 0.001). Levels of IL-10 were marginally higher in HLA A*01:01 homozygotes; however, the levels in both groups were extremely low (< 5pg/ml), and the higher mean in the A*01:01 homozygotes was most likely due to two outliers (see Figure 4.4). The IL-10 results are therefore not regarded as biologically significant. There were no significant differences in any of the other cytokines examined. At day 16, although IL-6 levels had fallen from the day 10 levels, higher levels continued to be observed in the HLA-A*01:01 homozygotes, although differences were no longer statistically significant.

At day 16, HLA-A*01:01 homozygotes demonstrated lower levels of secretion of IL-5 as compared with HLA-A*01:01/A*02:01 heterozygotes stimulated with

either A*01 expressing 721.221 LCLs (14.64 pg/ml vs. 75.38 pg/ml, p = 0.002) or A*02 expressing 721.221 LCLs (14.64 pg/ml vs. 90.03 pg/ml, p = 0.005). In addition, levels of IL-5 were lower in all stimulations of HLA-A*01 carriers performed with A*01 expressing 721.221 LCLs vs. those performed with A*02 expressing 721.221 LCLs (24.76 pg/ml vs. 90.03 pg/ml, p = 0.009).

Higher levels of IL-2 were seen at day 16 compared with day 10, as would be expected given that IL-2 was added to the cultures. However, higher levels were seen in the stimulations of all HLA-A*01 carriers performed with A*01 expressing 721.221 LCLs vs. those performed with A*02 expressing 721.221 LCLs (1972.19 pg/ml vs. 206.18 pg/ml, p = 0.040) even although the same amount of IL-2 was added to all cultures.

ELISA for TGF-β was performed as described in Section 4.2.5. TGF-β levels were below the dynamic range of the assay in 35 of the 36 samples tested, and comparisons between groups were not possible. This most probably resulted from the sample dilution, which was required to negate the presence of TGF-β in FBS in CCM.

4.4 Discussion

In the present study we sought to determine whether HLA-A*01:01-restricted EBV-specific CTL responses could be detected. Using sensitive methodologies and a peptide prediction strategy, no HLA-A*01:01-restricted CTL responses to EBV were detected in this study. This lack of response was observed in unstimulated BD-PBMCs and also following stimulation using EBV-infected LCLs to boost low-level memory-CD8 CTL responses.

The lack of demonstrable HLA-A*01:01-restricted EBV-specific CTL response may in part explain the elevated risk of EBV+ve cHL associated with HLA-A*01:01.

The increased disease risk associated with lack of an allele is perhaps surprising given that HLA-A*01:01-positive individuals should be able to present EBV peptides through 4 or 5 other class I alleles. However, this argument may be overly simplistic. The critical CTL response to EBV, which is reflected in disease risk, may be important at any stage from primary EBV infection to establishment of EBV+ve cHL. Although responses to EBV peptides restricted through at least 35 other class I alleles, including 13 HLA-A, 19 HLA-B and 3 HLA-C alleles, have been reported, one could hypothesise that the response to those peptides presented in the latent infection in general, or the latency II programme in particular, are most important in the context of EBV+ve cHL; there, therefore, may not be the redundancy in response traditionally assumed. Responses to the latency II peptides are much more restricted in the range of HLA-types through which a response can be generated; to date, responses to these proteins have been reported only for 8 B-alleles, 7 A-alleles and no HLA-C alleles (Hislop *et al*, 2007). In this context, lack of response through a single common allele (HLA-A*01:01) may become more important.

One of the mysteries of the class I HLA-associated risks of EBV+ve cHL has been a consistent reporting of association with HLA-B*08 (as discussed in the Introduction), an allele that elicits immunodominant EBV responses. As HLA-A*01:01 is in LD with HLA-B*08:01 (~50% of healthy HLA-A*01:01 carriers are B*08:01-positive, in-house data, unpublished), it has been intriguing that the strong responses expected through B*08:01 do not abrogate the risk associated with HLA-A*01:01. However, one of the most immunodominant CTL responses restricted through HLA-B*08:01, and to EBV in general, is to "RAKFKQLL", a peptide from the EBV lytic cycle protein BZLF1. It may be significant that B*08:01 has been shown to present only one latency II peptide for recognition by the CTL immune response; an epitope derived from EBNA1 which is reported to have a high level of sequence polymorphism adversely affecting presentation for CTL recognition (Bell *et al*, 2008) . This supports the hypothesis that CTL immune response to the latency II peptides at time of EBV+ve cHL disease development may be biologically relevant.

In a secondary analysis, cytokine excretion in response to stimulation with EBV was examined. This study is small and exploratory but the finding of different levels of cytokine secretion in response to stimulation with EBV-infected LCLs in HLA-A*01:01 homozygotes and HLA-A*01:01/A*02:01 heterozygotes is interesting

HLA-A*01:01 homozygotes generated much higher levels of IL-6 than HLA-A*01:01/A*02:01 heterozygotes following stimulation with autologous LCLs. IL-6 is a cytokine with potent antiviral activity, and would therefore be hypothesised to be associated with better responses and lower risk of disease. However, as discussed in Chapter 1, IL-6 has been implicated in the pathogenesis of cHL and other B cell neoplasms. Elevated levels of IL-6 were described in identical twins

of cases of cHL compared with matched healthy controls (Cozen *et al*, 2004), and in pre-diagnosis samples from cases of cHL compared with healthy individuals (Gaiolla *et al*, 2011). Both IL-6 and the IL-6 receptor have been shown to be expressed in cHL cell lines and by HRS cells in primary tissue where it is thought to act as an autocrine growth factor (Jucker *et al*, 1991). Should HLA-A*01:01 stimulate IL-6 in response to EBV peptides, particularly the latency II expression profile expressed by the HRS cell, it is biologically plausible that this could lead to elevated risk of EBV+ve cHL.

It should be pointed out that LCLs, particularly early-passage LCLs, are known to secrete IL-6 in response to EBV lytic peptides (Jones *et al*, 2007). The levels found in our study are in the range previously reported to be secreted by LCLs (Wroblewski *et al*, 2002). The LCLs used in our study were irradiated and therefore would be considered non-functional, and were washed before re-suspension, such that any differences noted would be unlikely to be due to LCL activity. It is possible, however, that the higher levels observed may be due to differential cell kill of LCLs in the stimulations. This was not directly assessed and this caveat should be borne in mind in interpreting these results.

Higher levels of IL-2 were also seen after 16 days of stimulation in A*01:01 homozygotes. Previously known as T cell growth factor, IL-2 is a cytokine secreted by CD4 and CD8 T cells. IL-2 helps drive a Th1 cytokine antiviral response, including activating CTLs; in particular, IL-2 is crucial for recall responses by CD8 memory T cells. However, it has become clear in recent years that one of the main functions of IL-2 is to drive a CD25+ve T_{reg} response, which may help limit autoimmunity (Malek, 2008). T_{reg} cells have been implicated in the pathogenesis of EBV+ve cHL (Assis *et al*, 2010). In addition, STAT5

phosphorylation is one of the main downstream events following IL-2 signalling and phosphorylated STAT5 has been shown to be present at high levels in the nuclei of HRS cells (Hinz *et al*, 2002). IL-2 levels have been shown to be higher in cHL patients versus controls (Viviani *et al*, 1998). In a study of cytokine responses to viruses (Makedonas *et al*, 2010), some individuals were shown to secrete IL-2, rather than IFN-γ from CTLs in response to EBV peptides. The HLA type of the individuals concerned was not given, but it is interesting to speculate that this could be associated with class I HLA type. Given the drive towards a T_{reg} response induced by IL-2, one might wonder that the higher levels of IL-2 seen in HLA-A*01:01 homozygotes in response to EBV in this study might mean reduced EBV-specific immunity at time of initial infection, or at time of EBV+ve cHL cancer development.

Lower levels of secretion of IL-5 were also seen in HLA-A*01:01 homozygotes in comparison to A*01:01/A*02:01 heterozygotes. IL-5 is one of the cytokines upstream in the JAK-STAT pathway (Murray, 2007), and is implicated in the proliferation and apoptosis resistance of HRS cells (Farrell & Jarrett, 2011). IL-5 is also secreted by HRS cells (Teruya-Feldstein *et al*, 2000) and is important in driving the eosinophilia associated with the disease. Thus, the finding of lower levels in the group more at risk of developing EBV+ve cHL is more difficult to reconcile. This finding in the context of the cytokine responses seen in HLA-A*02:01 carriers and in disease outcome is discussed further in Chapter 6.

Differential cytokine secretion in response to viruses in individuals associated with class I HLA phenotypes has previously been described. The TNF-α, IL-6 and IL-1β responses to smallpox vaccination showed an associated with HLA-B phenotype (Ovsyannikova *et al*, 2011). In a study of responses to the measles

vaccine, HLA-A*01:01 was associated with lower levels of IFN-γ secretion (Ovsyannikova *et al*, 2005). The authors of these studies suggest that levels of cytokine secretion in response to stimulation by viruses may be controlled by HLA genes or genes in close LD to these alleles. Although HLA class I phenotype is associated primarily with CTL response, it may be that some of the inter-individual variability seen with cytokines, many of which are implicated in autoimmune and malignant disease, is due to class I HLA phenotype.

Whilst this study is too small to offer conclusive findings, it generates interesting hypotheses for testing in future research. The absence of a detectable IFN-γ CTL response to EBV restricted through HLA-A*01:01 may be important. The understanding of the stage in cHL oncogenesis at which class-I HLA-associated risk is operating is not clear, and these findings favour the idea that the critical response may be to latency II antigens.

It is also possible to speculate that similar mechanisms may be operating in other EBV-associated malignancies, particularly those with HLA-class I associations. The most notable of these, NPC, is particularly associated with HLA-A*02:07 (Li *et al*, 2009b). In GWAS studies the association localises to the peptide binding groove of the HLA-A molecule (Tang *et al*, 2010) suggesting a critical role for the variable region and antigen presentation. The mechanisms behind this association, the tissue tropism of this malignancy and why the HLA allele associated with risk should be different to EBV+ve cHL are currently unexplained. It is possible that lack of CTL response to critical stage-specific antigens or differential cytokine secretion may also underlie the HLA-associated risks of NPC, and this might merit further study.

Further studies into the risks associated with the different class I allotypes are merited, as are studies elucidating the immune response to the latency II programme of EBV. The cytokine profiles observed in response to stimulation with EBV are exploratory, but intriguing. Further studies are required to clarify these findings and to study the mechanisms involved.

Chapter 5. Does class-I HLA phenotype influence outcome in Hodgkin lymphoma?

5.1 Introduction

A high proportion of patients with cHL are cured with standard treatment; > 90% in early-stage disease and > 75% in advanced stage disease (Connors, 2005; Townsend & Linch, 2012). The ability to predict outcome is important so that those patients who will not be cured by standard first-line treatment approaches can be targeted for alternative management, and to avoid over-treatment (and the attendant toxicities) in those patients with good outcomes. Established prognostication tools (Lister *et al*, 1989; Hasenclever & Diehl, 1998) employ clinical features of the disease at presentation to predict outcome and whilst useful, have limitations. The IPS (Hasenclever & Diehl, 1998) was designed to predict outcome in advanced stage disease. It is not currently used to treatment-stratify in the UK as it is insufficient to accurately predict those extremely poor outcome patients who may require a different front-line approach.

As a result, international efforts are focusing on finding and validating additional tools to refine and enhance current approaches to prognostication. Reinforcing the critical role of the immune system in the pathogenesis of cHL, discussed in the Introduction, it is note-worthy that the majority of the biologically-based prognostic approaches ("biomarkers") use features which characterise the immune response to the disease. Such approaches include prognostication based on the tumour microenvironment including tumour-associated macrophages (Steidl *et al*, 2010a; Sanchez-Espiridion *et al*, 2012), CTLs and T_{regs} (Oudejans *et al*, 1997; Alvaro *et al*, 2005) and also the peripheral blood lymphocyte/ monocyte ratio (Porrata *et al*, 2011). Additional studies have examined the prognostic importance of cytokines including: TARC (Weihrauch *et al*, 2005); IL-10 (Rautert *et al*, 2008) and combined cytokine profiles (Casasnovas *et al*, 2007).

EBV+ve cHL, in contrast to EBV-ve cHL, retains the ability to express class I HLA (Oudejans *et al*, 1996). As it is known that the risk of developing EBV+ve cHL is associated with class I HLA genotype, specifically the numbers of HLA-A*01:01 and HLA-A*02:01 alleles (as discussed in Chapters 1, 3 and 4) and that class I HLA is involved in the presentation of EBV peptides for recognition by the cellular immune response, it follows that HLA may be important in determining the immune response to the disease and therefore affect clinical outcome.

Previous studies, antedating modern HLA-typing techniques and treatment approaches identified certain HLA alleles as associated with clinical outcome in HL, as well as in NHL (Lu *et al*, 2011). In HL, the class II HLA allele DPB1*09:01 was associated with significantly shorter duration of remission (Oza *et al*, 1994). Patients with the HLA-A1 antigen (now A*01) were identified as having greater chance of cure and reduced chance of death from HL (Hafez *et al*, 1985). Indeed, "survivor bias", the inadvertent selection of survivors in early retrospective disease-association studies, identified both HLA-A 1 (now A*01) and B8 (now B*08) as particularly prevalent in long-term survivors of HL (Falk & Osoba, 1971).

Given these associations, a pilot study was performed to examine the association of HLA-A*01:01 and A*02:01 with clinical outcome in a large cohort of patients with cHL.

5.1.1 Aims & Objectives

This study aimed to determine if HLA-A*01:01 and A*02:01 alleles are a factor in determining clinical outcome in EBV+ve cHL.

5.2 Methods

5.2.1 Patient cohort

Patients with cHL were collated from two previous studies: the Scotland and Newcastle Epidemiological Study of Hodgkin's disease (SNEHD) (Jarrett et. al, Blood, 2005) and the "Investigation of the cause of Hodgkin lymphoma" (ITCH Study), an on-going study of patients principally from the West of Scotland. Both studies have ethical approval.

Cases were selected on the basis of availability of tumour EBV status, a suitable sample for HLA typing and clinical follow-up data and included 269 cases from the SNEHD study and 155 from the IPHL (total n = 424).

Clinical treatment decisions were made by individual physicians. HLA genotyping was performed as described in the Introduction to an intermediate resolution using allele-specific PCR (Gen-probe) and subsequent bead-based sequence specific oligonucleotide (SSO) assay (Luminex). Allele assignment was performed using Quicktype for Lifematch software (Gen-probe).

Clinical follow-up data were obtained through clinical records, liaison with treating physicians, and the Scotland and Newcastle Lymphoma Group (SNLG) database. Where the patients resided in Scotland, additional follow-up data regarding vital status, date of death and cause of death were available from the Information Services Division (ISD), NHS National Services Scotland, Edinburgh, UK. This was requested for 131 cases where follow-up was less than 96 months or where cause of death was unknown. These data are derived from death certificate information and from the Community Health Index (a register of all patients in NHS Scotland). Data regarding relapse and progression were not

systematically recorded at the LRF Virus Centre and the current cancer registry systems in Scotland do not systematically collect data on relapse or progression. As a result, EFS was not included in the analysis.

5.2.2 Statistical Analysis

The outcome of main interest was survival in EBV+ve cases. EBV-ve cases and all cases together were analysed for comparison and DSS were analysed by number of HLA-A*01:01 alleles, number of HLA-A*02:01 alleles, and by the presence or absence of HLA-A*01:01 or HLA-A*02:01. Certain other factors e.g. age, sex, disease stage at presentation were analysed to enable comparison with other data sets and to exclude confounding. Statistical analyses used Kaplan-Meier estimates of survival; survival curves were compared using the log-rank test. A p-value of < 0.05 is considered as demonstrating significant survival differences.

A Cox proportional hazard regression model was generated for analyses of OS and DSS with the association of the presence or absence of HLA-A*01:01 or HLA-A*02:01, the number of HLA-A*01:01 alleles and the number of HLA-A*02:01 alleles, following adjustment for histological subtype, clinical stage, age and sex. All analyses of histological subtype were restricted to NS and MC subtype. Hazard ratios are not reported due to the non-categorical nature of some of the variables. Two-sided p-values are reported. A p-value of < 0.05 is considered significant. All analyses were implemented using SPSS v.19.

5.3 Results

5.3.1 Description of Cohort

The analysis included 424 cases diagnosed between 1993 and 2008. The demographic make-up of the cases is summarised in Table 5.1. 137 cases (32.3%) had EBV+ve cHL. Median follow up was 92 months (range 1-226 months). As internationally defined (Section 1.4.1), 57.9% of patients had advanced stage disease. Histological subtype of disease was available for all 424 patients.

Table 5-1 Characteristics of cases included in the analysis of clinical outcome

		Cases (n = 424)	Missing (n)
Female, n (%)		191 (45.0)	0
Age (median, range)		34 (15 -78)	1
Age group, n (%)	**≤ 34 years**	215 (50.8)	See above
	35-49 years	92 (21.7)	See above
	≥ 50 years	116 (27.4)	See above
Follow up in months (median, range)		92.0 (1-226)	0
DEPCAT 5 (mean, range)†		3.35 (1-5)	129
EBV+ve cHL, n (%)		137 (32.3%)	0
Clinical Stage, n (%)	**I**	55 (17.4)	108
	II	135 (42.7)	
	III	73 (23.1)	
	IV	53 (16.8)	
B-symptoms, n (%)		138 (43.8)	109
Advanced stage disease, n (%)		183 (57.9)	108
Histological Sub-type, n (%)	**Nodular sclerosis**	302 (71.2)	0
	Mixed cellularity	95 (22.4)	
	Lymphocyte rich	13 (3.1)	
	Lymphocyte-depleted	2 (0.5)	
	cHL, not otherwise specified	12 (2.8)	

† DEPCAT (Carstairs deprivation category, defined in Section 2.3.1.4)

5.3.2 Survival Analysis

5.3.2.1 Analysis of all cases – OS

In the analysis of all cases, OS was worse with increasing age ($p < 0.001$) (Figure 5.1) and with male sex ($p = 0.039$, Figure 5.2). Accepted prognostic criteria such as advanced clinical stage were predictive of poor survival in all cases (advanced stage vs. early stage disease, $p = 0.033$, Figure 5.3). In contrast, histological subtype (NS vs. MC cases) was not found to be predictive of OS ($p = 0.469$, Figure 5.4). In unadjusted Kaplan-Meier analysis, EBV status of the cHL was predictive of OS with EBV+ve cHL being associated with poorer survival than EBV-ve disease ($p = 0.016$, Figure 5.5), although more EBV+ve disease was seen in the older adult group (50.9% of cases > 50 years having EBV+ve disease as compared to only 22.3% of those aged 34 years or less). There were no significant differences in OS by number or presence of HLA-A*02:01 and A*01:01 alleles (summarised in Table 5.2).

Cox proportional hazard regression analysis of OS identified age ($p < 0.001$) and clinical stage ($p = 0.005$) independently associated with OS in all cases.

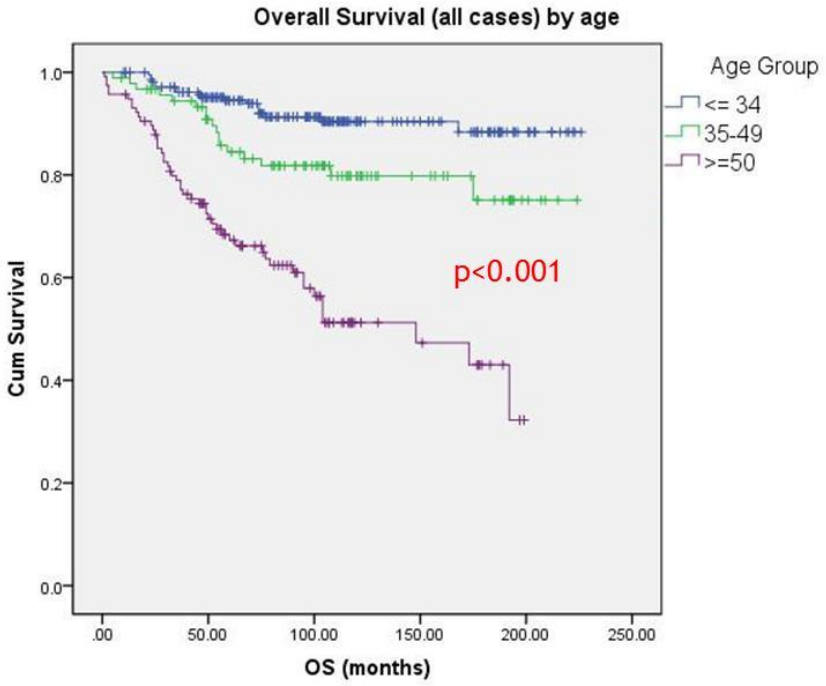


Figure 5-1 Overall survival in all cases by age group

OS, overall survival. Cases are stratified by age group: ≤ 34 years old; 35-49 years old; and ≥ 50 years old.

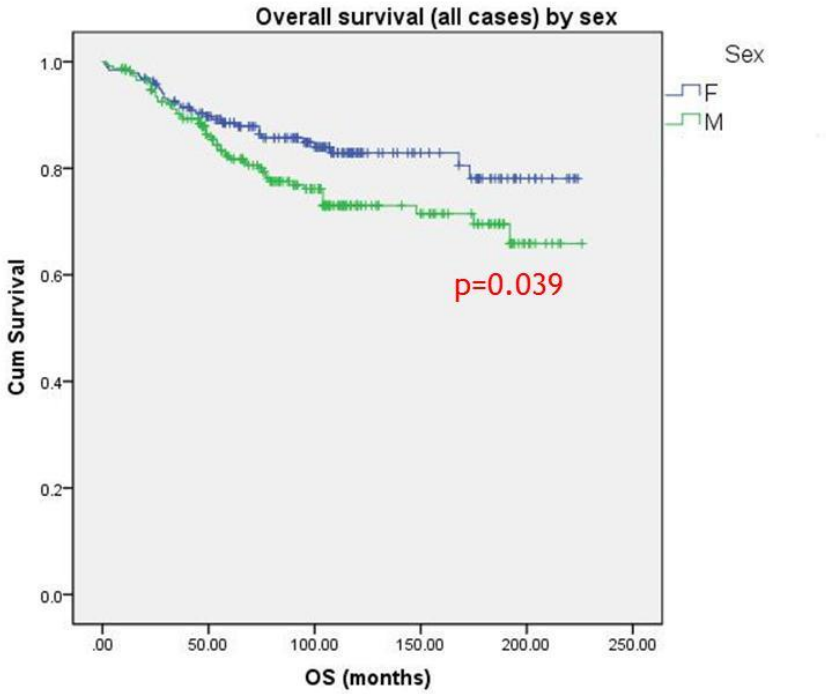


Figure 5-2 Overall survival in all cases by sex

OS, overall survival; F, female; M, male

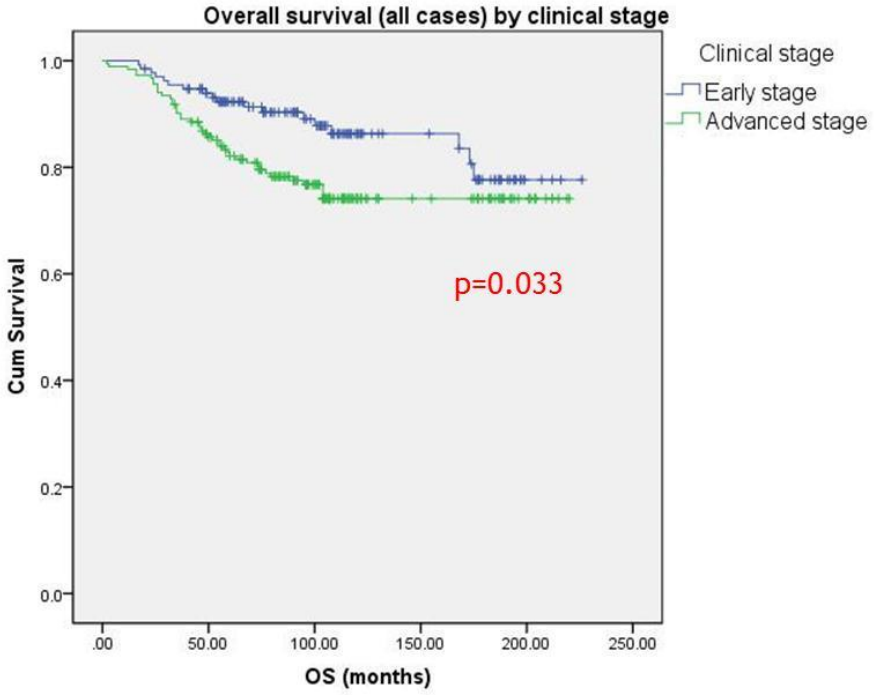


Figure 5-3 Overall survival in all cases by clinical stage of disease

OS, overall survival. Early vs. advanced stage disease shown.

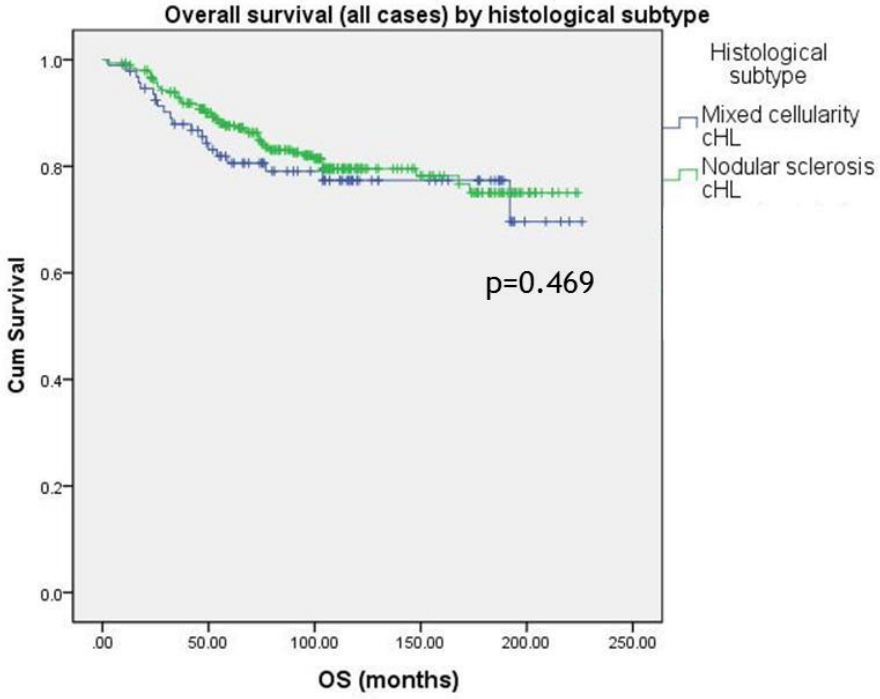


Figure 5-4 Overall survival in all cases by histological subtype of cHL

OS, overall survival. Analysis restricted to mixed cellularity and nodular sclerosis subtypes.

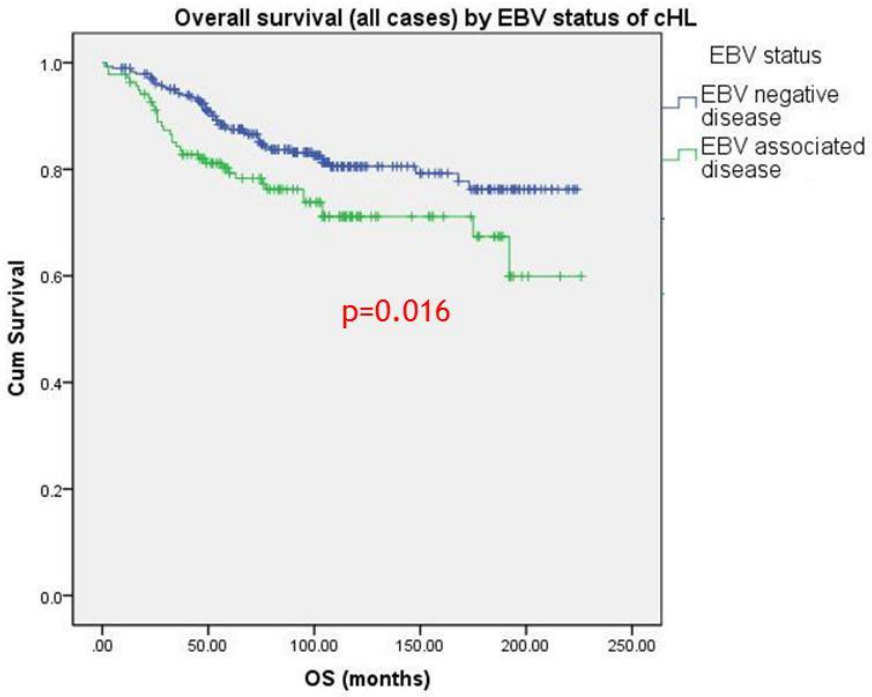


Figure 5-5 Overall survival in all cases by EBV status of the cHL

OS, overall survival

Table 5-2 Unadjusted log-rank comparisons of overall and disease-specific Kaplan-Meier estimates of survival in all cases by HLA-A*01:01 and HLA*02:01 genotype.

Factor	Overall survival	Disease-specific survival
	p-value	p-value
Presence of an HLA-A*01:01 allele	0.556	0.071
Number of HLA-A*01:01 alleles	0.792	0.158
Presence of an HLA-A*02:01 allele	0.307	0.996
Number of HLA-A*02:01 alleles	0.480	0.992

P-values are from the log-rank comparisons of overall and disease-specific Kaplan-Meier estimates of survival by a single variable as stated. A p-value of < 0.05 is considered significant.

5.3.2.2 *Analysis of all cases – disease-specific survival*

DSS was analysable for 358 of the 424 cases. Unadjusted log-rank comparison of Kaplan-Meier estimates of survival demonstrated that only advanced stage disease was associated with statistically significant poorer DSS (p = 0.001, Figure 5.6), with the majority of deaths due to cHL occurring before 5 years. DSS was not associated with EBV status of the disease (p = 0.634, Figure 5.7). Histological subtype was not predictive of DSS (p = 0.967, data not shown). In the analysis of all cases, neither number nor presence of HLA-A*02:01 alleles was associated with DSS. In the analysis of all cases, improved DSS was seen in carriers of an HLA-A*01:01 allele, approaching statistical significance (p = 0.071) (Figure 5.8). Cox proportional hazard regression analysis of DSS in all cases identified only clinical stage (p = 0.006) as independently prognostic of outcome.

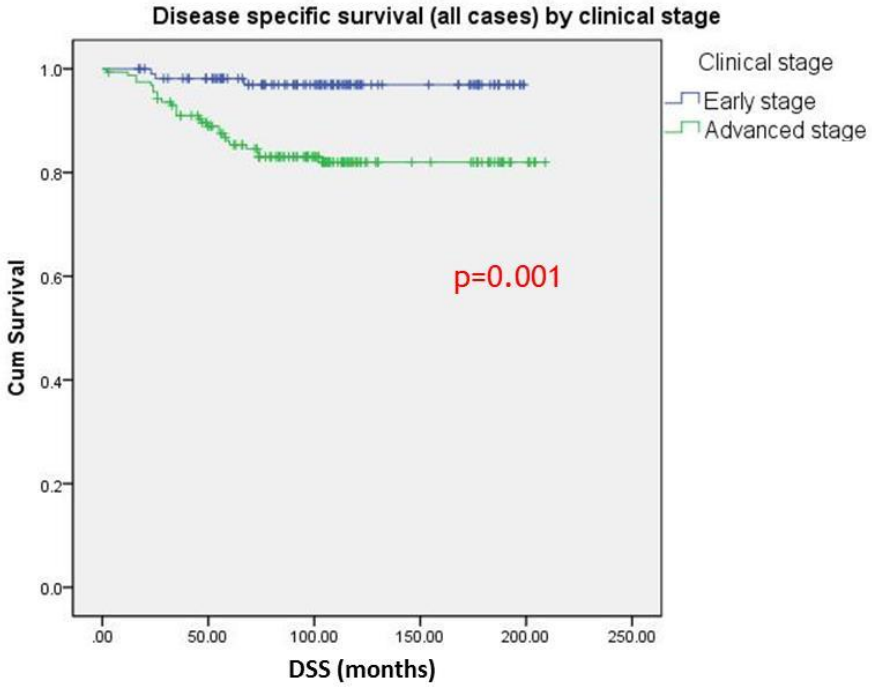


Figure 5-6 Disease-specific survival in all cases by clinical stage
DSS, disease-specific survival. Early vs. advanced stage disease shown.

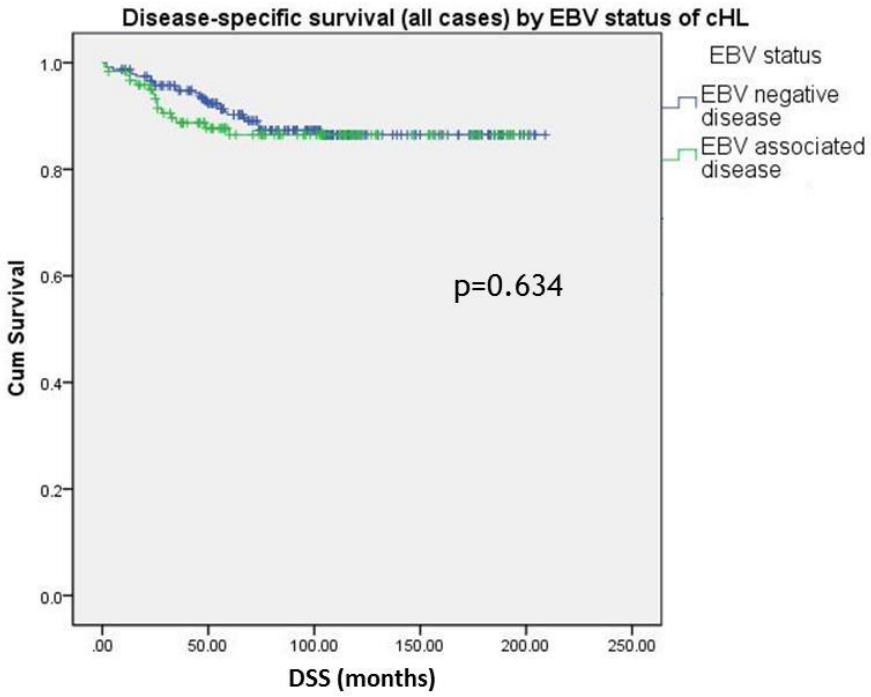


Figure 5-7 Disease-specific survival in all cases by EBV status of the cHL

DDS, disease-specific survival.

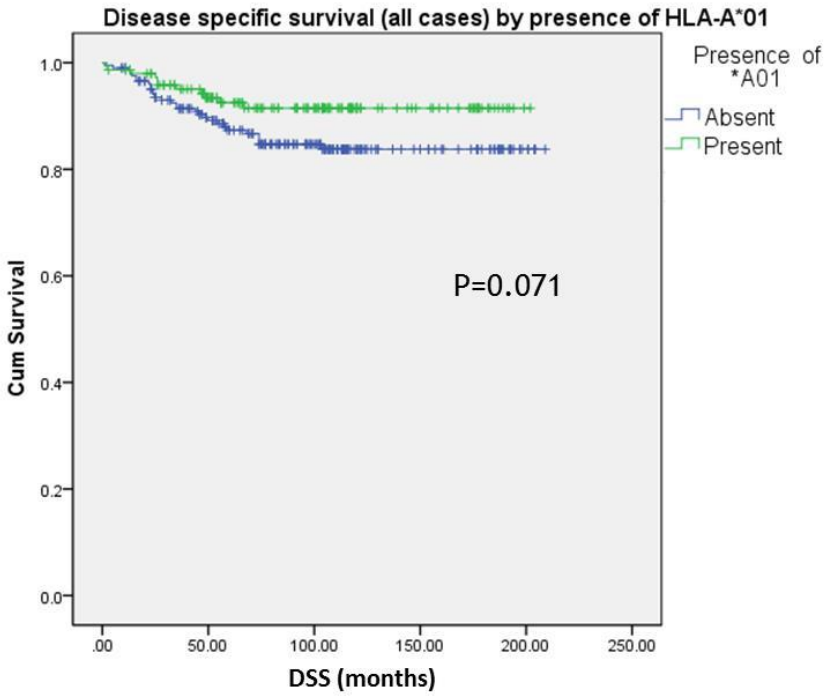


Figure 5-8 Disease-specific survival in all cases by the presence or absence of an HLA-A*01:01 allele

DSS, disease-specific survival.

5.3.2.3 Analysis of EBV-negative cases- OS

Unadjusted log-rank comparison of Kaplan-Meier estimates of survival demonstrated that only increasing age group ($p < 0.0001$) and male sex ($p = 0.003$) were associated with inferior OS in EBV-negative cases. Neither advanced stage ($p = 0.32$) nor histological subtype ($p = 0.61$) were associated with poorer OS in this group. Neither HLA-A*02:01 nor HLA-A*01:01 demonstrated any significant association with OS or DSS in EBV-negative cases (see Table 5.3.) Patients with EBV-ve cHL who had an HLA-A*02:01 allele had an improved OS and this difference approached statistical significance ($p = 0.066$, Figure 5.9). Cox proportional hazard regression analysis of OS in EBV-negative cases identified only age ($p < 0.001$) as independently prognostic.

Table 5-3 Unadjusted log-rank comparisons of overall and disease-specific Kaplan-Meier estimates of survival in EBV-negative cases by HLA-A*01:01 and HLA*02:01 genotype.

Factor	Overall survival	Disease-specific survival
	p-value	p-value
Presence of an HLA-A*01:01 allele	0.913	0.278
Number of HLA-A*01:01 alleles	0.757	0.536
Presence of an HLA-A*02:01 allele	0.066	0.239
Number of HLA-A*02:01 alleles	0.181	0.496

P-values are from the log-rank comparisons of overall and disease-specific Kaplan-Meier estimates of survival by a single variable as stated. A p-value of < 0.05 is considered significant.

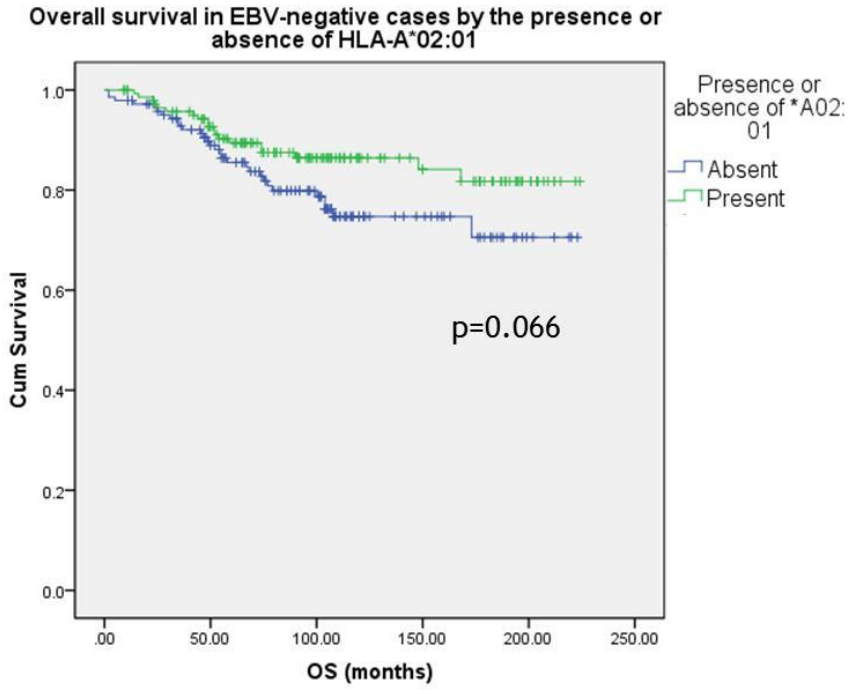


Figure 5-9 Overall survival in EBV-negative cases by the presence or absence of the HLA-A*02:01 allele

OS, overall survival.

5.3.2.4 Analysis of EBV-negative cases- DSS

Analysis of DSS in EBV-negative cases demonstrated that male sex (p = 0.003) and advanced stage disease (p = 0.023) were associated with poorer DSS (data not shown). Cox proportional hazard regression analysis of DSS in EBV-negative cases identified clinical stage (p = 0.014) as independently prognostic.

5.3.2.5 Analysis of EBV-associated cases – OS

HLA-A*02:01 carriers were under-represented in EBV+ve cases, 32.8% as compared with the 50.2% of EBV-negative cases (p = 0.001, by 2-sided Pearson CHI square analysis), as expected.

EBV+ve cHL cases also demonstrated poorer OS with increasing age (p < 0.001), with no long term survivors in the older adult age group (Figure 5.10). Poorer

survival with advanced stage disease was also seen (p = 0.023, data not shown). There was no significant association with male sex or histological subtype and OS in EBV+ve cases.

Inferior OS was observed with increasing number of HLA -A*02:01 alleles present (p = 0.017, Figure 5.11). Patients with EBV+ve cHL who carried a copy of HLA-A*02:01 demonstrated inferior OS which did not reach statistical significance (p = 0.184, Figure 5.12). Data are summarised in Table 5.4.

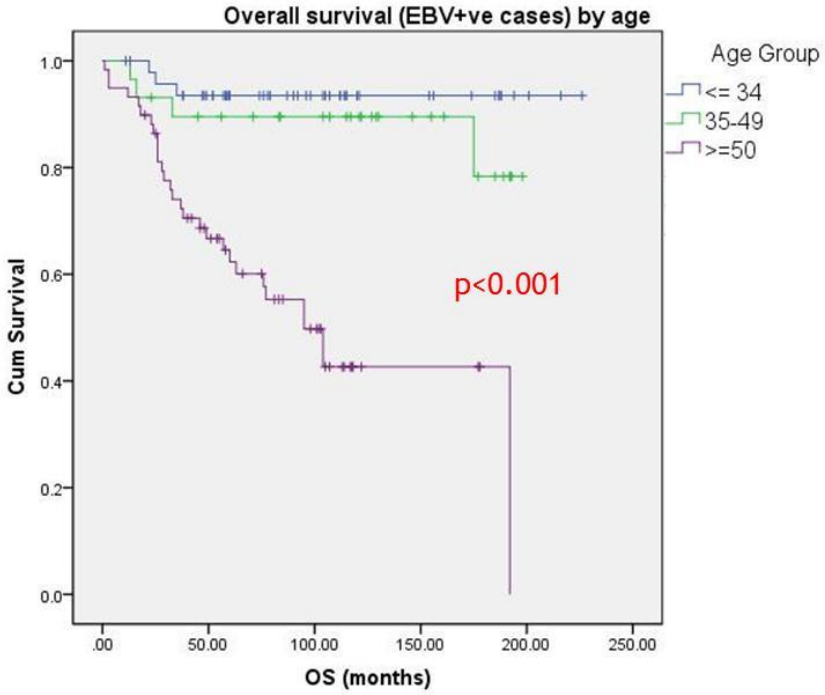


Figure 5-10 Overall survival in EBV-associated cases by age group

OS, overall survival. Cases are stratified by ≤ 34 years old, 35-49 years old and ≥ 50 years old.

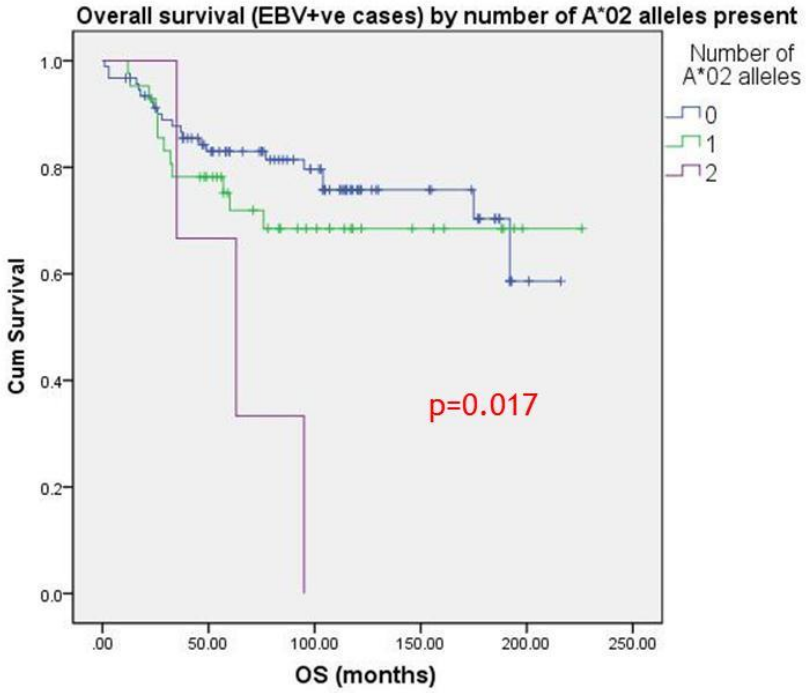


Figure 5-11 Overall survival in EBV-associated cases by number of A*02:01 alleles

OS, overall survival.

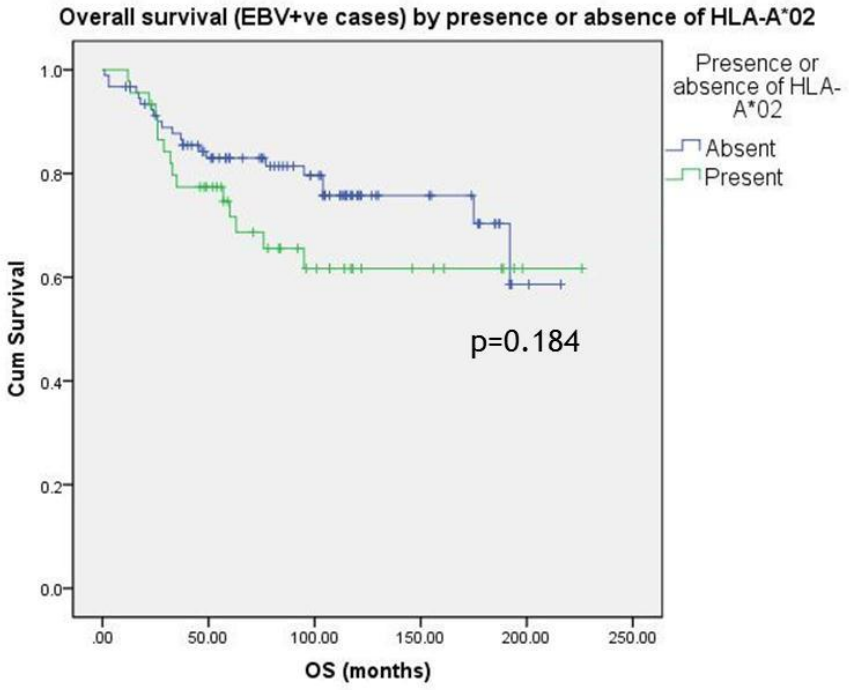


Figure 5-12 Overall survival in EBV-associated cases by presence or absence of an HLA-A*02:01 allele

OS, overall survival

A previous preliminary analysis with shorter follow-up presented at ASH 2012 (Farrell *et al*, 2012) demonstrated a significantly lower OS with the presence of HLA-A*02 (p = 0.04, Figure 5.13). The most likely reason for the loss of significance in the analysis with longer follow-up was a single death from cerebrovascular accident (stroke) at 192 months (> 16 years) from diagnosis in the HLA-A*02:01-negative group.

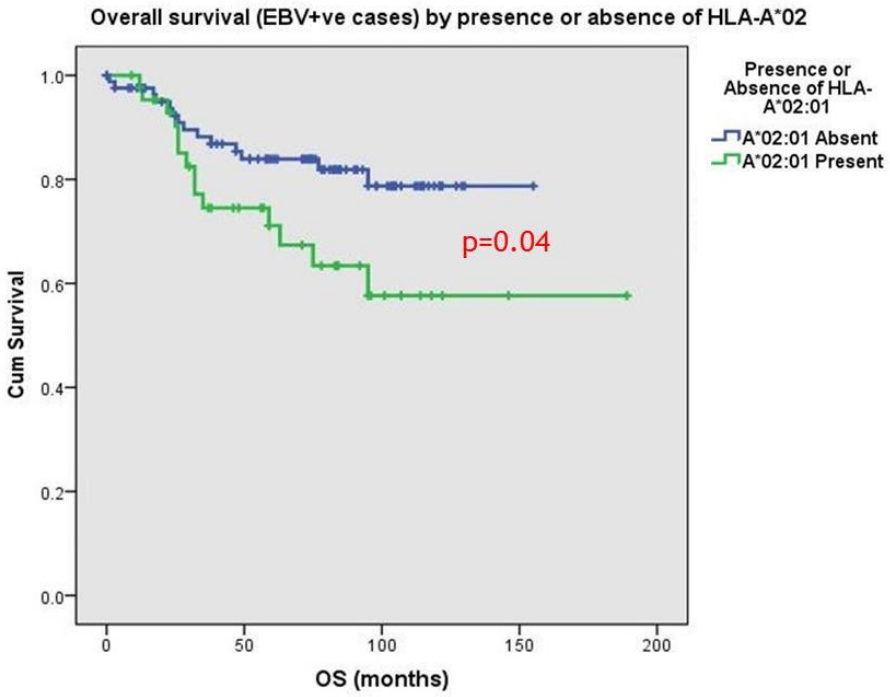


Figure 5-13 Overall survival in EBV-associated cases by presence or absence of HLA-A*02:01 in a prior analysis with shorter follow-up

OS, overall survival. Data shown as presented at ASH 2012 (Farrell *et al*, 2012). These data included fewer cases (n = 385) with shorter follow-up compared to the present analysis.

There was no statistically significant association between number of HLA-A*01:01 alleles present and OS, although there was only one death in HLA-A*01:01 homozygotes in the course of 192 months of follow up (91.7% OS at 192 months (n = 12), as compared with 61.8% in those with one HLA-A*01:01 allele (n = 43) or

58.1% in those with no HLA-A*01:01 allele (n = 78), Figure 5.14). Presence of an HLA-A*01:01 alleles was not significantly associated with OS in EBV+ve cHL.

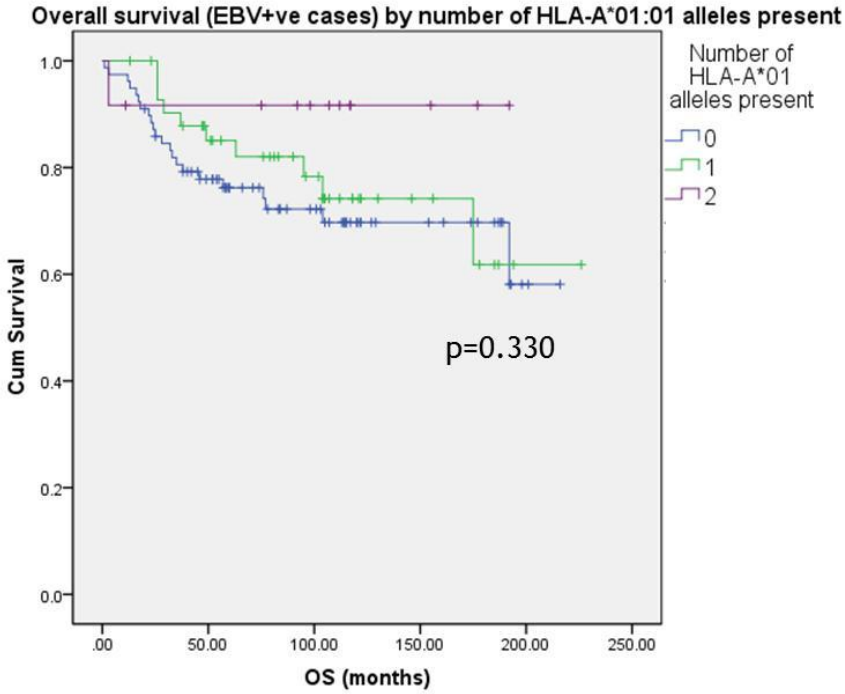


Figure 5-14 Overall survival in EBV-associated cases by number of HLA-A*01:01 alleles

OS, overall survival

Cox proportional hazard regression analysis of OS in EBV-associated cases identified increasing age (p < 0.001), presence of HLA-A*02 (p = 0.010), and increasing number of HLA-A*02 alleles (p = 0.047) were independently associated with poorer OS.

5.3.2.6 Analysis of EBV-associated cases – DSS

Log-rank analysis of Kaplan-Meier estimates of DSS in EBV+ve cases (122 cases analysable) demonstrated reduced DSS with advanced stage disease (p = 0.007). Improved DSS was seen with fewer A*01:01 alleles, although this did not reach

statistical significance (p = 0.219, Figure 5.15). Patients with an A*02:01 allele (p = 0.088, Figure 5.16) or greater numbers of A*02:01 alleles (p = 0.200, Figure 5.17) demonstrated poorer OS, although this did not reach statistical significance. In the Cox proportional hazard regression model of DSS in EBV+ve cases, only the presence of an HLA-A*02 allele was independently prognostic (p = 0.039); advanced stage disease p = 0.769 in this model.

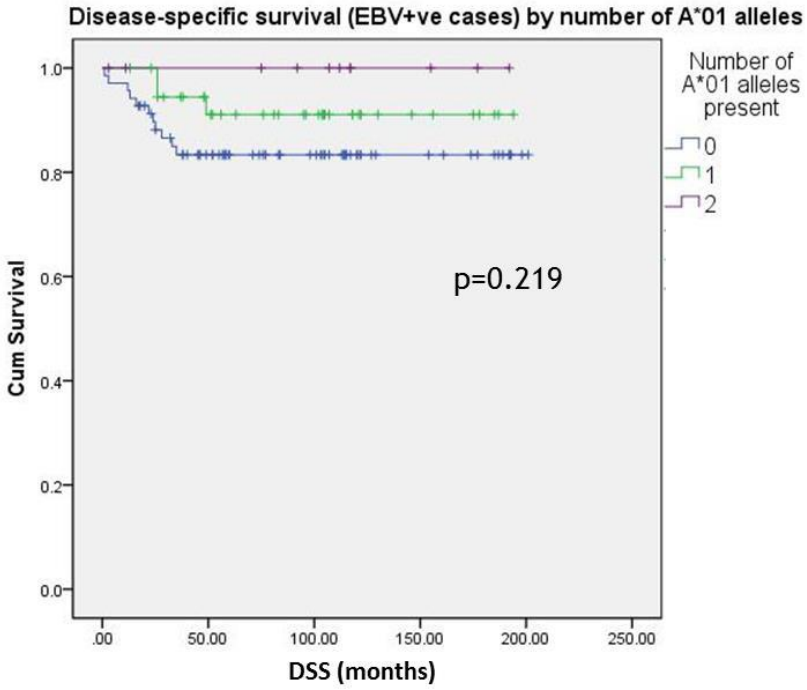


Figure 5-15 Disease-specific survival in EBV-associated cases by number of HLA-A*01:01 alleles

DSS, disease-specific survival.

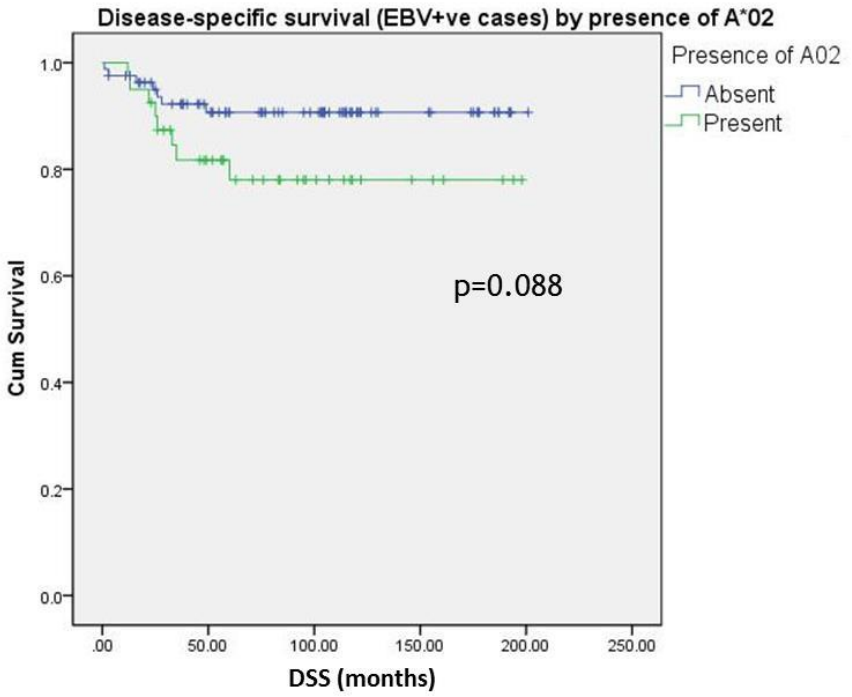


Figure 5-16 Disease-specific survival in EBV-associated cases by the presence or absence of the HLA-A*02:01 allele

DSS, disease-specific survival.

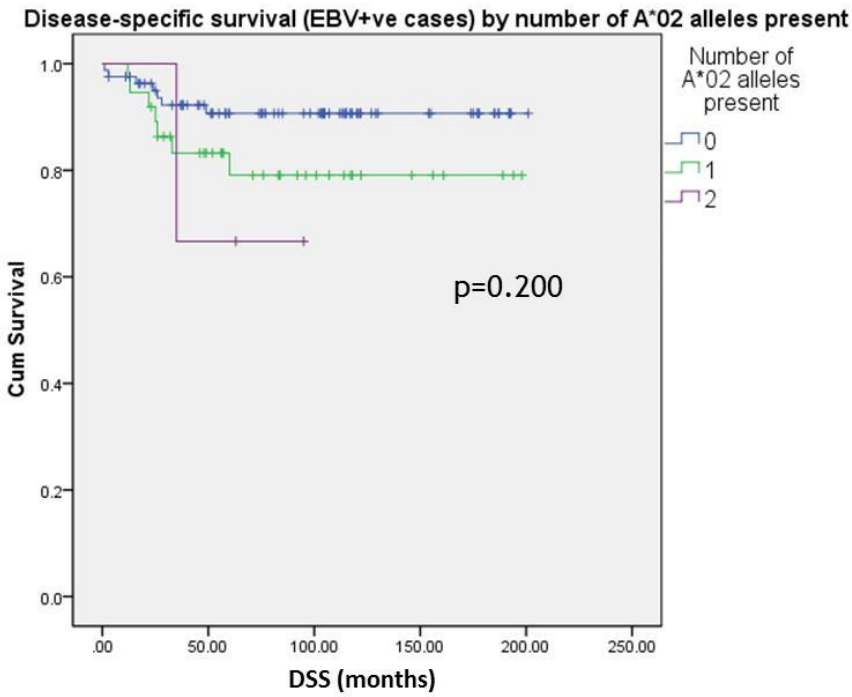


Figure 5-17 Disease-specific survival in EBV-associated cases by number of HLA-A*02:01 alleles

DSS, disease-specific survival

Table 5-4 Unadjusted log-rank comparisons of overall and disease-specific Kaplan-Meier estimates of survival in EBV-associated cases by HLA-A*01:01 and HLA*02:01 genotype.

Factor	Overall survival	Disease-specific survival
	p-value	p-value
Presence of an HLA-A*01:01 allele	0.229	0.146
Number of HLA-A*01:01 alleles	0.330	0.219
Presence of an HLA-A*02:01 allele	0.184	0.088
Number of HLA-A*02:01 alleles	0.017	0.200

P-values are from the log-rank comparisons of overall and disease-specific Kaplan-Meier estimates of survival by a single variable as stated. A p-value of < 0.05 is considered significant (indicated in blue).

5.3.3 Summary of Results

In this study of 424 adults with cHL from Scotland and the North of England, the clinical variables of age, sex and clinical stage were confirmed as prognostic. Histologic subtype (i.e. MC or NS subtype), still regarded as important to some investigators and physicians, was not significantly associated with outcome in any analysis. In contrast, EBV-status of the cHL was predictive of OS in univariate analysis, but was not independent of age, due to the higher proportion of older adults with EBV+ve disease. DSS was not associated with EBV status of the disease, suggesting that causes of death other than cHL (e.g. other cancers) account for the excess overall mortality in EBV+ve cases. This may suggest that EBV positivity in older adult cHL is as a surrogate of immune competence, and merits further study.

The finding that HLA-A*02:01 was under-represented in EBV+ve cases as compared with EBV-ve cases supports the previously published finding that this allele is protective against developing EBV+ve cHL (Hjalgrim *et al*, 2010).

Corroborating the findings of previous studies (Falk & Osoba, 1971; Hafez *et al*, 1985), but in a study conducted in the modern era with PCR-based HLA typing and modern therapeutic interventions, improved DSS in carriers of HLA-A*01:01 in all cases was observed.

In the present study HLA-A*02:01 was associated with inferior overall and DSS in EBV+ve cHL. In adjusted Cox proportional hazard regression analysis of OS in EBV+ve cases, only increasing age, presence of HLA-A*02:01 and increasing number of HLA-A*02:01 alleles were independently prognostic of inferior survival, following adjustment for histological subtype, clinical stage, age and sex.

In EBV-negative cases, the risk was in the opposite direction, with HLA-A*02:01 being associated with improved OS, approaching statistical significance.

5.4 Discussion

As HLA-A*02:01 is protective for developing EBV+ve cHL, we hypothesise that EBV+ve cHL arising in this 'protected' group is associated with greater biological or immunological dysfunction, resulting in poorer survival outcomes associated with HLA-A*02:01.

The non-significant association of HLA-A*01:01 with improved DSS in all cases supports previous studies, but is currently unexplained. Risk of disease development in these patients may be due more to poor immune control associated with HLA-A*01:01 (see Chapters 3 and 4) and not to other factors, so one might expect a better response to treatment.

Given the extremely poor outcomes seen in this study in HLA-A*02:01 carriers with EBV+ve disease (61.7% 10-year OS), it is possible that this group of patients is not currently being well-served by standard first-line therapy. HLA-A genotype may be helpful in identifying groups of patients who have inferior outcomes using standard therapy and may benefit from novel or cellular therapies.

Given the differences in survival observed by EBV status of disease, and the effect of HLA on survival in EBV-stratified cohorts of patients, this study supports the importance of stratifying cHL cases by EBV status in future clinical studies.

Although larger (n=424) than previously published studies of survival by HLA type in cHL (n = 52) (Hafez *et al*, 1985), DLBCL (n = 166) (Lu *et al*, 2011) or FL (n = 165) (Lu *et al*, 2011), I should emphasise that the conclusions drawn on survival in EBV+ve cases are based on analysis of the 137 patients with EBV+ve cHL included in the study. These numbers are small in comparison to the clinical studies used to generate prognostic scores e.g. of the 5141 patients included in

the IPS study (Hasenclever & Diehl, 1998) one would expect approximately 1700 EBV +ve cases, although it is noteworthy that EBV status was not included in the analyses in this paper. For this reason, larger studies within the context of clinical trials are required to extend these findings. It is hoped that the future research of the group will enable the analysis of survival by HLA in large prospective national clinical studies, in order that any differentials in outcome can be translated into clinical benefit for patients.

Chapter 6. General Discussion

6.1 Introduction

Over the last two decades, our knowledge of the pathophysiology of cHL has increased exponentially, improving our understanding of this heterogeneous disease. Epidemiological, molecular, microenvironmental and genetic findings are increasing our understanding of why some individuals develop cHL. Precisely how a ubiquitous virus such as EBV causes cHL in a minority of immune competent hosts remains an open question, although HLA type and the CTL response are likely to be central in this.

Clinical developments mean that the vast majority of patients with cHL are now cured of their disease; however, late toxicities of therapy remain a significant cause of morbidity in survivors. In addition, whilst the majority are cured, approximately 15-20% of patients, many of whom are young adults, will die of their disease. Clinicians treating this disease recognise that some cHL behaves in a more recalcitrant manner, proving stubborn in the face of intensive therapy. Much remains to be done to improve outcomes for this group.

The work of this thesis set out to address a number of specific questions regarding the role of HLA class I in the aetiology and clinical outcome of cHL. This Chapter summarises these findings and discusses the implications that this may have for further research in this area.

6.2 Does the presence of an HLA-A*01:01 allele modify the magnitude of the CTL response to HLA-A*02:01-restricted epitopes?

Our findings suggest that overall HLA-A phenotype does not significantly affect the EBV-specific CTL response restricted through HLA-A*02:01. It was demonstrated that responses in HLA A*02:01/ A*x heterozygotes were greater

than in the other groups studied but these findings had to be disregarded for reasons of confounding, and adjusted analysis was not possible. In valid comparisons between HLA-A*02:01/A*01:01 heterozygotes and HLA-A*02:01 homozygotes, no differences in CTL response as assessed by ELISPOT, CTL degranulation and IFN-γ secretion were demonstrated.

Greater EBV CTL responses were observed in samples from female donors and with shorter time-to-processing. Differences by sex have not previously been reported with regard to EBV responses, but may go some way to explaining the higher prevalence of EBV-associated malignancies in men as compared with women. In addition, this finding has potential implications for biological and clinical studies examining the EBV-specific CTL response. If this study was to be repeated or extended in the future, it could be suggested that groups should be matched for sex, or restricted to one sex, and should include only samples processed on the same day as collection.

The primary experimental question "*Does the presence of an HLA-A*01:01 allele modify the magnitude of the CTL response to HLA-A*02:01-restricted epitopes?*" was partly answered. Exploratory analysis of cytokine levels in response to stimulation with EBV peptides demonstrated significantly higher secretion of IL-10 (with a nearly 10-fold difference), IL-17 and IL-5 in response to stimulation with EBV peptides in HLA-A*02:01/A*01:01 heterozygotes, compared to other HLA-A*02:01 carriers. This suggests a possible effect of HLA-A*01:01 which might begin to explain some of the HLA-associated differences in risk of developing EBV+ve cHL. It is possible that HLA-A*01:01 is associated with an inhibitory immune response in the face of EBV, either directly, or through LD with genes in the extended MHC encoding involved in the cytokine response. Although this

study was small and exploratory, the magnitude of the differences observed, particularly for IL-10, is provocative and warrants further investigation.

6.3 Can any HLA-A*01:01-restricted EBV-specific CTL responses be detected or are any inhibitory cytokine responses detected?

Using sensitive methodologies and a peptide prediction strategy, no HLA-A*01:01-restricted CTL responses to EBV were detected in this study. This lack of response was observed in unstimulated BD-PBMCs and also following stimulation using EBV-infected LCLs to boost low-level memory-CD8 CTL responses.

The lack of demonstrable HLA-A*01:01-restricted EBV-specific IFN-γ CTL response may in part explain the elevated risk of EBV+ve cHL associated with HLA-A*01:01.

The critical CTL response to EBV, which is reflected in disease risk, may be important at any stage from primary EBV infection to establishment of EBV+ve cHL. However, one could hypothesise that the response to those peptides presented in the latency II programme are most important in the context of EBV+ve cHL. Our findings favour the idea that the critical response may be to latency II antigens. Responses to the latency II peptides are much more restricted in the range of HLA-types through which a response can be generated. In this context, lack of response through a single common allele (HLA-A*01:01) may become more important. Further studies elucidating the class I-restricted immune response to the latency II programme of EBV are merited.

In the analysis of cytokine excretion in response to stimulation with EBV-infected LCLs in HLA-A*01:01 homozygotes and HLA-A*01:01/A*02:01 heterozygotes,

differences in levels of cytokine secretion were demonstrated. HLA-A*01:01 homozygotes generated much higher levels of IL-6, a cytokine implicated in the pathogenesis of cHL, than HLA-A*01:01/A*02:01 heterozygotes following stimulation with autologous LCLs. Higher levels of IL-2 were also seen after 16 days of stimulation in A*01:01 homozygotes. IL-2 is known to drive a T_{reg} response, thus higher levels may contribute to a dampening of EBV-specific immunity contributing to development of cHL.

Lower levels of secretion of IL-5 were also seen in HLA-A*01:01 homozygotes in comparison to A*01:01/A*02:01 heterozygotes. At first this is more difficult to explain as IL-5 is implicated in the proliferation and apoptosis resistance of HRS cells. However, in the context of the improved DSS seen in carriers of HLA-A*01:01 (Chapter 5), this begins to make sense as lower levels in this group may suggest lower proliferation and increased apoptosis of the HRS cells. Functional studies to explore this should be performed.

Whilst this study is small and exploratory, the cytokine profiles observed in response to stimulation with EBV are intriguing. Differential cytokine secretion in response to other viruses by class I HLA phenotypes has previously been described, although the mechanisms involved have not yet been explained. Further studies should be performed to validate these findings and to study the means by which this operates.

6.4 Are HLA-A*01:01 and A*02:01 alleles a factor in determining clinical outcome in EBV+ve cHL?

In this study of 424 adults with cHL from Scotland and the North of England, we demonstrated that HLA-A*01:01 and A*02:01 are factors in determining clinical outcome in cHL.

Corroborating the findings of studies performed in a previous era, improved DSS in all cases of cHL (EBV+ve and EBV-ve) was observed in carriers of HLA-A*01:01. Despite being reported now in a number of studies, this finding is currently unexplained. Risk of disease development in these patients may be due solely or predominantly to poor immune control associated with HLA-A*01:01 (see Chapters 3 and 4) in the absence of other risk factors, and therefore one might expect a better response to treatment. Alternatively, these results might suggest a more important role of class I HLA in the tumour microenvironment than is currently presumed.

In the present study HLA-A*02:01 was associated with inferior OS and DSS in EBV+ve cHL. In adjusted analysis of OS, only increasing age, presence of HLA-A*02:01 and increasing number of HLA-A*02:01 alleles were independently prognostic of inferior survival. As HLA-A*02:01 is protective for developing EBV+ve cHL, we hypothesise that EBV+ve cHL arising in this 'protected' group is associated with greater biological or immunological dysfunction, resulting in poorer survival outcomes associated with HLA-A*02:01.

Given the extremely poor outcomes seen in this study in HLA-A*02:01 carriers with EBV+ve disease (61.7% 10-year OS), it is possible that this group of patients is not currently being well-served by standard first-line therapy. HLA-A genotype may be helpful in identifying groups of patients who have inferior outcomes using standard therapy and may benefit from novel or cellular therapies.

Although larger than all previously published studies of survival by HLA type in lymphoma, at 424 patients, these conclusions are based on analysis of only the 137 patients with EBV+ve cHL. These numbers are small in comparison to the clinical studies used to generate prognostic scores, e.g. the 5141 patients

included in the IPS study (Hasenclever & Diehl, 1998), although it is noteworthy that EBV status was not included in the analyses performed by this group. For this reason, larger studies within the context of clinical trials are required to extend these findings. It is hoped that the future research of the group will include the analysis of survival by HLA in the large prospective UK national clinical studies (e.g. NCRI RAPID and RATHL, discussed in Chapter 1) which have recently completed recruitment. If studies of outcomes in such large prospective trials confirm differential outcome by HLA-type, this could generate hypotheses for targeting of therapy which could be translated into clinical benefit for patients.

6.5 Concluding Remarks

The work of this thesis sheds further light on the central questions which remain at the heart of cHL research, namely, why do some people develop EBV+ve cHL, and why do some patients continue to die of their disease when the majority are cured.

A lack of a detectable HLA-A*01:01-restricted EBV-specific CTL response and differences in cytokine secretion in carriers of HLA-A*01:01 suggest biologically plausible mechanisms by which risk of developing cHL may be increased and merit further exploration.

Differential DSS and OS were observed, with improved DSS in carriers of HLA-A*01:01 in all cases, and inferior OS and DSS associated with HLA-A*02:01 in EBV+ve cases. These findings require confirmation, but potentially could explain some of the differences in outcome seen between individuals, and offer exciting possibilities for the targeting of therapy to improve clinical outcome.

Appendix 1: Suppliers

Supplier	Address
Abbot Diagnostics Ltd.	Abbott House, Vanwall Business Park, Vanwall Road, Maidenhead, Berkshire, SL6 4XF, UK
AID Diagnostika GmbH	Ebinger Strasse 4, D-72479 Straßberg, Germany
Alta Bioscience	University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK
Anachem Ltd.	Anachem House, 1 & 2 Titan Court, Laporte Way, Luton, Bedfordshire, LU4 8EF, UK
BD Biosciences	Edmund Halley Road, Oxford Science Park, OX4 4DQ, Oxford, UK
Beckman Coulter (UK) Ltd.	Oakley Court, Kingsmead Business Park, London Road, High Wycombe, HP11 1JU, UK
DAKO UK Ltd.	Cambridge House, St. Thomas Place, Ely, Cambridgeshire, CB7 4EX, UK
DJB Labcare Ltd.	20 Howard Way, Interchange Business Park, Newport Pagnell, Buckinghamshire, MK16 9QS, UK
E-biosciences Ltd.	2nd Floor, Titan Court,3 Bishop Square Hatfield, AL10 9NA, UK
Elkay Laboratory Products (UK) Ltd.	Unit E, Lutyens Industrial Centre, Bilton Road, Basingstoke, Hampshire, RG24 8LJ, UK
ENM Company	5617 Northwas Highway, Chicago, IL 60646-6135, USA
Fisher Scientific UK Ltd.	Bishop Meadow Road, Loughborough, LE11 5RG, UK
Gambro BCT	Lundia House, Unit 3 The Forum, Minerva Business Park, Peterborough, PE2 6FT, UK
GE Healthcare UK Ltd.	Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK
Gen-Probe Life Sciences Ltd.	Appleton Place, Appleton Parkway, Livingston, West Lothian, EH54 7EZ
Gilson Scientific Ltd.	20 Charles St. Luton, Bedfordshire, LU2 OEB, UK
Grant Inments (Cambridge) Ltd.	Shepreth, Cambridgeshire, SG8 6GB, UK
Graphpad Software Inc.	2236 Avendida de la Playa, La Jolla, CA 92037, USA
Greiner Bio-One Ltd.	Brunel Way, Stroudwater, Business Park, Stonehouse,GL10 3SX, UK
Haier	Westgate House, Westgate Ealing, London, W5 1YY, UK
Hawksley	Marlborough Road, Lancing Business Park, Lancing, Sussex, BN15 8TN, UK
HTZ Ltd.	3rd Floor, 55 Gower Street, London, WC1E 6HQ, UK
IBM UK Ltd.	UK Head Office, PO Box 41, North Harbour, Portsmouth, Hampshire, PO6 3AU, UK
Invitrogen Ltd.	3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK
Luminex B.V. Europe	Krombraak 13-15, 4906 CR Oosterhout NB, The Netherlands
Microsoft Corp.	One Microsoft Way, Redmond, WA 98052-6399, USA
Microzone Ltd.	4 Heath Square, Boltro Road, Haywarth Heath, West Sussex, RH16 1BL, UK
Millipore (UK) Ltd.	Suite 3 & 5, Building 6, Croxley Green Business Park, Watford, WD18 8YH, UK
Miltenyi Biotec Ltd.	Almac House, Church Lane Bisley, Surrey, GU24 9DR, UK

Supplier	Address
MVE Biological Systems	2200 Airpost Industrial Drive, Suite 500, Ball Ground, GA 30107, USA
New Brunswick Scientific/ Eppendorf Ltd.	Endurance House, Vision Park, Histon, Cambridge, CB24 9ZR, UK
Pall life sciences	Walton Road, Farlington, Portsmouth, PO6 1TD, UK
QIAGEN Ltd.	QIAGEN House, Fleming Way, Crawly, West Sussex, RH10 9NQ, UK
R&D Systems Europe	19 Barton Lane, Abingdon Science Park, Abingdon, Oxon OX14 3NB, UK
Royal Mail	Royal Mail, FREEPOST SCO6274, 21 South Gyle Crescent, Edinburgh, UK
Scientific Laboratory Supplies (SLS)	Ruddington Lane, Wilford Industrial Estate, Nottingham, NG11 7EP, UK
Sigma-Aldrich Company Ltd.	The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK
Thermo-Fisher Scientific Inc.	81 Wyman Street, Waltham, MA 02454, USA
Thomson Reuters	2141 Palomar Airport Road, Suite 350, Carlsbard, CA 92011, USA
vWR International Ltd.	Unit 15, The Birches, Willard Way, Imberhorn Industrial Estate, East Grinstad, West Sussex, RH19 1XZ, UK
Xstrahl Ltd. UK	The Coliseum , Watchmoor Park Riverside Way , Camberley Surrey, GU15 3YL, UK

Appendix 2: SHARE study questionnaire

The SHARE study – Study of Healthy Adult Responses to EBV

Study Questionnaire v2 10th July 2009 REC Ref: 08/S0709/149

SHARE study number:

Age:_____years Sex: M/F

Post code: _ _ _ _ _ (omit last two digits e.g., G61 1 or PA34 8)

Have you ever had glandular fever (infectious mononucleosis) Yes/No

If Yes: was this confirmed by a laboratory test Yes/No

What age were you at diagnosis: _____

Do you have any current symptoms of allergy Yes/No

Smoking

Do you smoke cigarettes at all nowadays Yes/No

Have you ever smoked regularly Yes/No

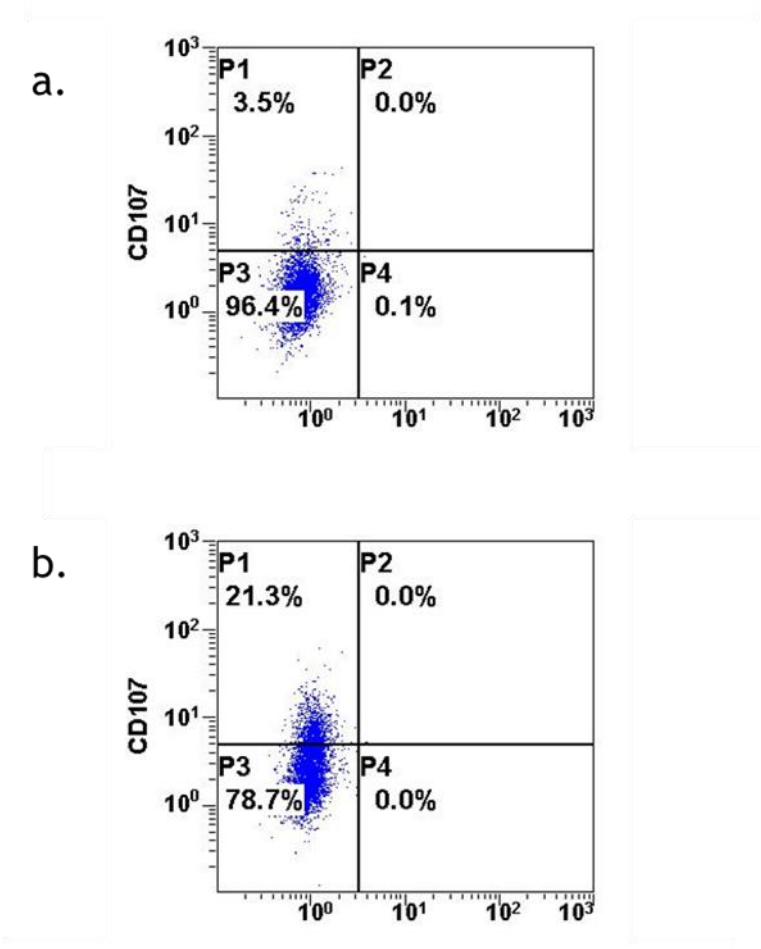
What age were you when you started smoking: _____

How many cigarettes do you/did you smoke per day:_____

Appendix 3: Amino Acid Code

One-letter abbreviation	Three-letter abbreviation	Full name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Glu	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Appendix 4: Gating strategy in CD107 flow cytometry



Gating strategy used in the assessment of CTL degranulation by flow cytometry for CD107. Representative results from one donor p10098 shown. The anti-CD017 is present for the duration of stimulation and binds the CD107 normally only transiently expressed on the cell surface prevents its re-internalisation by endocytosis. a) Negative control: B-depleted peripheral blood mononuclear cells (BD-PBMC) with culture medium plus DMSO only. b) BD-PBMC stimulated with EBV peptides, in this case peptide pool 2 (see Table 3.2) comprising latent peptides. Gate P1 comprises the positive events counted. The delta CD107 is defined as the change in CD107 expression following stimulation (P1 stimulated – P1 negative control). The gates used were the same for all stimulations and controls.

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