Epstein-Barr Virus Infection in Adult Renal Transplant Recipients

A Thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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The University of Manchester

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A Thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

Epstein-Barr Virus Infection in Adult Renal Transplant Recipients

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Abstract

Aims: To explore the clinical significance of EBV infection in adult renal transplant recipients when detected in the late post-transplant period.
Methods: (1) A prospective observational study recruiting 499 stable adult kidney transplant recipients with serial blood sampling for EBV DNAemia and assessment of clinical outcomes and associated factors. (2) A retrospective analysis of PTLD incidence, timing and outcomes in relation to EBV infection.
Results: EBV DNAemia in stable kidney transplant recipients is common, found in 46% of recruited individuals screened over 1 year, with persistent DNAemia seen in 10%. DNAemia prevalence increased significantly with time from transplant ($p<0.0001$) from 16% within 1 year of transplant to 66% in those transplanted for 20-24 years. High baseline DNA levels predicted persistence of DNAemia. Time adjusted analyses showed significant association of DNAemia with EBV seronegative status and previous PTLD and low DNAemia rates with Mycophenolate Mofetil (MMF) use and lymphopenia. The mechanism did not appear to be directly linked to MMF induced B cell depletion. Chronic high viral load detection was significantly associated with time from transplant, EBV seronegative status at transplant, ciclosporin use and plasma detection of DNA. No significant differences in overall patient survival at 3 years, clinical symptoms or clinical findings such as anaemia, thrombocytopenia or rate of decline in renal function were seen between stable transplant recipients with and without EBV DNAemia. PTLD incidence also increases with time from transplant and was greatest during the 10th-14th post-transplant years. Disease was EBV positive in 68% cases. No statistically significant differences in overall patient survival, or overall disease complete response rates were seen in relation to recipient EBV serostatus or EBV status of PTLD histology.
Conclusions: EBV DNAemia prevalence increases with time from transplant but was not associated with worse patient or graft survival or specific symptoms. PTLD incidence including EBV negative disease also increases with time from transplant but response rates and survival were not influenced by EBV serostatus or histological status.

Declaration of authorship

This PhD thesis is the work of David Muir Morton. The work included is the candidate's own, unless otherwise stated. No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Most of all I would like to thank my family, my wife Freya and the team, Finn, Tom and Hamish for putting up with my hours in the office and lack of play.

The Author

The author of this work is currently a Locum Consultant Nephrologist at Manchester Royal Infirmary. The research presented in this thesis was performed as part of a PhD at the University of Manchester undertaken September 2009-

September 2012. The author graduated from the University of Manchester with an MBBS in 2002 having achieved an MA (Hons) in History at the University of Edinburgh 1996. After obtaining the MRCP (UK) in 2006 I was appointed Specialist Registrar in Nephrology in 2006 and subsequently completed the MRCP Neph (2012) and obtained a Certificate of Completion of Training in Nephrology in 2013.

Role of the candidate in this PhD

All patient identification, data collection, data management, data analysis and paper preparation in relation to the PTLD studies presented below were performed by the author. Patient identification and recruitment of patients to the EBV study was carried out by the author and Stephanie Johnson. Blood sampling was performed by the renal transplant outpatient nurses. Blood sample extraction for EBV DNA (automated) and serological analysis (automated) of clotted samples for EBV antibodies was performed in the clinical HPA laboratory at Manchester Royal Infirmary. All EBV PCR optimisation experiments and analysis of study samples were performed by the author. Recruitment, blood sampling and analysis of plasma and whole blood samples for EBV DNA for individuals recruited to the phase 2 EBV study was performed by the author. Ultrasound examinations were performed by sonographers at Manchester Royal Infirmary after a study protocol was created by the author, Paul Taylor, Lynn Kenderdine and Mandy Wilde. Data collection, medical casenote review, data analysis and paper preparation for the EBV studies was performed by the author. Flow cytometry analysis of lymphocyte subsets was performed by Julie Adams with support from the author. Biostatistical support was

provided by Steve Roberts (University of Manchester) and supervision, review of experiments and paper review by Pamela Vallely, Mike Picton, Beatrice Coupes, Paul Klapper, Kate Ryan, Julie Adams, John Burthem, Richard Byers and Paul Brenchley.

List of Abbreviations and Symbols

µ	Micro
µl	Microlitre
Bp	Base pair
BL	Burkitt's lymphoma
CAEBV	Chronic active Epstein-Barr virus
CMV	Cytomegalovirus
CT	Cycle threshold
dH2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5' triphosphate
EA	Early antigen
ELISA	Enzyme linked immunosorbent assay
EBV	Epstein-Barr virus
EBER	Epstein-Barr virus encoded small RNAs
EBNA	Epstein-Barr nuclear antigen
FBC	Full blood count
GC	Germinal centre
HL	Hodgkin's lymphoma
HHV	Human herpes virus
HLA	Human leukocyte antigen
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
IM	Infectious mononucleosis
IL	Interleukin
LMP	Latent membrane protein
MHC	Major histocompatibility complex
ml	Millilitre

min	Minute
NFAT	Nuclear factor of activated T-cells
NPC	Nasopharyngeal carcinoma
NK	Natural killer
NF	Nuclease free
OHL	Oral hairy leukoplakia
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PTLD	Post-transplant lymphoproliferative disease
R	Receptor
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
SOT	Solid organ transplantation
TCR	T cell receptor
Th	T helper
TK	Thymidine kinase
TNF	Tumour necrosis factor
VCA	Viral capsid antigen

1. Introduction

1.1 Background and Context of the research

This PhD investigates the natural history and implications of EBV infection in adult renal transplant recipients focusing particularly on the late (> 1 year) post-transplant period.

Epstein-Barr virus (EBV) is a gammaherpes virus capable of causing disease in humans. Evidence of infection in humans (seropositivity) is found in 90% adults worldwide. After primary infection the virus establishes latency and persists lifelong in B lymphocytes. In the healthy human, numbers of infected B cells are controlled by host immune responses including T cells. In the transplant patient current models suggest that the administration of immunosuppression impairs T cell number and function (1) . This T cell impairment results in a failure to detect and destroy EBV infected B cells which may proliferate in an uncontrolled manner with subsequent development of life-threatening post-transplant lymphoproliferative disorder (PTLD). The area of research is important as PTLD is the second most common form of malignancy after skin cancer, occurring in transplant recipients, and survival after diagnosis is poor. EBV DNA is typically detected in blood at time of PTLD diagnosis in up to 80% cases but may also be seen in those without clinical evidence of disease.

The first year after transplantation is recognised as a high risk period for both EBV related illness and development of PTLD. Individuals without prior EBV infection who receive a kidney transplant from an EBV seropositive donor are at particular risk. This situation applies particularly to paediatric transplant populations where

rates of EBV seropositivity are typically lower than amongst adults. Consequently EBV related clinical guidelines have focused primarily on the first post-transplant year.

The prevalence and clinical implications of EBV DNAemia in adults particularly in the late post-transplant period are not yet well described or studied. EBV DNAemia may result in extensive investigation for lymphoma and pre-emptive treatments such as rituximab and withdrawal of immunosuppression which, while seeking to reduce PTLD related mortality and morbidity, may put individuals at risk of complications of immunotherapy and risk of graft rejection or loss. Patient and graft survival rates for adult renal transplant recipients are generally longer than those for recipients of other solid organs and consequently in the adult renal transplant population there is a group exposed to immunomodulatory medication for very long periods of time, some for over 30 years.

This PhD will research the epidemiology and prevalence of EBV infection in both the stable adult kidney transplant population and amongst those individuals who develop PTLD. Analysis will be made of the implications of time from transplant and duration of immunosuppression on EBV and PTLD epidemiology. Outcomes in relation to patient and graft survival and EBV related morbidity will be analysed in these groups and clinical and biological factors associated with EBV infection and PTLD investigated.

1.2 Key Research Question

What is the clinical significance of EBV infection in adult renal transplant recipients when detected in the late post-transplant period?

The focus of the PhD is based on the key research question above. To answer this question a series of 4 papers will be presented in alternative thesis format. These papers will follow a line of enquiry that will aim to answer a number of key clinical questions in a logical and continuous fashion. The clinical questions are presented below:

1.2.1 Key clinical questions

1. What is the prevalence of EBV DNAaemia in adult renal transplant populations?
2. Are rates of EBV DNAemia influenced by time from transplantation?
3. What clinical, viral, and immunological factors are associated with EBV DNAemia?
4. What are the clinical outcomes and implications of EBV DNAemia particularly in the late post-transplant period?
5. What is the incidence of PTLD in UK based adult kidney transplant recipients managed with immunosuppressive regimens following NICE guidelines?

6. Is EBV infection associated with incidence, timing or outcome of PTLD?

The 4 papers presenting the research and aiming to answer the research and clinical questions are as follows:

Paper 1: Epstein-Barr virus infection in adult renal transplant recipients.
This is a detailed analysis of the prevalence, associated factors, clinical outcomes and implications of EBV DNAemia in stable adult renal transplant recipients attending routine outpatient follow up.
This paper has been submitted to the American Journal of Transplantation (June 2013) and is currently under review.

Paper 2: Viral, immunological and clinical features of EBV infection in adult renal transplant recipients.
This paper investigates in more depth differences between individuals identified and described in the first paper and includes those with chronic high viral load detection, low level or transient DNAemia and those with persistently undetectable levels of DNA in blood.

Paper 3: Epidemiology of Post-Transplant Lymphoproliferative Disorder in Adult Renal Transplant Recipients.
This is an in depth description and analysis of cases of Post-transplant lymphoproliferative disorder occurring in renal transplant recipients from

Manchester Royal Infirmary 1969-2011. Analysis will be made of incidence rates, presentations, survival and prognostic markers with comparison to published standards in the literature. The prevalence and implications of Epstein-Barr virus positivity in these cases will be examined.

This paper has been published in the journal Transplantation:

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., et al. (2013). Epidemiology of Posttransplantation Lymphoproliferative Disorder in Adult Renal Transplant Recipients. *Transplantation*, *95*(3), 470–478. doi:10.1097/TP.0b013e318276a237

Paper 4: Post-Transplant Lymphoproliferative Disorder in Adult Renal Transplant Recipients: Treatment, Response, Survival, and Prognosis.

This paper analyses the outcomes and prognostic markers including relevance of EBV infection in patients from the Manchester Royal Infirmary transplant unit with PTLD.

2 Review of literature

2.1 Background

2.1.1 Kidney failure and renal replacement therapy

Kidney failure occurs in approximately 108 per million population (pmp) per year in the UK (2). The onset of kidney failure has serious implications for patient survival. Without renal replacement therapy progressive metabolic derangement and death occur. Renal replacement therapy in the form of peritoneal dialysis, haemodialysis and kidney transplantation prolong life and improve quality of life through removal of uraemic toxins and fluid. The population prevalence for adults receiving renal replacement therapy was 774 per million population per year (pmp) in 2008 (2). However, even with renal replacement therapy the renal registry data shows an increased age-standardised mortality ratio compared to the general population of 28.6 at age 30 years and 4.6 at age 80 years (3).

2.1.2 Kidney Transplantation

Kidney transplantation is the chosen form of renal replacement therapy for approximately 50% of current patients on renal replacement therapy and 2486 kidney transplants were performed in the UK 2008 – 2009 (2). Transplantation offers improvements in length and quality of life over peritoneal and haemodialysis. One year survival in the UK in 2008 for prevalent transplant patients was 97.7%

compared to 91.8% in prevalent dialysis patients aged less than 65 and 79.6% in dialysis patients over the age of 65 (3).

Graft survival is a measure of kidney transplantation outcome and has improved over the last 4 decades: 1 year survival rates have improved from approximately 60% (late 1970's) for cadaveric kidney transplants to 93% (2004-2007) for first deceased heart-beating kidney transplants. Similarly, living donor 1 year graft survival has improved from approximately 80% in the 1970's to 96% (2004-2007). Ten year graft survival has similarly improved from 40% (deceased donors) and 66% (living donors) in the late 1970's to 66% (deceased) and 70% (living) for the 1995-1997 cohort of transplant recipients (all data derived from NHS Blood and Transplant Statistics). Incidence of early acute rejection has also declined from 50% in the early 1990's to current rates of less than 10% at one year. These improvements in graft survival and reduction in early acute cellular rejection follow changes in immunosuppressive regimens and the introduction of increasingly potent agents. Acute cellular rejection is driven by the activated T lymphocyte (1). Introduction of more potent immunosuppressive agents and combinations of therapies has resulted in more effective inhibition of T cell activation and proliferation. These agents include the calcineurin inhibitors ciclosporin and tacrolimus, the antiproliferative agent mycophenolate mofetil and induction agents such as the CD25 monoclonal antibody basiliximab. More effective inhibition of T cell responses has reduced rejection rates and improved graft survival but, as T cells are necessary for the effective functioning of the innate, adaptive and humoral

immune responses, there is a consequent risk of compromise to the patient's defence mechanisms to identify and control microbial pathogens and abnormal cellular proliferations.

In AIDS (the acquired immunodeficiency syndrome) progressive depletion of T cells as a consequence of HIV infection results in a well-recognised ladder of opportunistic infections and malignancies. In solid organ transplantation the mechanisms behind the T cell dysfunction are different yet a similar spectrum of infections is experienced. For HIV patients the CD4 count is used to identify those at increased risk of specific infections. In solid organ transplantation infection is linked to dose and use of immunosuppressive agents. The first year after transplantation is typically a high risk period, so to reduce the risk of rejection T cell depleting induction therapies may be used and primary immunosuppressive drug concentrations are maintained at higher levels. Empirical prophylaxis is given during the first 6 months after transplantation to protect against specific opportunistic infections such as *Pneumocystis jirovecii* pneumonia or tuberculosis. In the immunocompetent patient primary infection with viruses such as cytomegalovirus, Epstein-Barr and varicella zoster result in well described clinical syndromes. The infections may result in illness but control is established and lifelong carriage of these herpes viruses occurs. In the transplant patient such infections can result in severe and life threatening illnesses that may be prolonged and difficult to control without reductions in the immunosuppressive burden and specific antimicrobial agents. The risk of malignancy, in particular for those with

aetiology linked to oncogenic viruses is also increased in solid organ transplant patients.

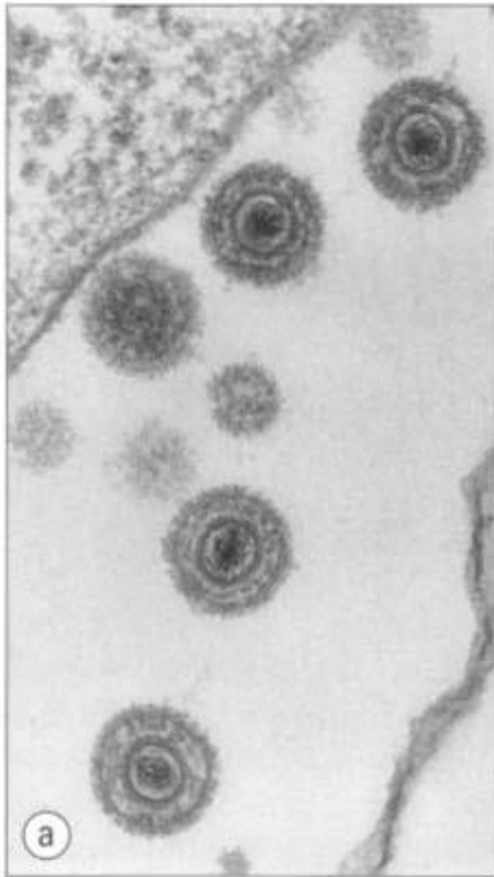
The risk of serious malignant and infectious complications in transplant patients is reflected in mortality data describing causes of death in those on renal replacement therapy in the UK during 2010 (4). These statistics show an increased risk of death for such individuals compared to the age matched general population. While cardiac disease is the leading cause of death for prevalent and incident haemodialysis patients in the UK, infection and malignancy currently account for 45% of deaths compared to 17% due to cardiac disease in prevalent transplant patients.

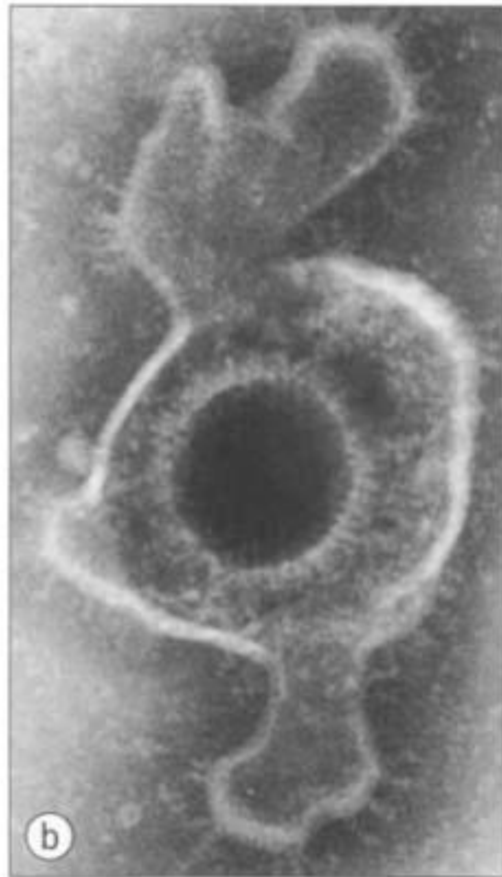
PTLD is the most common non-skin malignancy affecting recipients of renal transplants with a 12-fold increased risk compared to the general population (Opelz & Dohler, 2004). A third group of cancers including oral, lip, anal, cervical, vulval and vaginal cancers make up a further large proportion of reported malignancies. The incidence of these cancers compared to the general population is increased 25-fold for non-melanoma skin cancer, 14-fold for oral, tongue and lip, and 12-fold for cervical, vulval and vaginal (6,7). Implicated in the pathogenesis of PTLD and the other cancers listed above are oncogenic viruses including Epstein-Barr virus, human herpes virus 8 (Kaposi's sarcoma associated virus) and the human papilloma viruses. For example Epstein-Barr virus can be detected in approximately 70% of PTLD histological specimens (Caillard, Lelong, Pessione, &

Moulin, 2006). Impaired immune responses, in particular T cell function and surveillance as a consequence of the administration of immunosuppression results in a failure to detect and destroy infected cells. These factors lead to a greater number of cells being infected by these viruses than would be the case in a healthy individual. Infected cells may then undergo malignant transformation as a consequence of virally mediated signals, genetic mutations and impaired immune function. Unchecked proliferation of these abnormal cells may result in malignancy.

2.2 Herpesviridae

Epstein-Barr virus or human herpes virus 4 (HHV4) is one of 8 members of the *Herpesviridae* family known to infect humans. All *Herpesviridae* family members have virions or viral particles that share common properties. They are similar in size, 120-220nm in diameter and typically spherical in shape. The core of the virion is the viral genome consisting of a linear, double stranded DNA of 170,000 nucleotides encoding up to 200 genes (9). Surrounding the genome is an icosahedral capsid, 100-110 nm in diameter and containing 162 protein subunits called capsomeres. The tegument is a layer of viral proteins separating the capsid from the outer layer of the virion, the envelope (Figure 2.1). Contained in the envelope are a number of glycoprotein spikes that evenly cover the surface.





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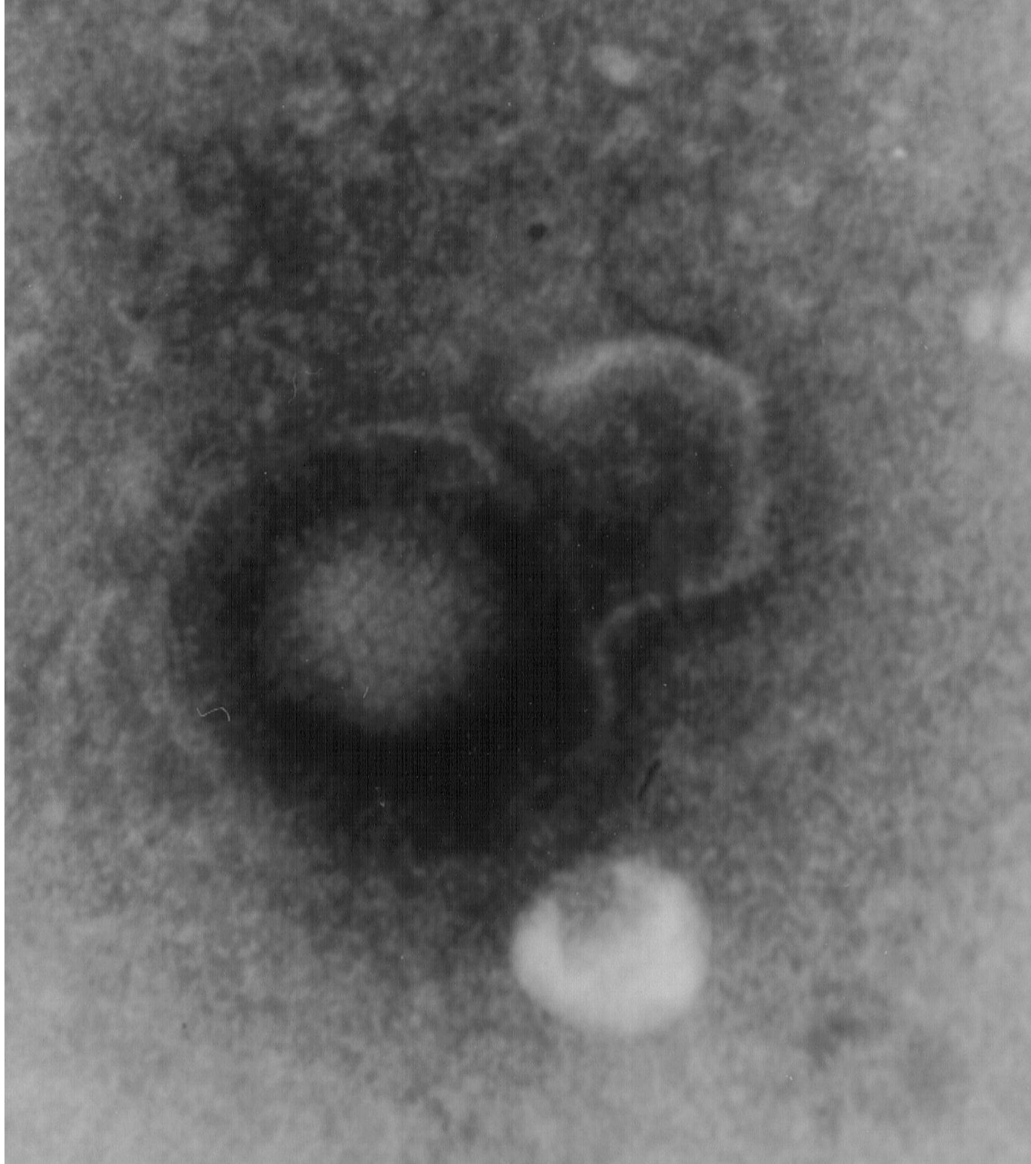

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Figure 2.1 Electron microscope images of enveloped herpesvirus particles. (a) Thin section. (b,c) Negative staining of round capsid containing genetic material surrounded by the tegument and encased in the outer layer of the virion, the envelope (Magnification ×140,000). Electron microscope images donated by Professor Paul Klapper.

The current classification of the *Herpesviridae* family is based on the International Committee on Taxonomy of Viruses report 2006 (10). Epstein-Barr virus (HHV4) is

a member of the Herpesviridae subfamily gammaherpesvirinae and genus lymphocryptovirus. Gammaherpesvirinae genera including EBV can infect epithelial cells and B and T lymphocytes. EBV only infects humans and establishes latent infection in B lymphocytes. The two other Herpes subfamilies include Alphaherpesvirinae and Betaherpesvirinae. Alphaherpesvirinae primarily establish latent infections in sensory ganglia. Genera include the Human herpes virus varicellovirus (HHV3 varicella zoster virus), and the simplex viruses (human herpes virus 1 / herpes simplex virus 1 and human herpes virus 2 / herpes simplex virus 2). Betaherpesvirinae include the genera cytomegalovirus (human herpes virus 5/ human cytomegalovirus) and Roseolovirinae including human herpes virus types 6 and 7 (HHV6 and HHV7). Latency is established in tissues including kidney, lymphoreticular cells and secretory glands.

2.3 Epstein-Barr Virus (EBV)

The Epstein-Barr virus particle received its name and was first referred to as "herpes-type" in 1964 (11). This followed demonstration of the virus using electron microscopy in a Burkitt's lymphoma derived continuous cell line (12). Epstein was a pathologist who suspected and subsequently proved that a viral agent was implicated in the aetiology of cases of lymphoma presenting in children in Uganda. These cases, often presenting as swellings in the jaw, were first described by Denis Burkitt, a British surgeon, in 1957 (13). Since the 1960's EBV has been associated with many other diseases. All these diseases typically involve EBV driven proliferation of lymphoid or epithelioid cells and include infectious mononucleosis, Hodgkin's lymphoma and nasopharyngeal carcinoma. In immunocompromised human hosts such as those with HIV infection or recipients of solid organ or stem cell transplants other conditions described include post-transplant lymphoproliferative disorder and oral hairy leukoplakia.

2.3.1 EBV Transmission

In western countries most individuals have been infected with EBV by time of adulthood. EBV can be detected in saliva and genital secretions. Exchange of these fluids through kissing or sexual contact results in transmission of the virus (14). Two types of EBV, currently described as EBV 1 (A) and 2 (B) have so far been identified. Individuals may be infected with one or both types but may also host one or a number of strains of these types. Primary infection may involve

acquisition of a single type or strain of the virus or multiple strains. Subsequent superinfection with other strains or types may also occur (15–17). The 2 types are distinguished on the basis of allelic polymorphism in their EBNA (Epstein-Barr nuclear antigen) genes (18). Strains of EBV 1 and 2 can be distinguished on the basis of latent gene (including EBNA and LMP1) sequence variations. (19). Type and strain patterns have been associated with geographical variation and there may also be links with specific diseases (20,21) EBV Type 1 infection is seen predominantly in Caucasian and South East Asian populations while Type 2 is more prevalent in equatorial Africa (22). In the UK a recent epidemiological study described a 77% EBV Type 1 prevalence in infected young adults, 17% Type 2 and 5% showing evidence of infection with both subtypes (23). While it seems that in the immunocompetent and healthy a number of strains of these types can be detected in a single individual, typically one strain predominates. In contrast, in the immunocompromised, particularly in those with high detectable EBV viral loads, multiple strains may be detected (18). Immune recognition and response to those variant subtypes and strains has been shown to differ (24). Primary infection with a Type 1 strain and development of cytotoxic T lymphocytes may not protect against subsequent later infection with a different strain or subtype. However, in the context of thoracic transplant recipients no acquisition of donor EBV strain was seen in 11 recipients, all of whom were EBV seropositive and retained their original EBV strain during follow up (25). Compartmentalization of different strains has also been noted. Oral cavity strains may differ entirely from predominant strains found in the blood and B lymphocytes in healthy individuals (15). In patients with

oral hairy leukoplakia EBV strains found in the abnormal epithelial tissues were similar to those in the oral cavity but often different to those found in B lymphocytes and blood in the same patients (26). Primary infection with type 1 is a risk factor for the development of infectious mononucleosis (27).

2.3.2 EBV genome structure

The EBV genome is a linear double stranded DNA molecule. It consists of approximately 172 kilobase pairs depending on the strain. The linear molecule has a Terminal repeat sequence at each end of approximately 540 basepairs. The genome is divided into short and long unique sequences separated by a series of tandem repeats including the IR1 or major internal repeat which contains the latency promoter Wp. Variation in tandem repeats allows the identification of different EBV strains. Variation between genomes may result in differences in biological activity or ability to cause disease between strains. Strains include the B95-8 strain, isolated from an individual with infectious mononucleosis and the first to be completely sequenced (28), the GD-1 strain, highly prevalent in Guangdong ,China, where the incidence of nasopharyngeal carcinoma is high and where GD-1 strain mutations are highly prevalent in such cases (21), and strain “i/xhol+” associated with cases of gastric cancer in Latin America (29).

2.3.3 EBV Infection

Exchange of saliva and genital secretions through kissing and sexual contact allows the transmission of Epstein-Barr virions from one individual to another (30). Contact of the virus with the oral mucosa and lymphoid tissue in Waldeyer's ring then results in infection of predominantly naive tonsillar B lymphocytes. Epithelial cells in the oropharynx can also be infected. Such epithelial cells may provide a site for active viral replication with subsequent release of virions back into the oral saliva or direct epithelial to B cell transmission of the virus (31). Evidence for the ability of EBV to infect epithelial cells is demonstrated by the detection of EBV DNA and lytic cycle EBV antigens in tissue samples taken from patients with oral hairy leukoplakia (32,33) and also in EBV positive nasopharyngeal carcinoma.

Current models suggest EBV enters cells via the following mechanisms. Firstly the EBV glycoprotein gp350/220, found in the virion envelope, binds to the B lymphocyte receptor CD21 (CR2 complement receptor) (34–37). Through the process of endocytosis the virion enters the B cell in a membrane vesicle. The viral envelope then fuses with the vesicle membrane with release of the viral nucleocapsid into the B cell cytoplasm (38). A second mechanism of entry into host cells involves direct fusion rather than endocytosis of the viral envelope with the host cell wall. This interaction involves the virion envelope complex gp25, gp42/38 (coded by the viral gene BZLF2) and gp85 (BXLF2 viral gene product) (37) The fusion method is seen in epithelial cell interactions where CD21 is not expressed or expressed only in small concentrations. There may be an epithelial

and T cell receptor that enables endocytosis of the virion but it has not yet been identified (39).

The passage of only a single EBV genome to a naive resting B cell nucleus results in B cell activation, proliferation and also protection from apoptosis. A newly infected B cell, once activated by antigen interaction typically migrates to the lymphoid follicle. Here it enters the germinal centre and may differentiate into either an antibody producing plasma cell or a resting memory cell that will re-enter the circulation (40). For these memory cells further antigen challenges in the future can trigger maturation into antibody producing plasma cells.

2.3.4 Latent EBV infection

Epstein-Barr virus as with other human herpes viruses has evolved to persist in the human host lifelong after primary infection, a process called latency. This involves the maintenance of the intact viral genome in the nucleus of specific host cells. A limited number of viral genes are expressed with the purpose of protecting the host cell from apoptosis and evasion of cellular immune responses. During latency there is an absence of viral reactivation or replication. Replication, necessary for the successful transmission of virus to other potential hosts, occurs during lytic cell cycles which may be initiated from time to time with the expression of increased numbers of viral genes and effective viral replication despite prompt responses by the host cellular immune system.

The linear EBV genome, upon successfully reaching the host cell nucleus, circularises to form an episome (extrachromosomal nucleic acid) (41). Multiple episomes may be present in each cell. Transcription of viral genes follows, initiated by the Wp promoter, a tandem repeat DNA sequence contained in an area of the genome known as IR1(major internal repeat) (42).

Latency is not a just a single state but rather a number of different states that involves the expression of a variety of numbers and classes of viral gene product (Table 2.1). Further these different patterns of viral gene and antigen expression may have specific implications in terms of biological activity. Associations have been made between specific latency states and a variety of disease conditions.

The latently infected B cell may express up to 11 genes (18). These include EBNA (Epstein-Barr nuclear antigen) 1, 2, 3A, 3B, 3C and 5 (LP), LMP 1, 2A and 2B, EBER 1 and 2 and BART's (BamH1A rightward transcripts). Limited expression of a small number of antigens may help avoid detection by host immune responses although cytotoxic T lymphocytes responses exist against certain latent antigens (43).

Further many of the products of particular latent genes directly or indirectly hinder the ability of the host immune system to identify and eliminate infected cells.

EBNA-1 protein expression is found in all latency states associated with disease. It is crucial for maintenance of the viral genome and can trigger expression of other

latent genes including LMP-1 and LMP-2B. Further the EBNA1 gene includes an internal glycine-alanine repeat (Gar) domain. This repeat sequence may inhibit proteasomal processing of EBNA-1 with subsequent reduction in available antigenic EBV peptide for presentation by MHC class 1 molecules (44). The GAr domain also appears to inhibit self-synthesis of EBNA-1 (45,46). Removal of GAr experimentally results in increased EBNA-1 antigen, and increased MHC-1 EBNA-1 peptide presentation and subsequent host T cell recognition.

EBNA-2, like EBNA-1 is a transcription factor capable of up-regulating viral genes involved in B cell immortalization and prevention of apoptosis including LMP-1 and LMP-2B (47). Along with EBNA -5 (LP) it is one of the first viral genes to be expressed and is crucial for B cell transformation.

EBNA-3 is subdivided into 3A, 3B and 3C and again these genes play a part in preventing B cell apoptosis (48).

EBNA-5 or EBNA –LP can upregulate LMP2A expression and is involved in lymphoblastoid cell line proliferation (49).

LMP-1 expression is in part triggered by initial expression of other early latent genes including EBNA-1. It is linked to B cell proliferation, maintenance of the latent state via inhibition of lytic cell cycle activators including BZLF1 (50) and has anti apoptotic effects including up-regulation of BCL-2 (51).

LMP-2A like LMP-1 again has a role in the persistence of the latent state.

EBER 1 and 2 are small EBV RNA. Like EBNA proteins they are widespread and seen in most latently infected cells. In tumours where Epstein-Barr virus may play an aetiological role, tissue can be examined at single cell level using in situ hybridization for EBER-1 transcripts. EBERs may also contribute to dysregulation of host immune responses by inducing expression of IL-10. IL-10 inhibits the effects of cellular immune responses including macrophage and dendritic cell function, can induce expression of LMP-1 in EBV infected cells and can promote B cell immortalization.

BART's BamH1A rightward transcripts are also small RNA molecules. Like EBER they are found in all forms of latency and are also detected in disease states including Nasopharyngeal carcinoma (52) and Hodgkin's lymphoma (53). Disease associations may relate to modulation of LMP-1 expression.

Table 2.1 Latency patterns of EBV infection

Latency pattern	Viral Protein expression	Associated disease
Latency 0	EBER BARTS's	Healthy individuals Resting B cells
Latency I	EBER EBNA-1 BARTS's	Burkitt's lymphoma
Latency II	EBER EBNA-1 LMP-1 LMP-2A BARTS's	Hodgkin's Lymphoma Nasopharyngeal carcinoma T-cell lymphoma
Latency III	EBER EBNA-1 EBNA-2, -3A, -3B,-3C, -5(LP) LMP-1 LMP-2A, -2B BARTS's	Infectious mononucleosis Post-transplant lymphoproliferative disorder

2.4 Immune responses to EBV Infection

Successful control of EBV infection by the human host requires a complex interaction between innate, adaptive, humoral and cellular immune responses. Understanding of these responses comes primarily from observations and investigations made in individuals undergoing infectious mononucleosis as most primary infections are asymptomatic.

2.4.1 Innate responses

The initial responses to early EBV infection are probably driven by the infected B cells and perhaps by myeloid dendritic cells. Recognition of viral nucleic acids by B cell receptors results in secretion of type I Interferons such as Interferon-α and –β and perhaps IL-12 as part of the innate immune response to viral infection (54). These cytokines, secreted by B cells and myeloid dendritic cells can contribute to the development of adaptive immune responses but also activate local NK cells. Type I interferons in the first 24 hours after infection can induce apoptosis of infected cells thereby limiting early replication and spread of the virus to other cells. The Interferons also increase expression of MHC class I molecules on uninfected cells which may protect them from activated NK cell activity. The Interferon-I effect seems to be short-lived and beyond 24 hours the immortalization of infected B cells and the transcription of latent genes including EBNA and LMP proteins protects these cells from apoptosis (55,56).

Protective effects of the type I Interferons are relatively short-lived, and the initial control of primary EBV infection is mediated by NK cells. These NK cells are able to kill virally infected cells and also drive developing adaptive responses through the secretion of cytokines (54). Infection of B cells by virus results in increases in MHC class I cell surface molecules and viral antigen presentation to allow targeting of these cells for destruction and stimulation of adaptive immune responses (57). Immunoevasive strategies of EBV include the ability to hinder and limit this MHC class I presentation through the transcription of viral genes thereby reducing the amount of viral peptide presentation to the host immune system. NK cells appear

to have adapted to this strategy and preferentially target infected cells with low MHC class I expression. NK cells bind to infected cells that are expressing viral antigens in the absence of MHC-1 molecules via the NK cell's killer activating receptor (KAR). This results in secretion of cytotoxic granules including perforin, granzyme, tumour necrosis factor and Fas ligand expression by the NK cells with subsequent lysis of the target cell (58). In the presence of MHC-1 expression, NK cell activity is down-regulated by engagement of the killing inhibitory receptors (KIR) and C-lectin molecules (NKG2/CD94) which bind to MHC molecules. This allows the NK cell response to be replaced by the MHC-mediated cytotoxic T-lymphocyte (CTL) response. Virus-infected cells in the oropharynx also secrete Interferon-α and -β which locally stimulate NK cell activity and hinder infected B cell immortalization and also secrete Interferon-γ which drives adaptive responses including activation of B and T cells (59).

Effective NK cell activity is important for the early control of primary EBV infection. Deficiency of this innate immune response can result in severe and life-threatening primary infection (60–62). In X-linked lymphoproliferative disorder failure of NK cells to identify infected B cells results in unchecked proliferation of infected B cells, and excessive cytokine secretion by activated T cells. The defect is due to a mutation or deletion in the SAP (signalling lymphocytic activation molecule (SLAM) -associated protein) encoding gene on the x chromosome (63,64). This molecule in combination with CD244, an NK cell surface molecule, is required for effective interaction with the B cell molecule CD48 (54,60).

2.4.2 Adaptive immune responses

As discussed above, following primary infection of the oropharyngeal mucosa the innate immune responses limit early viral replication and infection of B cells. As the infection progresses, release of cytokines from B cells, myeloid dendritic cells and NK cells begin to act to prime more specific adaptive responses.

2.4.2.1 CD8+ T cell responses

Antigen presenting cells (APC) including B cells and myeloid dendritic cells migrate from the oropharyngeal mucosa and sites of viral replication to the oropharyngeal lymphoid tissue of Waldeyer's ring. At the cell surface of these antigen presenting cells lytic and latent antigenic peptide complexed with MHC class I molecules is presented. Direct interaction between viral antigen-specific CD8+ T lymphocytes and the APC's occurs as a consequence of engagement of the MHC class I antigenic peptide complex on the surface of the APC, with the T cell receptor of the CD8+ T-lymphocyte alongside other cell adhesion molecules. This interaction results in T cell activation and proliferation with a subsequent clonal expansion of EBV peptide (epitope) specific cytotoxic effector CD8+ T cells (Figure 2.1). This expansion of the T cell population accounts for the atypical lymphocytosis associated with infectious mononucleosis. In the context of primary infection the majority of these CD8+ T cells recognise B cells presenting lytic antigens (50%) and a smaller proportion (1-5%) cells with latent antigens (65,66). Further it seems that the CD8+ responses are not directed in similar proportions against all lytic and

latent EBV antigens. Instead certain immunodominant antigens generate much greater responses than others. In the lytic setting immediate early antigens including BZLF1 and BRLF1 provoke the greatest responses while for latency EBNA-3A, 3B, and 3C dominate followed by LMP-2 and then to a lesser extent EBNA-1,2 and LMP-2 (66–68). As control of the primary EBV infection occurs the huge increase in CD8+ T cells falls with subsequent apoptosis of these predominantly lytic epitope specific cells. The proportion of these cells falls from 20-50% of the total CD8+ population to approximately 2-5% while the proportion of latent antigen targeted CD8+ cells remains fairly constant at 0.5-2% of the total CD8+ circulating population (18,69) (Figure 2.2).

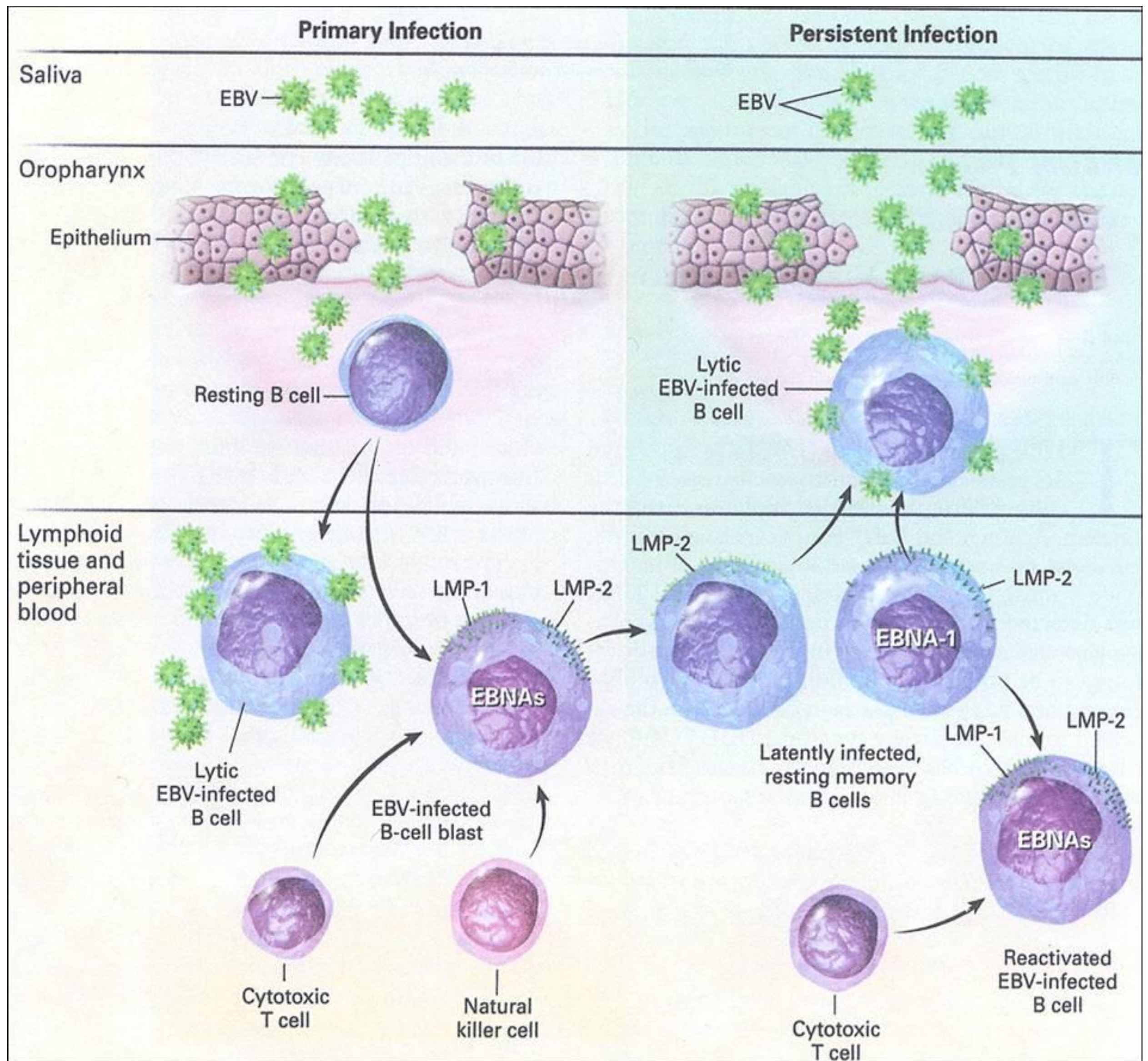


Figure 2.2 Model of Epstein-Barr (EBV) Infection in Humans

In the oropharynx, EBV directly infects resting B cells or infects epithelial cells, which in turn infect B cells. During primary infection, EBV-infected B cells undergo lytic infection with production of virus or express the full complement of latent viral proteins. The latter cells are kept in check by natural killer cells and cytotoxic T cells. After convalescence, EBV is present in the peripheral blood in latently infected memory B cells that express latent membrane protein (LMP) 2 and possibly EBV nuclear antigen (EBNA) 1. The latter cells can undergo EBV reactivation and express other latent viral proteins, resulting in their recognition and destruction by cytotoxic T cells. Some latently infected cells undergo lytic replication in the oropharynx, resulting in production of virus with shedding of virus into the saliva or infection of epithelial cells with release of virus. Adapted from Cohen with the permission of the publisher (70).

Reproduced with permission from (Cohen, J. Epstein-Barr Virus Infection. NEJM 2000; 343:481-492), Copyright Massachusetts Medical Society.

Presentation of EBV peptide is Human Leukocyte Antigen (HLA) class I restricted and HLA alleles may account for individual differences in anti-EBV immune responses. For example BZLF-1 and EBNA-3A are presented by HLA*08 and in such individuals with latent EBV infection up to 5% of all CD8+ cells will be directed against BZLF-1 and up to 1% against EBNA-3A (54). CD8+ CTL s against EBNA-1 appear in many to be hard to detect yet in certain individuals with HLA alleles including B*35 and B*07 such cells are present (57). Poor presentation of EBNA-1 by antigen presenting cells results in lack of T cell response to this antigen. The poor presentation is attributed to a gly-ala repeat domain which protects the protein against degradation in the proteasome prior to transportation to the nuclear endoplasmic reticulum for MHC-1 binding (45,54).

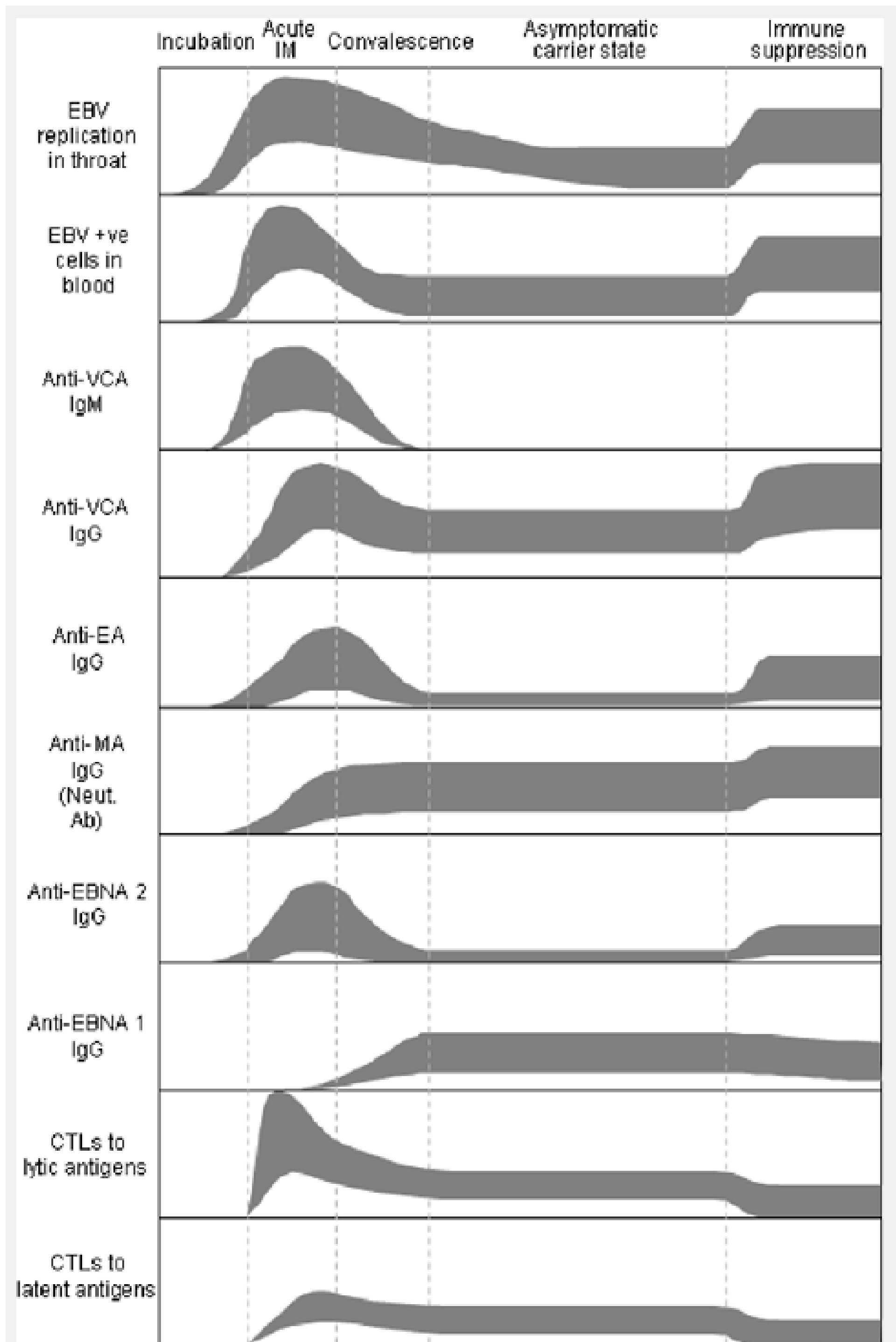


Figure 2.3 Parameters of the Epstein-Barr Virus (EBV) host balance

Parameters of the Epstein-Barr virus (EBV) host balance during the incubation, symptomatic, and convalescent phases of acute infectious mononucleosis (IM) (i.e., primary EBV infection), during subsequent asymptomatic virus carriage, and following the imposition of immunosuppressive therapy on an existing virus carrier state. For each parameter, shaded band represents the broad

range within which most infected individuals lie; where the band extends to the baseline, this indicates that the relevant responses are detectable in some, but not all, infected individuals.

Reproduced from Rickinson,A.B., Kieff,E.D. (2006) Epstein-Barr Virus. In Field's Virology. Snipe,D.M., Howley,P.M. (eds). Lippencroft, Williams & Wilkins, pp. 2657-2701(18).

2.4.2.2. CD4+ T cell responses

Antigen presenting cells bearing EBV peptides complexed to MHC class II molecules interact directly with T cell receptors on CD4+ T cells in the lymphoid tissues. These interactions typically result in a B cell-T cell conjugate with subsequent activation and proliferation of the CD4+T cells. Such activated cells provide a T helper 1 (Th1) response in the context of EBV viral infection (58). Cytokines such as IL-2 and Interferon-γ are secreted which promote the activation and differentiation of viral antigen specific CD8+ T cells into cytolytic effector cells. Further, the antigen bearing B cell is driven to differentiate into a plasma cell able to secrete IgM antibody. These B cells may also form germinal centres in the lymphoid follicles with subsequent switch from IgM antibody to high affinity IgG antibody production.

In primary EBV infection, unlike with CD8+ expansion, CD4+ T cell numbers do not seem to increase to any great extent and even in infectious mononucleosis total only approximately 0.3% of the total CD4+ population (54,71). In the latent post primary phase this number drops to only 0.1%, approximately one tenth of the circulating CD8+ cell numbers. The CD4+ cells although acting predominantly in a T helper 1 role may also be directly cytotoxic to EBV infected cells (72,73). The

immunodominance of EBV antigens also appears to be different with CD4+ cells recognising cells presenting EBNA-1 antigen while CD8+ positive cells against EBNA-1 appear to be very rare (72).

2.4.2.3 Latently infected B cell preference for Waldeyer's ring tissue

It seems that naive oropharyngeal B cells after infection with EBV virus migrate to the oropharyngeal lymphoid tissue. Interaction with CD4+ and CD8+ T lymphocytes results in differentiation into plasma cells capable of secreting antibody or latently infected memory B cells. These EBV infected memory B cells then leave the germinal centre in the lymphoid follicles and colonize the lymphoid tissue of Waldeyer's ring but may also enter and circulate in the peripheral blood compartment. Movement through the peripheral blood compartment may result in seeding of other secondary lymphoid tissue such as the spleen and mesenteric lymph nodes. While EBV infected memory cells can be found in secondary lymphoid tissues such as the spleen the concentration of such cells is approximately 20 times lower here than in the lymphoid tissue of Waldeyer's ring or in the peripheral blood compartment (18,74). This may suggest that latently infected memory B cells have evolved to preferentially migrate back to the tissue of Waldeyer's ring which is also the site of viral replication. The immune system seems to recognise the tendency for these cells to preferentially localise to Waldeyer's ring as up to 20% of tonsillar CD8+ T cells are EBV specific. These cells also appear to have evolved to preferentially migrate to the lymphoid tissue of Waldeyer's ring and tend to be CCR7 cell surface marker positive and also CD103

positive, a cell surface marker involved in retention of these cells at mucosal sites (75).

2.4.2.4 EBV Antibody responses

Individuals who have not been infected by the EBV virus have no serologically detectable antibody responses. Following primary infection a number of antibodies develop many of which persist for life and can be used to define the phase and presence of EBV infection (Figure 2.3). Responses can be divided into acute and persistent. During acute infection, IgM, IgA and high affinity IgG are directed against intracellular and virion surface antigens. Current models suggest antigen loaded B lymphocytes migrate from the oropharyngeal mucosa, the site of viral replication and primary infection, to the oropharyngeal lymphoid tissue of Waldeyer's ring. Here B cells with EBV antigenic peptide presented by Class II MHC molecules interact with CD4+ T cells. Such interaction results in the release of cytokines including IL-2 and Interferon-γ from the activated T cell. Activation of the B cells can promote their primary differentiation into IgM secreting plasma cells. B cells may also be driven to proliferate rapidly, forming germinal centres in the lymphoid follicles. Subsequently somatic point mutations, typically single nucleotide substitutions in the immunoglobulin heavy and light chain variable region exons result in changes to the B cell receptor affinity for the EBV antigens driving the process. B cells with B cell receptors displaying high affinity for the antigen are selected while those with lower affinity undergo apoptosis. These B cells with BCR with high affinity for EBV antigen then terminally differentiate into plasma cells

secreting antibodies. Typically these cells switch to produce high affinity IgG antibodies instead of the lower affinity IgM antibody produced in the early primary response. Other selected B cells differentiate into memory B cells specific for EBV antigen that can be activated upon later antigenic stimulation to secret antibody (58). It is the memory B cell population that supports latent EBV infection.

2.4.2.5 Acute phase responses

In the acute phase of EBV infection, by the time individuals develop symptoms, lower affinity IgM, and IgA and IgG antibodies can be detected to the intracellular EBV antigens Viral capsid antigen (VCA), Immediate early (IE) antigen and Early antigen (EA) and EBNA-2 antigen. IgG antibodies to viral glycoprotein antigens expressed on the cell surface including Membrane antigen (MA) and gp350 can also be detected in this early phase. These anti-glycoprotein antibodies have the ability to neutralise infected cells and non-cell associated virions inhibiting spread of viral infection (18). While antigens such as VCA and EBNA are intracellular it is likely that they are also expressed at the cell surface and reflect targets for cytotoxic responses directed against the infected cells.

2.4.2.6 Persistent phase responses

Resolution of symptoms in Infectious mononucleosis and the post primary infection period in those with asymptomatic infection is characterised by changes in the antibody response profile. The highest levels of antibodies, directed against latent

and lytic antigens, are found typically towards the end of the symptomatic lytic phase of infection. Following this, antibody levels on the whole fall and typically are maintained in a steady state for life although antibody titre levels will vary between individuals. Lower affinity IgM and also IgA antibodies to VCA tend to disappear. IgG to early, immediate early and EBNA2 antibodies also tend to fall to very low or undetectable levels while IgG to the intracellular latent antigen EBNA-1 now becomes detectable. Seropositivity, the marker of previous infection is typically demonstrated by the presence of IgG antibodies to VCA and EBNA-1 which persist throughout life. Individuals will also continue to produce constant levels of IgG neutralizing antibodies directed against MA and gp350 with current models suggesting these antibodies are designed to limit reactivations and control super-infections by new viral strains (18,76).

2.5 Epstein-Barr virus associated disease

EBV is associated with diseases in both the general population and in immunocompromised individuals and a summary of these conditions are presented in table 2.2. These diseases can result from primary infection or may develop post primary in those with reactivation of latent virus or chronic infection. Illnesses occurring as a result of primary and post primary infections may include lymphoproliferative disorders, malignant conditions and complications of the above such as hepatitis, splenic rupture or neurologic syndromes.

Table 2.2 EBV related disease

Condition	Cell type	Population at risk
Infectious Mononucleosis	Epithelial / Tonsil / B cell	Teenagers, EBV naive young adults
X linked lymphoproliferative disease	B / T cell	Children. Males with specific gene defect
Chronic Active EBV	T / NK	Children, Japanese, ?perforin gene mutation
EBV associated Haemophagocytic lymphohistiocytosis	T / NK / Macrophage activation	Infants, children Inherited or acquired Immunodeficiency
Hodgkin's disease	B cell	Children, elderly
Burkitt's lymphoma	B cell	African children
B lymphoproliferative disease	B cell	Primary Immunodeficiency states, HIV infected Ataxia Telangiectasia
Post-transplant lymphoproliferative disorder	B cell / Tcell / NK cell	Solid organ / stem cell transplant recipients
Smooth muscle tumours Leiomyosarcoma	Smooth muscle	Immunosuppressed
Nasopharyngeal carcinoma	Epithelial cell	Southern Chinese, Inuit
Oral hairy leukoplakia	Epithelial cell	Immunocompromised HIV infected
Gastric cancer	Adenocarcinoma	USA, Germany
T cell / NK cell lymphoma	T cell / NK cell	Immunocompromised Individuals with CAEBV HIV infected

2.5.1 Primary Infection

EBV infection predominantly occurs in children with the majority of adults worldwide demonstrating antibodies to EBV.

2.5.1.1 Congenital infection

Congenital and intrauterine infections are unusual as very few women enter pregnancy without serological evidence of previous EBV infection. In seronegative mothers experiencing primary infection during pregnancy there is evidence of vertical transmission of virus. However miscarriages and congenital abnormalities as a consequence of this transmission do not seem to occur (77).

2.5.1.2 Infants and children

Primary infection with EBV is common in developing countries during the first two years of life and consequently seropositivity levels are close to 100% in adults. In most children primary infection is clinically silent and mild. Maternal antibodies may protect the infant from infection for the first few months of life and may help reduce the degree of associated illness (78). Changing patterns of infection in the UK suggest primary infection rates in children may be falling with an increase in teenage rates and an association with more severe illness (79,80).

In the UK up to 75% of adults entering university are likely to be seropositive. Of the remainder 50% will seroconvert during their time at university but only a

relatively small proportion of these (23%) will develop illness such as infectious mononucleosis as a consequence of primary infection (14).

2.5.1.3 *Infectious Mononucleosis (IM)*

Infectious mononucleosis is perhaps the most well-known condition with which EBV is associated. It is an acute illness occurring in a small proportion of individuals experiencing primary EBV infection. A small proportion of cases of infectious mononucleosis can be caused by other agents including cytomegalovirus, HIV, HHV-6, mumps, toxoplasmosis and hepatitis. The illness is characterised by fever, lymphadenopathy and pharyngitis. The degree of symptoms may relate to the immune responses to the infection. EBV infection triggers a benign proliferation of EBV specific CD8+ cytotoxic T-cells directed against the infected cells (81). The blood film can demonstrate this lymphocytosis with many lymphocytes displaying “atypical” or large irregular nuclei. The T cell expansion and activation is accompanied by the production of cytokines including Interferon –γ and IL-2. Excessive cytokine production is associated with symptom severity (82).

The onset of symptoms follows an incubation period of 14-42 days and only 6% of affected individuals report contact with another symptomatic case. The initial prodrome consists of malaise, headache, fatigue and fever. The classical triad of high grade fever, lymphadenopathy and pharyngitis follows. Lymphadenopathy is typically found in the cervical, axillary and inguinal chains and may be symmetrical and non-tender. The pharyngitis typically involves the tonsils which may

oedematous, necrotic and covered in a white exudate. Petechial spots may be visible on the palate. Maculopapular rashes occur rarely as part of the illness (5%) but are common in those given ampicillin to treat suspected bacterial throat infections (90%). Malaise including nausea and anorexia are common. This may relate to cytokine production or a mild hepatitis (transaminitis) seen in up to 90% individuals although typically only 5% will become jaundiced. Benign lymphoproliferation also results in splenomegaly (50%) and hepatomegaly (15%) (83).

In the majority of cases the illness is self-limiting and resolves within 2 to 6 weeks. Relapses of IM, occurring within 12 months of the initial illness are reported but seem to occur infrequently. Complications of IM and primary EBV infection are uncommon but include the following (Table 2.3):

Table 2.3 Complications of primary EBV infection

System	Complication	Reference
Respiratory	Airway obstruction, Pleural effusions, Pneumonitis	(84,85)
Haematological	Haemolytic anaemia, Thrombocytopenia, Antiphospholipid syndrome	(85)
Renal	Acute renal failure, Interstitial nephritis, Glomerulonephritis	(86–92)
Neuro-psychiatric	Chronic fatigue, Depression Cranial nerve defects Meningo-encephalitis	(93–96)
Lympho-reticular	Splenic rupture	(97)
Digestive system	Hepatitis, esophagitis Autoimmune hepatitis	(98,99)
Dermatological	Maculopapular rash Erythema nodosum Genital ulceration	(100,101)

2.5.2 Chronic active Epstein-Barr virus (CAEBV)

The symptoms of EBV associated infectious mononucleosis can persist in a small subpopulation with serious and potentially fatal outcomes. This condition CAEBV is

characterised by a duration of symptoms of longer than 3 months. Persistent and high levels of viral DNA are detectable in peripheral blood samples and antibody responses are typically abnormal (102). It occurs predominantly in Japanese children. T and NK cells are often infected alongside B cells and infected cell type may relate to prognosis. Overall survival is poor at 40% mortality with death a result of complications including sepsis, lymphoma and haemophagocytic syndrome (62).

2.5.3 X linked lymphoproliferative (XLP) disease (Duncan syndrome)

This rare inherited condition affects young males. Primary EBV infection results in a severe acute infectious mononucleosis. There is then a subsequent failure by immune cells including NK cells to destroy EBV infected B cells which continue to proliferate. This infected B cell proliferation is associated with excessive and dysregulated production of cytokines including Interferon-γ by activated T cells (61). Fulminant IM can result with mortality of up to 96%. Bone marrow failure and lymphoma can occur in those who survive the initial illness. The cause of the disease has been identified as a mutation/deletion in the SAP (signalling lymphocytic activation molecule (SLAM) -associated protein) encoding gene on the X chromosome (63)(64)(Sayos, Wu et al. 1998; Bottino, Parolini et al. 2001). This protein has a role in regulating T cell activation and in enabling NK cells to identify EBV infected B cells. Abnormalities or loss of the gene result in the uncontrolled cytokine production and NK cell defects (62).

2.6 Epstein-Barr virus infection in Adult Renal Transplant Recipients

Epstein-Barr virus causes diseases in the general population such as infectious mononucleosis and is implicated in the pathogenesis of many other conditions including malignant lymphoma. A similar range of EBV associated diseases occur in the transplant population with the most serious manifestation, Post-transplant lymphoproliferative disorder. This condition is a spectrum of disease which includes T and B cell lymphoma and is associated with EBV infection in approximately 80% of cases (103).

Approximately 90% of adults in the UK will have evidence of previous infection by Epstein-Barr virus at the time of transplantation. In 136 adults worked up as potential renal transplant recipients at Manchester Royal Infirmary in 2008 92% were EBV seropositive, 96% Varicella Zoster seropositive and 57% cytomegalovirus seropositive (unpublished data). Primary infection after transplantation in our adult population is therefore uncommon with only 8-10% at risk.

The regulation and control of Epstein-Barr virus is disturbed in the transplant patient as a result of the administration of immunosuppression. In a non-immunosuppressed setting latently infected B cells are targeted by CD8+ T cells. NK and CD4+ T cells are also involved in the recognition and destruction of these cells. The same host immune responses alongside antibodies produced by B cells can target infected B cells in the lytic phase of EBV infection either during primary infection or reactivation. Current immunosuppressive agents used in renal

transplantation are detailed below. These agents through different pathways inhibit T cell activation and proliferation. A consequence of this impaired T cell function is a loss of control over the number of B cells latently infected with Epstein-Barr virus. The result is an increase in numbers of infected cells, poorer control of both primary EBV infection and reactivation, and the potential development of malignant conditions in the context of uncontrolled B cell proliferation.

2.6.1 Transplant Immunosuppression

The immunosuppressive agents used in adult kidney transplantation with an overview of their mechanism of action are given below. Since 2004 the current standard immunosuppressive regimen in our unit consists of basiliximab induction therapy followed by tacrolimus maintenance in combination with mycophenolate mofetil and prednisolone. More recently a practice of steroid withdrawal is followed at the end of the first week. Anti-thymocyte globulin is reserved for those with steroid resistant or vascular rejection. Ciclosporin was our maintenance agent of choice in the 1990's and is still used for those intolerant of agents such as tacrolimus while azathioprine and prednisolone were the agents of choice in the 1980's. Azathioprine remains an alternative agent for those with intolerance of other agents.

The article by Philip Halloran in the New England Journal of Medicine (2004) provides an excellent overview of the mechanism of action of these agents (1).

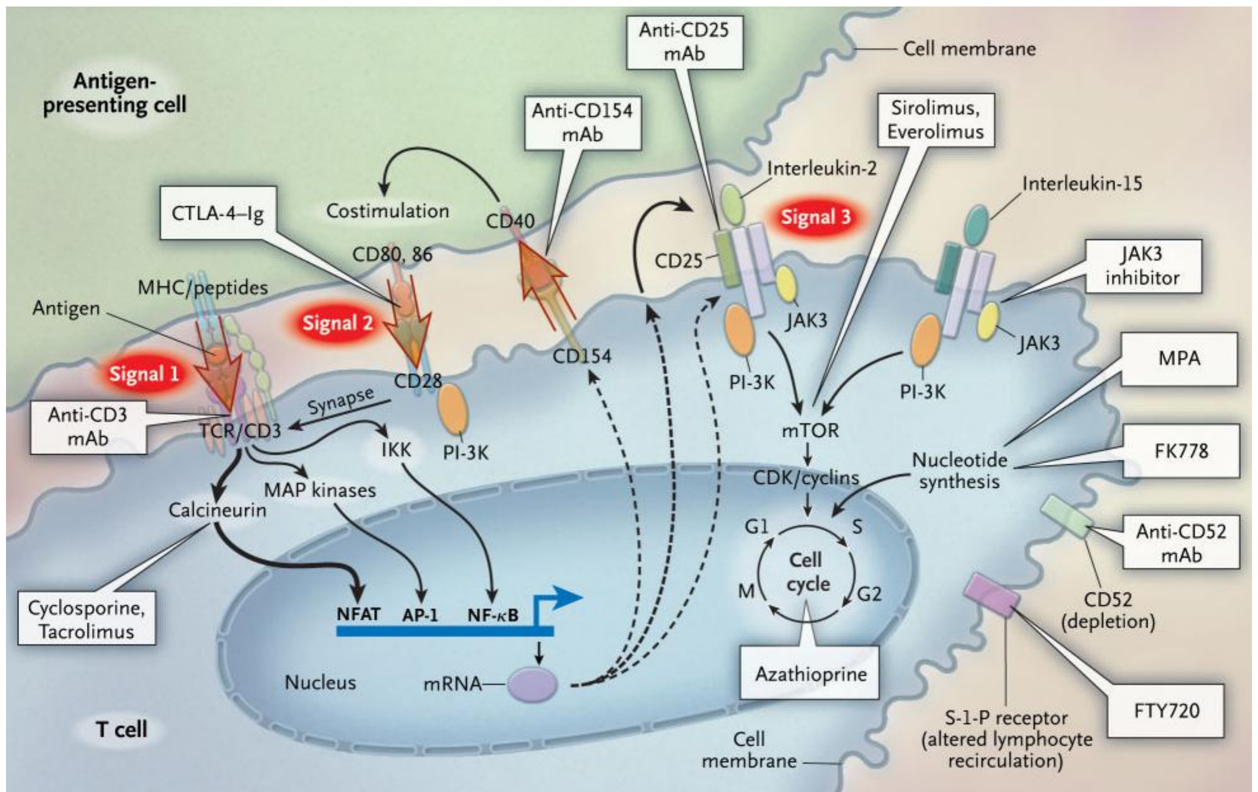


Figure 2.4 Individual Immunosuppressive Drugs and Sites of Action in the Three-Signal Model Reproduced with permission from (Halloran, P, F. Immunosuppressive Drugs for Kidney Transplantation. NEJM 2004; 351 :2715-2729), Copyright Massachusetts Medical Society.

In brief basiliximab is a mouse-human monoclonal antibody to the α chain (CD25) of the IL-2 receptor expressed on activated T cells. Administration of a dose of 20mg on day 0 and day 4 and binding of the antibody to the receptor results in inhibition of IL-2 mediated activation of lymphocytes. Tacrolimus and ciclosporin are both calcineurin inhibitors. Calcineurin activation in T cells results in NFAT regulated production of cytokines such as IL-2 which drive further T cell activation and subsequent proliferation. The calcineurin inhibitors act by binding intracelleular

immunophilin proteins, cyclophilin (ciclosporin) and FK506-binding protein 12 (FKBP12)(tacrolimus). Binding of these proteins results in the formation of complexes that bind calcineurin and thus inhibit IL-2 transcription and T cell activation. Mycophenolic acid (active metabolite of prodrug mycophenolate mofetil) inhibits inosine monophosphate dehydrogenase, an enzyme required for purine (guanosine nucleotide) synthesis. Proliferation of both T and B cells is inhibited and antibody production by B cells is also inhibited. Azathioprine is an antimetabolite. It is metabolised to 6-mercaptopurine which when activated results in the production of 6-thioguanine nucleotides (6-TGNs) and 6-Methylmercaptopurine nucleotides (6-MeMPNs). These are cytotoxic nucleotides that may be incorporated into DNA resulting in cell death (6-TGNs) or inhibition of de-novo purine synthesis. Thus azathioprine has anti-proliferative effects on both T and B cells and inhibits antibody production by B cells. The azathioprine antimetabolite 6-mercaptopurine is inactivated by two pathways including oxidation to 6-thiouric acid catalysed by Xanthine oxidase and methylation to methyl-6-MP catalysed by thiopurine S-methyltransferase (TPMT). Inhibition of Xanthine oxidase by agents such as allopurinol and homozygosity or heterozygosity for non-functional TPMT gene alleles results in impaired inactivation of azathioprine and myelotoxicity. Sirolimus is a target of rapamycin pathway inhibitor that like tacrolimus binds to the immunophilin FKBP12. In this situation the resulting complex inhibits cytokine, including IL-2, driven T cell proliferation via the target of rapamycin pathway but has no effect on the calcineurin pathway. Along with T cell antiproliferative effects it also inhibits antibody production from B cells.

Glucocorticoids such as prednisolone have multiple effects, many mechanisms not fully understood but include anti-inflammatory and T cell activation inhibition. Lastly ATG or anti-thymocyte globulin is a polyclonal Immunoglobulin G derived from rabbits and horses that targets human T-cell surface markers including CD3, CD45 and CD2 resulting in profound T cell lysis, depletion and altered function.

2.6.2 EBV related clinical scenarios in adult renal transplant recipients

In table 2.4 below predicted EBV related clinical scenarios following transplantation in relation to EBV pre-transplant serostatus are presented.

Table 2.4 EBV related clinical scenarios in adult renal transplant recipients

EBV Serostatus at time of transplant	EBV Clinical Scenario after transplant	Proportion patients affected
Seronegative (5-10%)	Remain seronegative	5-10%
	Primary Infection	50-100% of seronegative
	Infectious mononucleosis	?25% of those with primary infection
	Fulminant Primary infection or associated PTLD	Rare
	PTLD	Hazard ratio 3-6X that of seropositive recipients
Seropositive 90-95%	Latent carriage (undetectable viral loads)	>50%
	Asymptomatic DNAemia (transient)	Up to 50%
	Asymptomatic DNAemia (persistent) (Chronic high viral load)	?10%
	Symptomatic DNAemia	5-10%
	PTLD	1-3%
	EBV related malignancy E.g. leiomyosarcoma	Rare

2.6.3 EBV related malignancy and PTLD

The most serious consequence of EBV infection in the transplant population is malignancy. PTLD represents the 2nd largest group of malignancies after skin cancer.

2.7 Post-transplant lymphoproliferative disorder (PTLD)

The incidence of PTLD is 1-3% in adult renal transplant populations (5,104,105) and 2.5% in kidney pancreas populations (106). The incidence is highest in the first year, 0.25-0.46 cases per 100 persons per year dropping to 0.13-0.25 cases per 100 persons per year subsequently (5,8,105). Relative risk of lymphoma compared to the general population at 10 years is 11.8 (5). Those <10years and >60years may have a greater risk presumably due to EBV seronegativity in the young and an age related increased risk of cancer and wane in immune function in the elderly. 1 and 5 year survival is typically poor ranging from 40-60% and 30-60% respectively (5). In renal transplantation mean delay from transplantation to PTLD diagnosis is 76 months and a bimodal distribution of cases has been seen in the ANZDATA series with a peak in the first two years followed by a second peak between 5 and 10 years (8,105).

Approximately 1400 patients in the UK receive a kidney transplant per year currently compared to 644 liver, 129 heart, 143 lung and 3 heart-lung (2008-2009) (NHS Blood and Transplant statistics). Adult transplantation has a 5 year graft survival of 83-89% and patient survival of 88-95%. This contrasts to 5 year patient survival in liver, heart, lung and heart lung populations of 76%, 71%, 54% and 50%

respectively. (NHS Blood and Transplant statistics) Though the incidence of PTLD is lower than in other solid organ transplant populations in view of the larger follow up populations and longer graft and patient survival, renal and renal transplant physicians are likely to be involved in the care of a greater number of PTLD cases per year than their colleagues working with other organs.

PTLD is primarily a histological diagnosis and is categorised according to the WHO classification (107,108) set out below (Table 2.5). Small case series have been reported in which affected individuals present with an early or polymorphic lesion. Subsequent biopsies in cases of disease relapse, recurrence or progression have shown more serious or malignant lesions including Hodgkin like or monomorphic type PTLD (109)

The PTLD classification may therefore represent the progression of a disease entity with the accumulation of genetic abnormalities and proliferation of more malignant cells over time resulting in disease that becomes more difficult to treat and cure.

Table 2.5 WHO classification of PTLD (2008)

Class	Incidence	Sub classification	Characteristics
Early lesion	5%	Infectious mononucleosis like Plasmacytic hyperplasia	Reactive appearance Preserved normal lymph node architecture
Polymorphic	10-20%		Loss of normal lymph node architecture Variable mixture of cell types
Monomorphic	70%	B cell neoplasms: Diffuse large B-cell, Immunoblastic, Centroblastic, Anaplastic Burkitt lymphoma Plasma cell myeloma T cell neoplasms: Peripheral T cell lymphoma NK cell type	Destruction, malignant invasion of node and surrounding tissue. Monoclonal, Chromosomal and genetic abnormalities may accumulate
Hodgkin or Hodgkin like lymphoma	5-10%		May resemble early or polymorphic lesions Reed Sternberg cells present

2.7.1 EBV and PTLD

Current diagnostic guidelines stipulate that PTLD tissue is stained for the presence of EBV. The most sensitive method is In situ hybridisation for EBV encoded small

nuclear RNA (EBER). Less sensitive is immunohistochemical staining with antibodies directed against latent EBV genes including EBNA 1 and EBNA2 and LMP-1 (110) in PTLD tissue (8). EBV negative PTLD is found. In some series it occurs later and is associated with a poorer prognosis (111)(112). EBV negative disease may represent the occurrence in a transplant setting of lymphoma that would otherwise occur in the general population. It might be predicted that those with advanced age would therefore have a greater risk.

Current models suggest in the transplant setting a greater number of cells in the infected host are infected with EBV than in the non-immunosuppressed setting where 1-50 per million circulating B cells are infected. Infected cells may also include abnormal cells with genetic abnormalities which if not for the immortalizing effects of EBV and impaired immune responses in the context of immunosuppression would otherwise have undergone apoptosis (62). EBV positive PTLD therefore represents classically the uncontrolled proliferation and malignant transformation of EBV infected B cells. The proliferation occurs as a result of the immunoevasive strategies of the virus and effects of viral genes in combination with the impairment of predominantly T cell immune responses. Malignant transformation occurs as a consequence of the proliferation of infected immortalized genetically abnormal B cells and the acquisition of new genetic and perhaps epigenetic abnormalities.

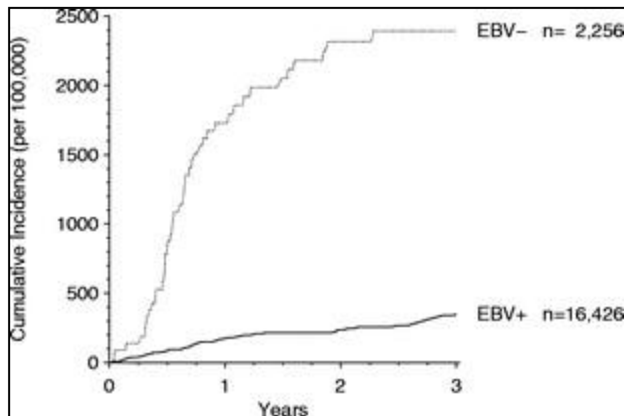
2.7.2 Risk factors for PTLD

2.7.2.1 EBV seronegativity

PTLD may occur in the setting of EBV reactivation post-transplant (113). However EBV seronegativity and primary EBV infection following the introduction of immunosuppression are associated with an increased PTLD risk ratio of 8.9 in some studies (104,114–116). Higher risk groups include paediatric transplant populations (incidence 4-22% versus 1-2% in adults) where a greater proportion of individuals are seronegative at time of transplantation. EBV seropositivity is approximately 90-95% in adults, 75% at age 18, 50% at age 5 and 16% at age 4. The first year after transplantation in particular is a time of high risk for PTLD in the seronegative. Seroconversion rates during this period range from 50-100% (117,118). Source of EBV acquisition may include a graft from a seropositive donor and intimate contact with those shedding the virus. House in 23 seronegative adult kidney transplant recipients, reported seroconversion at one year of 14/23 including 12/19 (63%) where a seronegative recipient received a kidney from a positive donor and 3/4 (75%) in negative donor - negative recipient combinations (118). EBV seronegativity appears to carry an increased risk of PTLD not just within the first year after transplantation but also late. This could be due to late primary infection but may reflect an "enduring" consequence of even early primary EBV infection (104,114). Individuals may carry more than one strain of the Epstein-Barr virus. To date it appears seropositive transplant recipients do not acquire new strains of EBV from the donor kidney (119). An increased risk of PTLD development in the seropositive as a result of acquisition of a different strain

from the donor would therefore appear unlikely. PTLD is now recognised to arise from donor lymphocytes, typically earlier in onset with the majority presenting with graft involvement, as well as from recipient lymphocytes (120). High levels of PTLD incidence have been reported in adult kidney transplant recipients (11.2%) in the United States. However seronegative rates for EBV and CMV in this population were surprisingly high at 65% and 80% respectively which may account for the high incidence (121). Recent registry data publication from the Collaborative Transplant Study reported that the risk of Non Hodgkin lymphoma (NHL) is increased for kidney transplant recipients (n=18,682) across all age groups who are EBV seronegative pretransplant (116). Multivariate Cox regression for NHL incidence reported a Hazard ratio of 6.5 for EBV seronegative (n=2256) patients at 3 years ($p<0.001$) compared to the EBV seropositive pretransplant (n=16,426) (Figure 2 A below). Figure 2 B below shows how the risk of PTLD is greatly increased for EBV recipient negative individuals receiving a kidney from an EBV positive donor. Risk for Donor negative recipient negative and donor negative recipient positive is 0 per 100,000 although only small numbers in each group. Donor positive recipient positive have an increased risk although far less than that for donor positive recipient negative.

A



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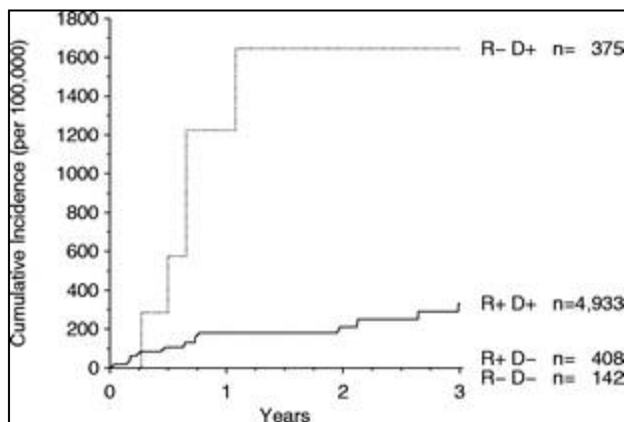

Figure 2.5 Pre-transplant serostatus and cumulative incidence of non-Hodgkin lymphoma in kidney transplant patients

Figure 2.5 A Cumulative incidence of non-Hodgkin lymphoma in kidney transplant patients according to recipient pretransplant Epstein-Barr virus (EBV) serostatus (log-rank P<0.001).

Figure 2.5 B Cumulative incidence of non-Hodgkin lymphoma in recipients of kidney transplants according to pretransplant recipient (R) and donor (D) Epstein-Barr virus serostatus.

Reproduced with permission from Epidemiology of Pretransplant EBV and CMV Serostatus in Relation to Posttransplant Non-Hodgkin Lymphoma. Opelz, Gerhard; Daniel, Volker; Naujokat, Cord; Dohler, Bernd Transplantation. 88(8):962-967, October 27, 2009

DOI: 10.1097/TP.0b013e3181b9692d

No significant increased risk for NHL was found on the basis of CMV serostatus pre transplant and antiviral prophylaxis for CMV was not shown shown to be protective. Doucette et al recently presented data to show that EBV viral load monitoring of high risk Donor seropositive, recipient seronegative adult and pediatric solid organ transplant patients can decrease the incidence of early PTLD. The study compared PTLD incidence in a historic cohort of transplant recipients 1996-2001 (n=80) and a monitored cohort (n=108) 2002-2007. All patients received antiviral prophylaxis for 14 weeks posttransplant. Viraemia was managed at the discretion of the responsible clinicians and responses included reduction in immunosuppression, antiviral treatment or no intervention. Incidence of early PTLD was reduced by 80% in this monitored cohort compared to the historical group (122).

2.7.2.2 Immunosuppression

Complete remission of disease may occur in up to 40% PTLD cases with the partial or complete withdrawal of immunosuppression alone. Registry data has attempted to find associations between specific agents, combinations of therapy, doses received and duration of therapy (5,123–126). It is logical to hypothesize that the more effective an agent in terms of prevention or treatment of rejection, the more effective is its suppression of T cell activation and proliferation and therefore so the greater is the risk of development of opportunistic infection and malignancy and the greater the risk of PTLD. The results of analyses are often conflicting. In adult renal transplant recipients an increase in PTLD incidence was seen with the

introduction of calcineurin inhibitors (105). However the CTS data published in 2004 analysing 145 104 cadaveric kidney transplant recipients showed no difference in incidence between ciclosporin, steroid and azathioprine and steroid and azathioprine alone regimens (5). Tacrolimus maintenance treated patients had a greater risk of PTLD than those receiving ciclosporin.

Use of agents including OKT3 and ATG for induction therapy and in the setting of acute steroid resistant cellular or vascular rejection is associated with an increased risk of early PTLD (within the 1-2 years after treatment). This increased risk does not continue to be apparent beyond this early period (5,105). No increased risk of PTLD is associated with the IL-2 receptor blocker basiliximab (5). Recent papers also suggest the antiproliferative mycophenolate mofetil may not increase risk of PTLD development but may even be protective in terms of reducing the incidence of EBV viraemia post-transplantation (124,127–129).

Incidence of PTLD is higher in combined Heart and Lung (2-9%) and multi organ (11-33%) than in kidney (2%) or liver transplants (2%) (5). This has been attributed to greater use of induction agents such as ATG or OKT3. The increased risk may relate to the quantity of lymphoid tissue received with the transplant organ.

2.7.2.3 HLA and donor recipient mismatch

Increased risk of PTLD is associated with higher levels of HLA match in EBV D+/R- lung transplant recipients, mismatch at the HLA-B locus in lung transplantation (130), and with specific HLA alleles in some studies. High risk has been associated with HLA A-26 and B-38 in the recipient in North American solid organ transplant

populations. Poorer prognosis has been associated with HLA B-44 and DR-15 with response to immunosuppression (131). ANZDATA (105) demonstrated no increased risk in association with HLA mismatches.

HLA class 1 alleles and polymorphisms including HLA-A*01are associated with the development of Hodgkin's lymphoma and Infectious mononucleosis following primary EBV infection (132). Genetic variation here may result in impaired or variable immune responses to EBV infection as a result of differences in HLA class 1 molecule ability to present viral peptides for recognition to cytotoxic T lymphocytes. More recently a case control study by Reshef et al in 110 solid organ transplant recipients with PTLD and 5601 controls without PTLD found HLA-A26 was associated with PTLD (OR 2.74, p=0.0007), and A26 and B38 associated with EBV positive PTLD (OR 3.99, p=0.001) while A1, B8, DR3 appeared to be protective (OR 0.41, p=0.005) (131).

2.7.2.4 Immune responses: Defects and Genetic polymorphisms

Polymorphisms resulting in reduced expression of IFN-γ have been seen in a study involving solid organ transplants. An association with PTLD was found in these low expressors. The findings were not reproduced in other studies and appeared variable in relation to the organ transplanted (133).

Defects in cellular immunity have also been found in individuals with nasopharyngeal carcinoma and EBV positive lymphoma in nontransplant populations. CD8+ EBNA1 CTL frequencies were significantly lower seen in cases of NPC compared to healthy individuals while LMP-1 specific CTL's were detected

in similar frequencies. Selective EBNA-1 specific CD4+T cell deficiency was seen in all EBV positive lymphoma cases unlike in EBV negative or lymphoma in the general population (134–136). In paediatric liver transplant recipients a polymorphism of the Sic11a1 gene is associated with development of PTLD and high EBV viral loads (137).

Polymorphisms of the lytic gene BZLF1 promoter region have been identified in DNA obtained from peripheral blood lymphocytes. Patients with EBV positive tumours including PTLD had different "malignant" polymorphisms and EBV strains as opposed to "nonmalignant" strains seen in healthy controls (138). More recent studies suggest variations in Tumour Necrosis Factor –α loci and high plasma TNF-α levels associated with these variant alleles may be risk factors for PTLD (139).

2.7.3 Clinical Presentation

Presentations are varied. Disease can present with allograft involvement and dysfunction. Oropharyngeal symptoms and cervical lymphadenopathy with or without B symptoms including fever, malaise, weight loss and sweats can be found particularly in early or polymorphic disease. Extranodal and widespread monomorphic disease may present subacutely or acutely with gastrointestinal or respiratory tract obstructive symptoms.

2.7.4 Treatment

Treatment based on current BCSH (2010) guidelines involves stepwise reduction of immunosuppression (140). Single agent rituximab (375mg/m2 weekly for four weeks) is recommended for patients with clinically low risk PTLD who fail to respond adequately to reduction in immune suppression. Rituximab plus anthracycline based chemotherapy (e.g. R-CHOP) is recommended for patients with PTLD who fail to achieve an adequate remission, progress despite previous reduction of immunosuppression and single agent Rituximab or have clinically aggressive lymphoma or critical organ compromise.

2.7.5 Non PTLD EBV related malignancy post-transplantation

Smooth muscle tumours including leiomyosarcomas associated with Epstein-Barr infection have been reported in lung, heart and kidney transplant recipients as well as in patients with the acquired immunodeficiency syndrome (141).

2.7.6 EBV related graft dysfunction

EBV has been associated with acute hepatitis and prolonged graft dysfunction in liver transplant recipients (98,142,143). In lung transplantation fever, fatigue, eosinophilia, the bronchiolitis obliterans syndrome and graft dysfunction with decreases in FEV1 have been reported (144).

2.7.6.1 Renal allograft dysfunction

PTLD presenting as renal allograft dysfunction is well documented. Deterioration in function may result from disease confined to the allograft, extrinsic compression or invasion from extranodal disease and as a consequence of immunosuppressive withdrawal (145–147). Graft infection has also been seen in cases of PTLD where disease was not only found near the allograft but also distant from the graft. In these cases EBV DNA and RNA was found localised to proximal tubular cells. In 8 cases EBV infection of the graft was seen up to 42 months before diagnoses of PTLD were made (148).

Type 1 membranoproliferative glomerulonephritis has been associated with primary EBV infection in native kidneys with positive immunostaining for EBV in glomerular cells (149).

2.7.7 EBV related clinical syndromes

Primary infection and EBV reactivation may be detected on the basis of serology and detection of viraemia. Individuals may often be asymptomatic or present with mild non-specific symptoms such as fatigue, low grade fever, sweats, oropharyngeal symptoms including pain and swollen glands. Complications of primary infection as seen with Infectious mononucleosis in the general population may also be seen. These may include haematological abnormalities, haemophagocytosis, pneumonitis, hepatitis and encephalitis is also reported in a renal transplant recipient (150).

2.7.8 Detection and implications of Epstein-Barr Virus

2.7.8.1 Serology

Current European Transplant society, North American and the BCSH guidelines on PTLD (2010) recommend the screening for recipient and donor EBV antibodies pre transplant. This allows the identification of D+R- and D-R-individuals at risk of primary infection and higher risk of PTLD post-transplant (151–153). In the UK the Health Protection Agency National standards unit recommends screening for IgG antibody titres to the Epstein-Barr nuclear antigen complex 1 (EBNA-1) and Viral capsid antigen (VCA) is performed conventionally. In the non-transplant setting IgG to EBNA are detectable 6-12 weeks after the onset of symptoms following primary infection. They are a marker of previous infection and persist for life. IgM and IgG to VCA appear at the start of the clinical illness in primary infection. VCA IgG persists for life and IgM antibodies, the markers of acute infection, typically are no longer detectable at 3 months. IgG antibodies to EBV early antigen (EA) can also be identified in some individuals in acute infection at the onset of clinical symptoms. The subset EA IgG anti-D is detectable and typically clears with the resolution of the illness. In PTLD persistence of EA has been noted (152,154). Surveillance of the seronegative using serial screening for VCA, EBNA or EA antibodies to identify transplant recipients experiencing primary infection and to potentially highlight those at risk of EBV driven PTLD has not been standard practice to date. Serology can be unreliable in the immunocompromised. In comparison to the general population transplant recipients who seroconvert while

immunocompromised may display abnormal or deficient antibody responses. IgM antibodies in particular may fail to appear and IgG appearance can be delayed (155). Individuals with low EA IgG titres in the presence of high EBV viral loads may be at greater risk of PTLD than those with high viral loads and high EA titres (154).

The classical serological assay for infectious mononucleosis, the heterophile antibody test or “monospot” can often give false negative results in the young, old and in immunosuppressed transplant recipients. A positive result in a patient with typical symptoms and signs suggestive of IM involves the agglutination of horse or sheep erythrocytes classically or more recently the agglutination of latex beads coated in bovine heterophile antigens when mixed with the patient’s serum or plasma.

2.7.8.2 Seroconversion

Despite the limitations of serology in diagnosing acute EBV infection after transplantation, research studies have provided data describing seroconversion rates in different populations.

In adults EBV seronegative rates of approximately 90-95% are found pre transplant. In adult renal transplant populations published seroconversion rates for the EBV naive are 50% at 90 days and 61% at 1 year including 12/19 (63%) D+R- and 3/4 (75%) D-R-. Similiar findings in paediatric liver transplant populations are reported including 50% seroconversion by 3 months and 89% by 1 year. Crawford et al in their study of EBV seroconversion in University students in Edinburgh found

that of 510 seronegative students entering university 46% of those retested 3 years later had seroconverted. Only 27 % of these had developed Infectious mononucleosis and illness (27).

2.7.9 EBV Viral load assessment

Post-transplant care involves the careful surveillance for and treatment of opportunistic infections. This includes the prevention, detection and appropriate management of viral infections such as EBV and cytomegalovirus. Quantitative viral DNA measurement in peripheral blood samples is now standard practice in transplant units. Circulating EBV DNA may be first detected in primary infection, and may spike to high levels after which a low level steady state will be reached in the healthy carrier. In cases of CAEBV persistent high levels of viral DNA may be found and will be associated with illness. In the transplant recipient viraemia likely will precede serological evidence of primary or reactivated EBV infection. High levels and rapid rises of viral DNA are found in disease states including PTLD. Viraemia may also be found in association with transient reactivation, in the absence of symptoms or illness, and may even reflect effective immunosuppression.

However difficulties in interpreting single EBV DNA measurements exist. No international reference standard for the measurement of EBV DNA existed until recently (156). Reporting units vary from copies per ml to copies per microgram of DNA to copies per 100,000 leucocytes in different laboratories and inter and intra laboratory variability is high with poor reproducibility (157). Further there is no

consensus as to what level of viral DNA represents a disease state and should trigger alarms and potential clinical interventions. The ideal medium in which DNA should be quantified is also unclear. In the healthy individual EBV post primary infection should only be detectable in intracellular compartments and whole blood assays. Levels should be low or undetectable although transient rises in the context of other illnesses or stress may be found. Detection of free viral DNA in plasma may be found but is unusual. Its presence may reflect escape from damaged or lysed cells during storage or transport. However the presence of DNA in plasma has also been identified as a potential biomarker for PTLD in some studies.

2.7.9.1 Definitions of EBV Syndromes Post-transplant

Attempts have been made to provide definitions for EBV disease, primary infection, viral reactivation and for chronic high viral load carriage after transplantation.

EBV related disease:

Disease has been defined as “clinical illness consistent with EBV infection in a transplant patient and supported by serological and PCR evidence of EBV infection occurring in the absence of extra-nodal mass lesions or histological evidence of PTLD. Examples include patients with fever, rash, hepatitis, enteritis, adenopathy or tonsillar hypertrophy in association with primary EBV seroconversion, IgM reactivation or high viral loads” (Bingler, Feingold et al. 2008)(152).

PTLD:

PTLD is a histological diagnosis classified currently according to the WHO criteria (107).

EBV Reactivation:

Reactivation has been defined previously as a quantitative PCR level above 40,000 genome copies / ml or two consecutive rising viral loads, the last above 10,000 genome copies / ml under stable immunosuppression (158–160).

Chronic high viral load carriage:

A proposed definition for the chronic high EBV load carrier is high load measured on >50% of samples for >6 months following either asymptomatic infection or complete resolution of EBV disease / PTLD (161).

2.7.9.2 EBV DNA quantification as a biomarker for PTLD

Difficulties exist in applying the results of many published studies investigating EBV viral loads to adult renal transplant populations. Many of the studies looking at EBV infection and reactivation involve small numbers of patients and describe heart, lung, liver and paediatric populations. Incidence rates of PTLD historically have been greater in these populations than in adult kidney allograft recipients. Further periods of greatest risk are often the first year after transplantation in paediatric and heart and lung populations. Studies involving serial monitoring for EBV typically follow patients through the first 1-2 years after transplantation, with little information available about late EBV infection. This is important as most cases of PTLD in adult renal patients occur “late” i.e. more than one year after transplantation, with a mean time to presentation of approximately 70 months. The

prevalence of EBV infection in the whole transplant population and in particular in patients many years out from time of transplantation is not known.

2.7.9.3 Sensitivity and specificity of viral load assessment for PTLD

Negative predictive values of up to 100% are reported in intestinal transplant populations who have either undetectable or low EBV viral loads during the first 6 months after transplantation (162). Interestingly the 5 patients in this cohort of 30 who developed PTLD were all seropositive pre transplant and all experienced initial high viral loads within 3 months of transplantation.

Attempting to set a threshold of viral load with the hypothesis being that those above have a greater risk of PTLD than those below again is problematic. Again low loads have high negative predictive values above 90% but positive predictive values can be poor, <30% in some studies (163). Pre-emptive immunosuppression reduction has been performed following the finding of 2 serial viral loads > 4000 copies/microgram DNA in paediatric liver transplant recipients. This strategy resulted in a reduction in PTLD incidence from 16% to 2% with viral load monitoring predicting risk of PTLD with a PPV of 56%, NPV of 100%, sensitivity of 100% and specificity of 83% (164).

While EBV positive PTLD is associated with high viral loads, false reassurance may be taken in some cases from a viral DNA negative peripheral blood sample. Although uncommon, EBV tissue negative PTLD and a small number of tissue positive PTLD cases, up to 53% in some series will have undetectable EBV viral loads at time of diagnosis (165).

Sustained high viral loads are associated with pretransplant seronegativity and primary infection rather than reactivation post-transplant. A persistently high viral load would also appear to pose a greater risk of PTLD development than transient viraemia (166,167).

2.7.9.4 Implications of chronic high viral load carriage

The implications for patients with persistent chronic high viral loads but little clinically to suggest PTLD is unclear. Data is lacking as to what constitutes a higher risk individual with a greater likelihood of developing EBV related illness. Those with acute or persistent viraemia in the context of clinical findings consistent with PTLD including B type symptoms, lymphadenopathy and oropharyngeal or gastrointestinal symptoms need thorough investigation and close surveillance. Reduction of immunosuppression in the asymptomatic but viraemic may seem logical. The presence of EBV DNA may constitute an overimmunosuppressed state and there may be an increased long term risk of PTLD development (146,168). Pre-emptive reduction of immunosuppression however particularly if asymptomatic and without evidence of other opportunistic infections, may result in poorer long term graft survival with returns to dialysis and decreases in long term survival (146).

It is interesting as illustrated in Bingler's study in 71 paediatric heart transplant recipients that of 20 high load carriers identified in his study 8 had suffered previous PTLD, 7 had prior symptomatic EBV related disease and 5 had no current or prior history of EBV related illness. Six of eight with previous PTLD developed a

disease recurrence of which 4/6 had a different WHO histological classification. Only 7/22 with prior EBV related illness and only 1/5 with no history developed PTLD (161). Such findings would encourage close long term surveillance of those treated and cured of PTLD and would favour more aggressive intervention including reduction of immunosuppression as opposed to monitoring for high viral load carriers. Again the implications and relevance of these studies are still unclear in adult renal transplant recipient populations however.

Table 2.6 Summary of findings for previous studies of EBV DNAemia in solid organ transplantation

Study	Population	Method	Time period	Viraemia incidence	PTLD incidence	Finding
Bamoulid (2013) (Bamoulid et al., 2013)	Adult kidney transplant recipients transplanted 2002-2010 n=383	Prospective monitoring. EBV D+/R- receive IV IG, IS reduction if persistent viraemia, pre-emptive rituximab for n=3	First year EBV screening PCR in wholeblood	40% at 1 year EBV D+/R- 67% 6% chronic viraemia >6months ATG use associated with viraemia	0%	EBV infection associated with opportunistic infection and graft loss. *Relevant to UK, 72% simulect treated, pre-emptive IS reduction may contribute to graft loss*
San-Juan (2012)(Fernández-Ruiz, San-Juan, & Aguado, 2013)	81 Solid organ transplant recipients n=51 Kidney transplanted 2003-2004 All EBV seropositive	Retrospective observational study	EBV wholeblood screening for 6 months post transplant Median follow up 67 months	84% overall 49% persistent >30days	0%	No association graft function or graft loss. Association EBV with IS related adverse events *No kidney specific EBV rates or breakdown*
Holman (2012)(Holman, Karger, Mullan, Brundage, & Balfour, 2012)	SOT and HCT recipients 185 adult kidney recipients	Retrospective analysis post transplant wholeblood quantitative EBV tests and association with PTLD DNAemia defined >1000 copies/ml wholeblood	7.5 year study period	30% for adult kidney transplant recipients 49% paediatric	6.2% Paediatric 2.2% adult kidney	PTLD risk increased with higher viral loads. *More detailed organ specific data would be useful. No data mean time from transplant*
Martin (2011)(Martin et al., 2011)	EBV D+/R- adult kidney transplant n=34 97% ATG induction	1st year prospective EBV monitoring Rituximab given to symptomatic or persistently viraemic	1 year study period	20/34 (61%) viraemia in 1st year. 50% symptomatic	6/20 with viraemia given rituximab and no PTLD. 6/34 lost to follow up and 3/6 PTLD and graft loss	1st year monitoring of D+/R- and intervention with rituximab may reduce PTLD incidence

Study	Population	Method	Time period	Viraemia incidence	PTLD incidence	Finding
Holmes (2009)(M. V Holmes et al., 2009a)	Adult kidney transplant recipients n=115 9% EBV seronegative 98% Basiliximab	Prospective EBV monitoring 1st 90 days post transplant. Wholeblood EBV pcr	90 day EBV screening then up to 2 year follow up period	56% (>100 copies/ml). Seronegative 50%, seropositive 55%	No PTLD 6 month-2 year follow up	MMF treated individuals lower incidence of viraemia
Tanaka (2011)(Tanaka et al., 2011)	Pediatric EBV seronegative kidney transplant recipients n=13. Basiliximab induction	Prospective EBV monitoring. IS reduction if persistent (>1000 copies/ml 6 months) DNAemia	Follow up 1-5 years	50% persistent DNAemia > 6 months	No PTLD	No difference in graft function in those with and without IS reduction
Smith (2010)(Smith et al., 2010)	Pediatric kidney transplant n=55 55% EBV seronegative	Prospective monthly EBV screening of plasma samples for 2 years post transplant	2 years	Subclinical infection 36% overall, median onset 192 days D+/R- 68% D+/R+ 26%	4 cases PTLD	Subclinical EBV and CMV infection associated with chronic allograft injury and increased GFR decline
Toyoda (2008)(Toyoda, Moudgil, Warady, Puliyanda, & Jordan, 2008)	Kidney transplant n=98 adult n=58 children Transplanted 1988-2000	Prospective EBV PCR screening Adults only if clinical indication MMF reduced 30% if viremia, stopped if persistent Wholeblood PCR	4 months screen (children)	9% DNAemia overall Children 12/58 21%, adults 2%	No adult cases 3/12 (25%) children with viremia No PTLD if EBV PCR negative	2 adults cleared EBV following IS reduction
Li (2007)(L. Li et al., 2007)	Pediatric kidney transplantation n=102	Prospective EBV screening to 36 months post transplant	80+-21 months	38% viremia 4% EBV related disease Seronegative recipients 85% Seropositive 20%	4 cases	Increased incidence of viremia in those receiving steroids 57% v 20% if steroid free. No association MMF, AZA, sirolimus Reduced 6 year graft survival if EBV viremia and poorer 3 year graft function

Study	Population	Method	Time period	Viraemia incidence	PTLD incidence	Finding
Bingler (2008)(Bingler et al., 2008)	Pediatric Heart transplant n=20 chronic high viral load carriers (8 PTLD history) 51 controls low or absent EBV	Retrospective analysis. EBV screening in PBMC's and wholeblood	Median 6.7-8.2 years	94% CHVL carriers EBV seronegative at transplant v 75% those with absent or low viral loads	9/20 (45%) CHVL developed PTLD 2/51 (4%) low or absent EBV developed late PTLD	CHVL is predictive of denovo or recurrent PTLD
Doesch (2008)(Doesch et al., 2008)	Stable adult Heart transplant recipients n=172	Prospective EBV screening, threshold 100 copies/ml	6 months from recruitment	67% viremia at recruitment median 4.9 yrs post transplant (range 1.1-16.9) Persistence over 6 months 21%	3 cases, 2 EBV negative, median follow up 4 years	EBV DNAemia associated with CNI therapy, higher ciclosporin levels, azathioprine and absence of MMF.
D'Antiga (2007)(Antiga et al., 2007)	Pediatric liver transplant n=34 Seronegative	Long term follow up EBV screening PBMC's for DNA 5 samples per year mean IS reduction if >1000 copies/10^5 PBMC	Median 5.8 years (R 1.5-17.7)	PCR 66% viremia if seronegative 0% if seropositive	3 PTLD, all seronegative	Seronegative patients at increased of viremia and sustained viral loads
Green (2009)(M Green, Soltys, Rowe, Webber, & Mazareigos, 2009)	Pediatric liver transplant recipients n=196 Chronic high viral load carriage n=36/196 75% seronegative	Retrospective review EBV PCR Wholeblood copies/ml	Long-term post-transplant EBV monitoring	18% CHVL carriage 21/36 EBV disease or PTLD preceded subsequent CHVL carriage	1 case (2.7%) CHVL carriers	23/36 resolved CHVL carriage without sequelae

2.7.9.5 Compartment analysis

Debate continues over the relative sensitivity and specificity of viral DNA detection in the intracellular and plasma compartments in relation to development of PTLD. A prospective study demonstrated the presence of persistent detectable free plasma EBV DNA in 13/15 cases of PTLD. Plasma DNA was not seen in controls, those without PTLD or EBV negative PTLD. The PPV of 100% and NPV of 86% for plasma DNA presence and PTLD was calculated. The absence of intracellular EBV DNA was highly predictive of freedom from PTLD. However the presence of intracellular EBV DNA, even persistent in nature was seen not just in PTLD patients but also in healthy controls and was less specific. Plasma EBNA PCR positivity had the greatest PPV (100%) and specificity (100%)   Wagner et al also demonstrated that in PTLD patient's plasma analysis for viral DNA was more sensitive and specific than analysis of peripheral blood mononuclear cells (169–172). In other studies the greater sensitivity of whole blood samples compared to plasma for the detection of EBV DNA outside of PTLD has been shown (173). Stevens et al carried out serial whole blood sampling for EBV viral DNA in 103 lung transplant patients. 6 patients with PTLD were identified. 78% of blood samples including pre-diagnosis (50/64 78%), time of diagnosis and post diagnosis samples were EBV positive and above a cut off value of 2000 copies per ml blood. Only 4/117 (3.4%) samples in patients without PTLD were EBV positive. All PTLD patients had simultaneously obtained serum and whole blood samples during follow up. None of the serum samples had evidence of EBV DNA despite high viral loads on whole blood samples (174).

2.7.9.6 Prevalence of viraemia

Viraemia is associated with pre-transplant seronegativity, young age, Tacrolimus, ciclosporin-Azathioprine regimens and multiple doses of ATG (129,146,168,175) Patients receiving Mycophenolate Mofetil (MMF) appear to have a lower incidence of viraemia than those not receiving MMF (129).

Symptomatic viraemia also is associated with primary infection to a greater extent than reactivation (176).

Seroconversion of the small proportion of EBV naive transplant recipients appears to occur predominantly in the first year. Studies involving serial screening for viral DNA have tended to focus on the early transplant period. Less is known about the late transplant period in terms of incidence and prevalence of viraemia. In the available literature in heart and lung transplant patients, up to 80-90% of patients may have evidence of viral DNA in at least one blood sample in the first few months after transplantation. The number of positive samples declines with increasing time from transplantation down to 47% of patients tested between 12 and 24 months (177). A more recent study in renal patients following 115 new transplant recipients for 90 days found 64/115 (56%) had whole blood PCR positivity for EBV DNA during follow up with detectable virus persisting for 6-77 days. 55% of the 103 EBV previously infected patients had reactivation and 50% of the EBV negative patients experienced a primary infection after transplantation (129).

Late viraemia has been recognised in one study following 75 lung transplant recipients where increasing time from transplant was recognised as the sole risk factor for reactivation of EBV in multivariate analysis (178).

2.7.9.7 Detectable Viral loads and risk of rejection

Retrospective studies in lung transplantation have shown patients with whole blood EBER positive PCR tests have statistically lower incidences of Grade 2 or higher allograft rejection than patients without PCR positivity over 2 years of follow up after transplantation. An increased risk of infections was not observed in the same EBV positive cohort (146,168,179). Pre-emptive reduction of immunosuppression again in lung transplant recipients in the context of 2 or more consecutive EBV viral loads with a rising trend measured 2-4 weekly did not result in acute rejection or reduced graft survival (159).

Green et al has reported that in 7 paediatric orthotopic liver transplant recipients with PTLD, all cleared their EBV viral loads from high level to < 200 genome copies per 10^5 ml with treatment. The mean time to clearance 18.8 days, corresponded with the mean time to rejection 13.8 days and all with rejection had EBV DNA levels < 100 genome copies per 10^5 ml at time of rejection episode (180). Stevens in a follow up of 103 lung transplant recipients found that in patients without PTLD, viraemia (>2000 copies per ml) was extremely rare and seen in only 4/117 (3.4%) serial whole blood samples. Further no transplant recipient treated for rejection displayed levels of EBV DNA> 2000 copies per ml (181).

2.7.9.8 Pre-emptive management of EBV DNAemia

Concerns relating to the increased risk of PTLD in transplant patients with EBV infection have resulted in pre-emptive strategies in adult renal transplant recipients such as the administration of antiviral agents and therapies such as intravenous immunoglobulin to high risk EBV donor seropositive recipient seronegative patients peritransplant. Further in the setting of detectable or rising viral loads reduction of Immunosuppression and rituximab have now been used and published in at least two reports in the literature (146,182). In the Bamoulid paper individuals treated with rituximab did not develop PTLD under follow up but in all EBV DNA levels returned to levels similar to pre therapy following reconstitution of B cell numbers. These strategies have been applied previously in the stem cell transplant population and paediatric liver transplant populations. Reduction in frequency of PTLD has been reported in liver patients (164). Other small studies report reduction in viral DNA levels with immunosuppression reduction and antiviral agents in 36% of their patients with further sustained reductions of up to 82% affected following the administration of CMV –IgG containing anti –EBV antibodies. Longer term follow up data looking at this uncontrolled population is not available but no acute rejection was seen (183). In paediatric renal transplantation reduction of immunosuppression (halving of azathioprine dose) has been performed where there was evidence of infectious mononucleosis complicating primary infection but not for asymptomatic reactivation. No PTLD was seen during follow up, and graft

function did not deteriorate as a consequence of reduced immunosuppression (176).

Rapid decline in viral loads (>50% within 72 hours) in stem cell transplant patients treated for PTLD has been associated with good clinical outcome while poorer rates of decline or rise in viral load were associated with poor response to treatment and outcome (158). Such findings have been reproduced in EBV positive lymphomas in immunocompetent patients where again DNA levels "paralleled the clinical course becoming undetectable in remission and remaining elevated in refractory disease" (184).

2.7.9.9 Use of antiviral therapy in EBV related disease, PTLD and chronic high viral load carriage

Antiviral therapy including aciclovir and ganciclovir has been used to both treat and prevent PTLD. Higher risk D+ R- solid organ transplant individuals receive antiviral prophylaxis in some units. Current guidelines suggest antiviral treatment may prevent PTLD (level II evidence) but there is no evidence for their use in the absence of other strategies such as reduction of immunosuppression or Rituximab. (level III evidence) (185). In immunocompetent individuals with infectious mononucleosis meta-analysis data suggests acyclovir does not reduce incidence of symptoms or adverse events but may reduce early viral shedding when compared to no antiviral therapy (186). Aciclovir and Ganciclovir are both purine nucleoside analogues. They are phosphorylated in EBV infected cells expressing Thymidine kinase and BGLF4 to monophosphate forms and then triphosphate

metabolites which are incorporated by viral DNA polymerases into viral DNA with subsequent apoptosis of the infected cell. Thymidine kinase and BGLF4 are both lytic gene products and are not expressed in latently infected cells and are not generally expressed in EBV positive malignancies occurring in latently infected and transformed B lymphocytes. Ganciclovir has been used successfully in combination with Arginine Butyrate in refractory EBV positive lymphoma. Arginine Butyrate induces Thymidine kinase expression in latently infected B cells thereby sensitising these cells to ganciclovir and subsequent apoptosis (187). In a series of 11 renal transplant patients with diffuse B cell lymphoma, acyclovir and reduction of immunosuppression were used to successfully induce complete remission in 10 (91%) cases. Interestingly all these cases expressed thymidine kinase in the PTLD tissue and CD8+ cells directed against BZLF-1, another lytic antigen, expanded in 2 of those successfully treated (188). Reports of successful treatment of PTLD expressing BZLF1/ZEBRA with Foscarnet and with antiviral agents and IV immunoglobulin are also available (189–191). In retrospective case control studies use of prophylactic acyclovir has been shown to reduce risk of PTLD particularly in the first year (192). Ganciclovir accumulates more and persists longer in infected cells and has been shown in animal models and in humans to reduce risk of tumour development to a greater extent than acyclovir (193,194). Most recently a single centre trial reported it's experiences, in EBV Donor +ve Recipient –ve new adult kidney transplant recipients, of using pre-emptive rituximab therapy to prevent PTLD in those individuals with persistent viraemia in the first year after transplant. In this study EBV D+R- individuals routinely received Ganciclovir then

Valganciclovir for 3 months after transplant as antiviral prophylaxis aagianst EBV. 11/20 (55%) of these individuals developed EBV viraemia in the context of primary infection while on prophylaxis. No obvious effect was observed either in preventing primary infection related viraemia or in managing the viraemia with antiviral prophylaxis. Recipients were followed for one year after transplant with regular screening for EBV viral loads. Viraemia developed in 20/33 recipients followed of which 6 received rituximab, 5/6 1 dose and 1/6 2 doses before clearance of detectable viral loads. Among the remaining 14 with viraemia not treated with rituximab 4 developed PTLD including 3 not on the screening program and a fourth CNS PTLD 30 days after negative PCR testing (182).

2.7.10 EBV gene expression

PTLD and other EBV related diseases including Burkitt's lymphoma and Hodgkin's lymphoma have been associated with different patterns of viral gene expression. Classified as latency type 0, 1, 2 and 3, these patterns involve the expression of mRNA for different EBV associated latent antigens. PTLD has been associated with latency type 3, an unrestricted form of latency characterised by the expression of the majority of latent genes. Difficulties remain that such a pattern of expression is not sensitive and specific for PTLD and can be found in healthy recipients with high viral loads (195). However a recent review highlighted more recent studies suggesting high viral load carriers may be differentiated from those with PTLD and EBV related disease with expression of LMP-1 and LMP-2a in the first group as opposed to expression of LMP-1, EBNA-1 and EBNA-2 in those with PTLD

(196,197). High load carriers also seem to carry higher numbers of EBV genomes in their infected B cells (20-30 copies) than those with undetectable or low viral loads (1-2 copies) (198). Monomorphous single site proliferations showed a trend to less lytic positivity. DNA microarray gene expression profiling has been used now to attempt to identify those at risk of EBV lymphoproliferation after transplantation. Analysis demonstrated genes that were over suppressed and suppressed that allowed the clustering of patients with high and low viral loads. This data was presented in abstract form at the World Transplant Congress 2006. (Khodai-Booran, Beyene et al. 2006)

2.7.11 Immune markers of EBV infection

Optimal transplant care involves an individualised approach to recipients in particular in relation to immunosuppressive regimens and doses. Golden goals are low incidence of acute rejection, excellent long term graft survival and freedom from opportunistic infections and malignancy. Clinical and laboratory markers of levels of global and in this context EBV specific immunosuppression are needed to reduce infection related morbidity and mortality.

Control of herpes viruses in transplant recipients is predominantly related to cellular immune function and in particular to effective T lymphocyte function. Reactivation of EBV and poor control of viraemia has been associated with low number and poor function of EBV specific T lymphocyte populations. Positive predictive values of up to 100% for PTLD have been reported in studies where transplant recipients have high EBV viral loads in the setting of low numbers of

EBV specific CD8 cytotoxic T lymphocyte numbers. Impaired function of these T lymphocytes measured by the ability of such cells to produce cytokines such as Interferon γ when stimulated with EBV peptides are found in transplant patients compared to healthy controls (199–201). Regression of PTLD following treatment including reduction of immunosuppression has been associated with increases in number and of such Interferon-γ secreting cells (202). Undetectable or minimal CD4+ and CD8+ T cell responses to latent viral antigens in cases of PTLD are associated with poor outcome and peripheral low absolute CD4+ T cell numbers may be a marker of those with increased risk of PTLD particularly in the context of high viral loads (203).

Assessments of EBV related cellular immune function have typically been performed in research laboratories and are not generally available in everyday clinical practice. Global and EBV specific T cell function has been assessed using the Cylex Immuknow assay and a T cell memory assay. T cells obtained from peripheral blood were stimulated with PHA and ConA and subsequent ATP production measured. Patients with PTLD and asymptomatic patients with persistent high EBV viral loads had lower levels of ATP release following T cell stimulation than those with undetectable or low levels of viraemia. The authors concluded the low levels of ATP in the chronic high viral load group and the PTLD patients may be due to T cell exhaustion (204).

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3.0 Methods and Materials

3.1 Study populations

The papers presented in the results section of this thesis report research involving two study populations including stable adult kidney transplant recipients recruited from clinic and kidney transplant recipients with a previous diagnosis of PTLD.

3.1.1 Epstein-Barr virus infection in adult renal transplant recipients

3.1.1.1 Patient identification and recruitment

To investigate the prevalence and clinical implications of EBV DNAemia in stable transplant recipients a single centre prospective study of Epstein-Barr virus infection in adult renal transplant recipients was performed. This study was a prospective, observational cohort study in two stages. Stage 1 of the study aimed to classify the recruited adult renal transplant population at Manchester Royal Infirmary (MRI) over 1 year on the basis of their EBV serology and PCR for viral DNA. This was to enable a description of the prevalence and clinical features of active EBV infection in the whole population. Stage 2 aimed to investigate in more detail clinical, viral and immunological differences between individual patients in response to EBV infection.

3.1.1.2 Recruitment and Consent

All adult renal allograft patients with a functioning graft attending routine outpatient follow up at Manchester Royal Infirmary between 22nd February 2010 and 13th September 2010 were invited to participate, 896 patients at time of study recruitment. Participants were 1month to 33 years post-transplantation. Inclusion criteria included age >18 years and the presence of a functioning graft at recruitment.

3.1.1.3 Study Methodology

Whole blood EDTA was collected and analysed for the presence of EBV DNA at recruitment and at 3 monthly intervals for 1 year. A quantitative real-time PCR assay as described by Niesters et al was used with the target gene BNRF-p143 (205). DNA extracts were batched and stored at -80^{0}C, with PCR amplification and detection of EBV DNA subsequently performed using the Applied Biosystems 7500 Fast Real-time PCR. The threshold of sensitivity for detection of EBV DNA was 1000 copies/ml blood. Samples with no detectable EBV DNA or concentrations measured at < 1000 copies/ml were classified as undetectable. Prevalence of EBV DNAemia was calculated for the recruited population at baseline and then patterns of EBV DNAemia over time analysed for all recruited individuals with 3 or more samples obtained over a period greater than 6 months. Blood samples were also taken at recruitment for analysis of EBV serostatus (the presence of IgG antibodies to EBV VCA and EBNA-1 antigens (U/ml)) using the DiaSorin S.P.A LIAISON

analyzer**.** Demographic, transplant and immunosuppressive details were obtained from the medical records at the time of recruitment.

Patients completed a survey of clinical symptoms including fever, night sweats, weight loss, loss of appetite, sinus congestion, anorexia, swollen glands, sore throat, loss of appetite and abdominal pain (152). A Karnofsky and ECOG score was carried out at baseline.

Patterns of EBV DNAemia over time were analysed in the 446 individuals with multiple blood samples. Individuals with <3 samples (n=53) were excluded from this analysis. On the basis of previous studies we predicted individuals would fall into 4 classes including those with no detectable DNA (NDNA), transient detection (TDNA)(<75% samples with detectable DNA), persistent DNAemia (PDNA) (≥75% samples with detectable DNA), and chronic high viral load carriage (CHVL) (≥3 samples over >6 months with ≥10,000 copies/ml DNA)(161,206).

Analysis of factors predicted to be associated with the presence of EBV DNAemia included demographic details including age and time from transplant, immunosuppressive agent exposure, rejection history, biochemical and haematological parameters including white cell differential, renal graft function, and a history of post-transplant complications including herpes virus and other opportunistic infections, and occurrence of non-melanoma skin cancer since transplantation.

Participants were followed up until 22nd February 2013 with subsequent analysis of patient and graft survival, decline of graft function, and development of PTLD in

relation to EBV DNA detection at recruitment and subsequent EBV DNAemia patterns.

3.1.2 Stage 2

Following completion of Stage 1 and analysis of the data a meeting of the Study Monitoring Group was convened to identify the patient subgroups for further study. We recruited a sub-population of 60 patients and invited them to participate in Stage 2. This part of the study was a nested case-control study investigating viral and immunological differences between individuals with high persistent EBV viral load detection, low level and transient detection and those with persistently undetectable levels of EBV DNAemia in blood during follow up.

3.1.2.1 Stage 2 Methodology

Recruited individuals included 21 with persistent high viral load detection and a further 20 with low level and transient DNAemia and 19 with persistent undetectable EBV DNA levels matched for age and time from transplant where possible. The study involved a single clinic assessment where a further whole blood EDTA sample was obtained. The plasma compartment was separated from the sample then extraction performed for EBV DNA from both the blood and plasma compartments. In addition whole blood (EDTA) was obtained for lymphocyte subset analysis using flow cytometry. During the assessment recruited individuals also underwent an ultrasound examination of the lymph node chains in the neck including anterior and posterior triangles, parotid, submandibular and submental areas as well as a physical examination.

3.1.2.2 Ethical approval

Ethical approval for the study was obtained from the research ethics committee, REC reference 09/H1013/71, and by the Central Manchester Foundation Trust research and development department, R & D PIN R00909. The ethical application was completed as part of this PhD. Recruitment of participants following written informed consent in line with good clinical practice standards and recommendations commenced on the 22nd February 2010.

3.1.3 Post-transplant lymphoproliferative disorder in adult renal transplant recipients

3.1.3.1 Patient identification and recruitment

A second study population was identified to perform a retrospective analysis of cases of Post-transplant lymphoproliferative disorder (PTLD) presenting in adult individuals with kidney transplants under the care of the Manchester Royal Infirmary transplant unit. Cases were identified following referral to our regional Haemato-oncology service, from regional pathology databases, and from the NHS Blood and Transplant service records. Histological diagnosis was confirmed by regional haemato-pathologists where tissue blocks were available, and classified according to the 2008 WHO classification as early, polymorphic, monomorphic or Hodgkin's disease (108). Multiple myeloma was excluded. The presence or

absence of EBV in tissue was confirmed using LMP-1 immunohistochemistry and / or EBER in situ hybridization. Recipient EBV serostatus was obtained from medical records where available, or by analysis for IgG antibodies to EBV VCA and EBNA-1 antigens in archived serum samples from the Regional Tissue Typing Laboratory. To access patient records at Salford Royal and Royal Preston approval was sought from the Caldicott guardian at these sites and honorary contracts were obtained from each trust to allow audit of patient records and clinical details. In each centre the clinical audit team was informed and provided local assistance in obtaining patient records. The renal physicians responsible for the care of the patients involved were also informed of the audit in each centre.

3.1.3.2 Ethical approval

Ethical approval was then obtained for review of all identified PTLD biopsy specimens for clarification of diagnosis in relation to the 2008 WHO PTLD classification including EBV tissue status (GM West NW REC 9 ref: II/NW/0001, Protocol 'Pathology of PTLD v1')(CMFT R and I pin R01441). Further approval (Ethical amendment) was also granted to allow testing of stored frozen serum samples obtained at time of transplant and at time of PTLD presentation for the presence of IgG antibodies against EBV antigens VCA and EBNA-1. Informed consent was obtained from all living subjects for review of tissue blocks and analysis of stored serum for EBV antibodies.

3.2 Suppliers and Manufacturers

Applied Biosystems	7 Kingsland Grange, Woolston, Warrington, WA1 4SR UK
BD Biosciences	2350 Qume Drive, San Jose, CA 95131
DiaSorin S.p.A	Strada per crescentino, 13040 Saluggia (Vercelli) Italy
Eurofins MWG Operon	Anzingerstr. 7a 85560 Ebersberg, Germany
Proimmune	The Magdalen Centre, Oxford Science Park, OX4 4GA
Qiagen	Qiagen house, Crawley, West Sussex, RH10 9NQ

3.2.1 Consumables and equipment

Applied Biosystems 7500 Fast Real-Time PCR system

ARTUS Real time EBV PCR diagnostic kit

BD FACS DIVA software

DiaSorin S.p.A LIAISON analyzer

Qiagen BioRobot MDx

3.2.2 Reagents and solutions

Taqman Fast Universal PCR Master Mix (2X), No Amperase UNG (Applied Biosystems).

BD Biosciences Multitest 6-color TBNK reagent

Proimmune Pro5® MHC Class I Pentamers

3.3 Molecular methods

3.3.1 Deoxyribonucleic acid (DNA) extraction

Extraction of Epstein-Barr virus DNA from EDTA whole blood research specimens was performed using the Qiagen BioRobot MDx. This is a fully automated process. In brief 300 µl samples are aliquoted into 2 mL bar code labeled tubes. The extraction procedure is as follows and taken from the departmental guidelines for the Qiagen BioRobot MDx (Abbott 2007).

"Samples are lysed in the presence of Protease and Buffer AL containing Carrier RNA and the Internal Control oligonucleotide. Ethanol is added to optimise binding conditions then the lysates are transferred to a QIAamp 96-well filter plate where free nucleic acids are bound to the filter membrane under vacuum pressure. The nucleic acids are purified by washing with buffer AW1 followed by buffer AW2 and finally ethanol under vacuum pressure. The plate is dried to remove any residual ethanol (which could inhibit downstream PCR) then the nucleic acids are eluted in buffer AVE, which is overlaid with Top Elute Fluid in order to enhance recovery."

3.3.2 Polymerase Chain Reaction (PCR)

3.3.2.1 Real-time Taqman assay

Real-time PCR assay methodology was based on the procedure described by Niesters et al in the Journal of Clinical Microbiology (Niesters, van Esser et al. 2000). An error was identified in the forward primer sequence reported by Niesters

et al. His paper reported a forward primer sequence (5'->3') GGA ACC TGG TCA TCC TTG C (19). For our experiments the EBV p143 forward primer with amended sequence (5'->3') GGA ACC TGG TCA TCC TTT GC (20) (100μM) and the EBV p143 reverse primer, sequence (5'->3') ACG TGC ATG GAC CGG TTA AT (20) (100μM) were used. The primers encode a protein, BNRF-1 p143, found in the tegument of the EBV virion, the area separating the capsid around the viral genome and the virion envelope. An EBV p143 probe was used with sequence (5'->3') CGC AGG CAC TCG TAC TGC TCG CT with a FAM reporter molecule at the 5 end and a TAMRA quencher at the 3 end. 25 ul volume reactions were used for PCR amplification. A 74 base pair DNA product was generated. For each reaction we used forward and reverse primer concentrations of 0.5 micromolar, 0.2 micromolar probe concentration, Taqman fast universal PCR master mix 2x (applied biosystems) and 5 μl of isolated DNA. Samples for EBV DNA testing after extraction were batched and frozen at -20^{0}c. These samples were thawed, remixed and centrifuged before each experiment. Reactions were performed in duplicate for each specimen and positive and negative controls were included in each run. An internal control was added to every sample at the start of the DNA extraction process to control for inhibitory factors. A pumpkin DNA plasmid was obtained from Eurofins, plasmid name pEX-A-IC DNA. Internal control primers had gene sequence. For the EBV quantification standard curve generation a 10 fold dilution series of the plasmid pEX-A-EBV standard Niest, gene size 78 base pairs, produced by Eurofins was used with concentrations from 10^8 to 10^3 copies/ml (Appendix 9.1). The positive control used was the 1st WHO International Standard

for Epstein-Barr Virus for Nucleic Acid Amplification Techniques (National institute for biological standards and control) NIBSC code: 09/260, "a whole virus preparation of the EBV B95-8 strain (type 1) [1,2], formulated in a universal buffer comprising Tris-HCl, human serum albumin (HSA) and trehalose". Negative control was nuclease free water. Runs were performed in 96 well optical reaction plates with optical caps. PCR amplification and detection was performed on the Applied Biosystems 7500 Fast Real-time PCR. The standard PCR cycling program consisted of 45 cycles of 15 s at 95°C and 60 s at 60°C.

Table 3.1 25 µl EBV Polymerase Chain Reaction mix

	Sequence 5'->3'			Supplier
Forward primer	GGA ACC TGG TCA TCC TTT GC (20)	0.5 µM	0.625 µl	Eurofins Mwg/operon
Reverse primer	ACG TGC ATG GAC CGG TTA AT (20)	0.5 µM	0.625 µl	Eurofins Mwg/operon
Probe (100 pmol/l)	CGC AGG CAC TCG TAC TGC TCG CT (23)	0.2 µM	0.05 µl	Applied biosystems UK
Taqman Fast master mix 2x			12.5 µl	Applied Biosystems UK
Nuclease free water			6.15 µl	
DNA			5µl	

3.4 Serological methods

Quantitative assessment of specific IgG antibodies to EBV Viral capsid antigen (VCA) and Epstein-Barr nuclear antigen-1(EBNA-1) in human serum was performed using the LIAISON indirect chemiluminescence immunoassay (CLIA) and was performed on the LIAISON analyzer. In brief after collection serum

samples were bar coded, batched and stored deep frozen at -20^{0}c. Following thawing and remixing specimens were introduced into the analyzer reaction module. 250 microlitres of serum was required for performance of the assay. Magnetic particles coated with EBV VCA antigen (p18 synthetic peptide) (solid phase) are then dispensed into the reaction. During an initial incubation VCA antibodies in the samples bind to this solid phase. In a second incubation an isoluminal-antibody conjugate (mouse monoclonal antibody to human IgG linked to an isoluminal derivative) reacts with these bound VCA antibodies. Wash cycles after the incubations remove unbound material. Starter kit reagents are then added thus inducing a chemilumiscence reaction. The light signal emitted reflects the quantity of isoluminal-antibody conjugate, and consequently the quantity of VCA IgG present in the sample, and is measured as Relative light units (RCU) (DiaSorin 2008). VCA antibody level is reported in U/ml with a range of 0-750. Antibody levels >20 U/ml for VCA are classified as positive and <20U/ml negative. If VCA antibodies are detected indirect chemiluminescence immunoassay is then performed for detection of IgG to EBNA-1 using the same methodology. Reference ranges for EBNA-1 include a range from 0-600 where <5 U/ml is negative and 5-20 U/ml an equivocal result.

3.5 Flow cytometer analyses

Whole blood (EDTA) samples were analysed for the frequency of CD3, CD4, CD8, CD19 and CD56 staining lymphocytes on a single spot assessment, using multicolour flow cytometry (Appendix 9.3). In brief for labeling of cell surface

markers 25 microlitres of whole blood was incubated with 10 microlitres of BD Bioscience 6-color T, B, NK reagent (combined monoclonal antibody) for 30 minutes at room temperature. BD FACS Fix/lysing solution was added with further sample incubation for 10 minutes. Samples were centrifuged and the supernatant removed and the cells resuspended in 0.3ml of 0.5% paraformaldehyde solution and analysed as soon as possible using a FACS CANTO II flow cytometer and BD FACS DIVA software.

3.6 Ultrasound examinations

Ultrasound examinations for detection of occult lymph nodes in the neck were performed by three accredited NHS sonographers in the ultrasound department at Manchester Royal Infirmary using service machines. High resolution ultrasound was performed using a high-frequency transducer (>7.5MHz). A protocol was generated whereby systematic screening of submental, submandibular, parotid, cervical, supraclavicular fossa and posterior triangle lymph node chains was performed. Site and size of of nodes >5mm (short-axis diameter) were reported (Appendix 9.2).

3.7 Statistical methods

Statistical methods are given in brief below for studies involving both EBV DNAemia in stable adult kidney transplant recipients and for incidence and outcomes of transplant recipients with PTLD. More detailed statistical information is

given in the methodology section of each paper presented in the results section. In all studies Groups were compared using mean and standard deviation for normally distributed variables and median and interquartile ranges for skewed data. Group comparisons used Chi-square and Fishers exact testing for categorical variables and Students 2 sided t-test for normally distributed continuous variables and Mann-Whitney for non-parametric data. Statistical analyses were performed using SPSS.

3.7.1 Epstein-Barr virus infection in adult renal transplant recipients

Logistic regression was used to estimate the odds ratios and 95% CI for dichotomised factors, both unadjusted and allowing for time from transplant, as a cubic function (the functional form was selected on the basis of the Akiake information criteria in a preliminary analysis). These analyses were conducted in R v2.15.

Participants were followed until 22nd February 2013, with subsequent analysis of patient and graft survival, graft function, and development of PTLD in relation to EBV DNA detection at recruitment and subsequent EBV DNAemia patterns. Kaplan-Meier survival curves and log-rank tests were used to analyse differences between groups. Individuals with a history of PTLD prior to recruitment were excluded from analyses of PTLD development during the follow up period (Graphpad Prism 5 software).

3.7.2 Post-transplant lymphoproliferative disorder in adult renal transplant recipients

We calculated the incidence of PTLD in our adult transplant population as number of cases over number of patient years at risk and then as cases per 1000 patient years. Poisson distribution 95% confidence intervals were calculated for the estimated rates. Incidence of PTLD in our adult transplant population was compared with age standardised rates of Hodgkin's lymphoma and non-Hodgkin's lymphoma in the general population (aged greater than 19 years) in England (total population n=39,104,000) for the calendar year 2008. Lymphoma incidence in the general population was obtained for ICD 10 code C81 (Hodgkin's) and ICD codes C82-85 and 96 (non-Hodgkin's) from the North West Cancer Intelligence Service (personal communication) and UK Cancer Intelligence Service report (2011) for cases occurring in 2008 and registered by September 2010 (16).

Group comparisons between early and late onset presentations and disease presenting in EBV seronegative and seropositive recipients used Chi-square testing for categorical variables and Students 2 sided t-test for continuous variables.

Kaplan-Meier time to event curves were used to graphically illustrate the cumulative time to presentation for EBV seronegative and seropositive recipients (Fig 4.3.2a) and tissue EBV positive and tissue EBV negative recipients (Fig 4.3.2b). Comparison of the curves used the log-rank test. Univariate analysis of factors associated with survival with adjustment for performance status and Ann Arbor stage, (since these parameters are known to affect outcomes), was

performed using Kaplan-Meier plots and the log-rank test. A p value of <0.05 was accepted as significant. Statistical analyses were performed using SPSS (IBM SPSS Statistics 20.0.1 March 2012) and Graphpad Prism 5 software.

4.0 Results

4.1 Epstein-Barr Virus Infection in Adult Renal Transplant Recipients

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Running title: EBV in Renal Transplantation

Key words: Epstein-Barr Virus, PTLD, renal transplantation, immunosuppression

Abbreviations:

ATG	anti-thymocyte globulin;
CI	confidence interval;
EBNA	Epstein-Barr nuclear antigen-1;
EBV	Epstein-Barr virus;
eGFR	estimated glomerular filtration rate
HR	hazard ratio
HL	Hodgkin's lymphoma;
IQR	inter-quartile range
LDH	lactate dehydrogenase
MMF	Mycophenolate Mofetil
NHL	non-Hodgkin's lymphoma;
NICE	National institute for clinical excellence;
OR	odds ratio
PTLD	post-transplant lymphoproliferative disorder
Sd	Standard Deviation
VCA	viral capsid antigen

4.1.1 Abstract

Epstein-Barr virus (EBV) DNAemia in the first year post-organ transplantation has been studied extensively. There is a paucity of data on the prevalence and sequelae of EBV infection in adult renal transplantation beyond the first year. However, Post-Transplant Lymphoproliferative Disorder (PTLD) in this population tends to present later, with 50% of later cases associated with EBV infection. This single-centre study examines the relationship between EBV DNAemia and demographic, immunosuppressive, haematologic, and infection-related parameters in 499 renal transplant recipients between 1 month and 30 years post-transplant. Participants were tested repeatedly for EBV DNAemia detection over 12 months and clinical progress followed for 3 years. Stratifying the group into detectable or undetectable EBV DNAemia at recruitment, in an analysis adjusted for time from transplant, 5 variables showed significance; EBV seronegative status at transplant ($p=0.014$), non-white ethnicity ($p=0.012$), mycophenolate mofetil use ($p<0.0001$), lymphopenia $<1x10^9$ ($p=0.045$), and PTLD diagnosis ($p=0.002$). Neither patient nor graft survival was significantly different between the 2 groups. The rate of decline of kidney function was not associated with either EBV DNAemia at recruitment or the persistence of EBV DNAemia. EBV DNAemia at recruitment was predictive of PTLD during the follow-up period, with a hazard ratio of 6.2 (CI=1.1-36.0, $p=0.04$).

4.1.2 Introduction

Current guidelines recommend screening for EBV DNA in blood in high risk recipients for the first year after transplantation (151,207–209). In these patients pre-emptive reduction of immunosuppression and treatments such as rituximab may reduce the incidence of early EBV related disease and PTLD (146,164,182). In adult kidney transplant recipients PTLD is a late event with only 15% of cases in our centre presenting during the first year (104,210). The prevalence of detectable EBV DNA in those attending follow up clinic in the late post-transplant period is unclear, and the logistics of screening and monitoring for EBV DNA in blood in these patients challenging. In those with late detectable EBV DNA and no current evidence of clinical disease or PTLD, the implications of DNAemia are uncertain. Therefore, we performed a prospective observational study in 499 adult kidney transplant recipients, clinically stable at the time of recruitment, of which 92% were beyond the first post-transplant year. We analysed the prevalence of EBV DNAemia at recruitment, and subsequent patterns of DNAemia over a one-year period. Factors associated with EBV DNAemia, patient and graft survival, and the incidence of PTLD were analysed after 3 years of follow up.

4.1.3 Methods

4.1.3.1 Patient group

Kidney transplant recipients followed up at Manchester Royal Infirmary between 22nd February 2010 and 13th September 2010 were invited to join the study. Participants were between 1month and 33 years post-transplantation. Inclusion

criteria were age >18 years, the presence of a functioning graft, and a recent stable clinical course.

4.1.3.2 Sampling and PCR

Whole blood EDTA was collected and analysed for the presence of EBV DNA at recruitment and at 3 monthly intervals for 1 year. DNA extracts were batched and stored at -80^{0}C, and a quantitative real-time PCR assay with the target gene BNRF-p143 (205), was used with the Applied Biosystems 7500 Fast Real-time PCR (Appendix 9.1). The threshold of sensitivity for detection of EBV DNA was 1000 copies/ml blood. Samples with no detectable EBV DNA, or concentrations measured at <1000 copies/ml, were classified as undetectable. Blood samples were also taken at recruitment for analysis of EBV serostatus (the presence of IgG antibodies to EBV VCA and EBNA-1 antigens (U/ml)) using the DiaSorin S.P.A LIAISON analyzer**.** Demographic, transplant and immunosuppressive details were obtained from the medical records at the time of recruitment.

4.1.3.3 Symptoms assessment

All recruited individuals were invited to complete a survey of clinical symptoms associated with EBV and PTLD including fever, night sweats, weight loss, loss of appetite, sinus congestion, anorexia, swollen glands, sore throat, loss of appetite and abdominal pain (152). Physician assessed Karnofsky and ECOG scores were completed at baseline for each individual.

4.1.3.4 Patterns of EBV DNAemia

Prevalence of EBV DNAemia was calculated for the study population at recruitment (baseline), and then patterns of EBV DNAemia over time analysed for

recruited individuals with 3 or more samples, obtained over a period of greater than 6 months (446 individuals). We assigned individuals to one of 4 classes, including those with no detectable DNA (NDNA), transient detection (TDNA)(<75% samples with detectable DNA), persistent DNAemia (PDNA) (≥75% samples with detectable DNA), and chronic high viral load carriage (CHVL) (≥3 samples over >6 months with ≥10,000 copies/ml DNA)(161)(206). Individuals with <3 samples (n=53) were excluded from this analysis.

4.1.3.5 Analysis of factors associated with EBV infection

Analysis was made of factors predicted to be associated with the presence of EBV DNAemia including age and time from transplant, EBV recipient serostatus at transplantation, immunosuppressive agent exposure, rejection history, biochemical and haematological parameters, including white cell differential and renal graft function, and post-transplant complications including opportunistic infections and non-melanoma skin cancer.

4.1.3.6 Statistics

Groups were described using mean and standard deviation or, where the data were skewed, median and interquartile ranges. Group comparisons used Fishers exact test for categorical variables and t-tests or Mann-Whitney for ordinal data. Statistical analyses were performed using SPSS (IBM SPSS Statistics 20). Logistic regression was used to estimate the odds ratios and 95% CI for dichotomised factors, both unadjusted and allowing for time from transplant, as a cubic function (the functional form was selected on the basis of the Akiake

information criteria in a preliminary analysis). These analyses were conducted in R v2.15.

Participants were followed until 22nd February 2013, with subsequent analysis of patient and graft survival, graft function, and development of PTLD in relation to EBV DNA detection at recruitment and subsequent EBV DNAemia patterns. Kaplan-Meier survival curves and log-rank tests were used to analyse differences between groups. Individuals with a history of PTLD prior to recruitment were excluded from analyses of PTLD development during the follow up period (Graphpad Prism 5 software).

4.1.3.7 Ethical approval

Research Ethics Committee (REC) review and approval for the study (Greater Manchester NEREC 09/H1013/71) was obtained. Written informed consent was obtained from all subjects participating in the study.

4.1.4 Results

We recruited 499 stable transplant recipients with a mean age of 51 years (range 20-81) and a median time from transplantation at recruitment of 7 years (IQR=3-12). The majority, 81%, were first transplant recipients with 62% male gender and 93% of white ethnicity. Demographic and transplant details are shown in Table 4.1.1.

Table 4.1.1 Patient demographic and transplant details

Characteristic	All patients n=499
	N %*
Mean age (diagnosis) (sd) (range)	51.4 (13) (20-81)
Median time from transplantation (years) (IQR)	7 (3-12)
Transplant history	
Ist transplant	404 (81)
Transplant number 2,3	94 (19)
Male gender	310 (62)
Ethnicity	
White	465 (93)
Black	8 (2)
Asian	26 (5)
Female	189 (38)
Median Creatinine (IQR)	133 (107-173)
Median egfr (IQR)	44.3 (33-57)
Time of transplantation Serology	
EBV Recipient Seronegative	54 (12)
EBV Recipient Seropositive	397 (88)
EBV data missing	48
CMV Recipient seronegative	146 (50)
CMV Recipient seropositive	148 (50)

CMV data missing	205
Cause of ESRD	
Diabetes Mellitus	58 (12)
Polycystic	85 (17)
GN/Autoimmune	142 (28)
Reflux/CPN/Cong	109 (22)
Hypertension/Renovascular	29 (6)
Other/unknown	76 (15)
Basiliximab induction	225 (45)
Treated rejection	105 (21)
ATG (rejection)	32 (6.4)
PTLD diagnosis prior to recruitment	22 (4)
Treated CMV disease	68 (14)
Current Diabetes Mellitus	95 (19)
Immunosuppression at recruitment	
Tacrolimus	278 (56)
Ciclosporin	127 (25)
CNI containing	405 (81)
Monotherapy	109 (22)
Dual therapy	276 (56)
Triple therapy	110 (22)
Azathioprine	110 (22)
Mycophenolate mofetil	205 (41)
Sirolimus	12 (2)
Prednisolone	265 (53)

*Number and percentage are given for each characteristic unless otherwise stated.

4.1.4.1 EBV serostatus

At recruitment 441 (90%) of individuals were EBV seropositive, with detectable EBV antibodies to Viral Capsid Antigen (VCA); 49 (10%) were seronegative, 9 had no serological analysis performed, and 110 (22%) had detectable VCA antibodies, but were Epstein-Barr Nuclear Antigen (EBNA) antibody negative or equivocal.

4.1.4.2 Symptoms assessment

Results from the survey of clinical symptoms associated with EBV and PTLD, including fever, night sweats, weight loss, loss of appetite, sinus congestion, anorexia, swollen glands, sore throat, loss of appetite and abdominal pain showed no detectable differences between those patients with persistently undetectable, persistently detectable, or high EBV viral loads. Overall survival was significantly lower in those with symptoms at recruitment which included weight loss (86.4 v 96.3%, p=0.002), loss of appetite (90.7 v 96%, p=0.011), ECOG performance status ≥2 (88 v 96.1%, p=<0.0001), and Karnofsky score <80 (88.4% v 96.3%, p=<0.0001).

4.1.4.3 Prevalence and patterns of EBV DNAemia

EBV DNA was detectable (>1000 copies/ml blood) in 153/499 (31%) individuals and undetectable in 346 (69%) at baseline assessment. Viral loads were of a magnitude of >1000 copies/ml in 94 (19%), >10,000 in 47 (9%) and ≥100,000 in 12 (2%) individuals. During the 1 year study period a total of 232/499 (46%) of individuals had 1 or more samples with EBV DNA ≥1000 copies/ml.

A median number of 4 samples, range 3-10, was obtained from each patient with a median time of 113 days (IQR=70-138 days) between sampling points. Patterns of

DNAemia are shown in Table 2. In the 53/499 patients with insufficient samples for pattern analysis, reasons for inadequate sampling included death (n=10), return to base hospital (n=23), return to dialysis (n=2), and missed sampling/lost to follow up (n=18).

Table 4.1.2 Patterns of EBV DNAemia

Pattern of DNAemia	N (%) n=446
No detectable DNA (NDNA)	234 (52)
Transient detection (TDNA)	139 (31)
Persistent DNAemia (PDNA)	42 (9)
High viral load carriage (HVL)	31 (7)

Patterns of EBV DNAemia observed in 446 individuals with 3 or more samples analysed for EBV DNA over a period greater than 6 months

The proportions of patients with detectable EBV DNA at recruitment who also had detectable DNA at the end of the sampling period are shown in Table 4.1.3. The higher the initial viral load the more likely the individual was to have a detectable viral load at the final sampling point, (OR=5.9, 95%CI 2.7-12.8, p=<0.0001), for those >10,000 (log 4) copies/ml v 1000 (log 3) copies/ml.

Table 4.1.3 Recruitment EBV DNAemia status and subsequent DNAemia status at study end

EBV DNAemia (n=446) At recruitment	N	EBV DNAemia At end of study	N (%)
Detectable	135	Detectable	67/135 (50%)
Detectable log 3-3.9	83	Detectable	28/83 (34%)
Detectable log 4-4.9	42	Detectable	30/42 (71%)
Detectable log 5+	10	Detectable	9/10 (90%)
Undetectable	311	Undetectable	290/311 (93%)
Undetectable	311	Detectable	21/311 (7%)

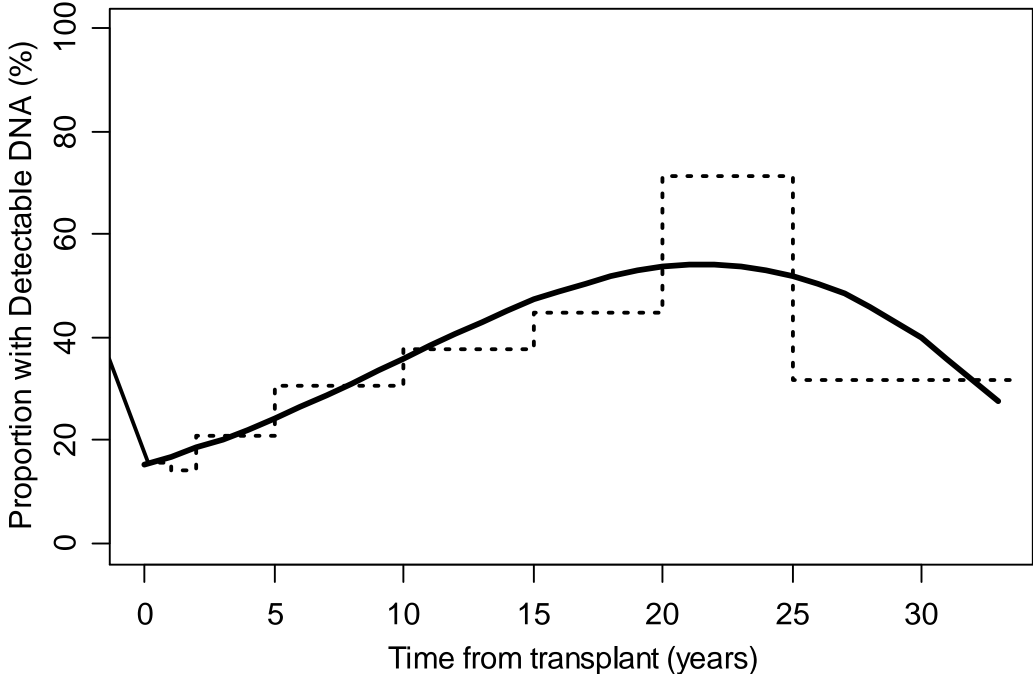
At recruitment, 49 individuals were EBV seronegative (undetectable VCA antibody), 9 of whom had detectable EBV DNA, and 4 had persistent or chronic high viral loads during the study period.

4.1.4.4 Influence of time from transplant on EBV DNA detection

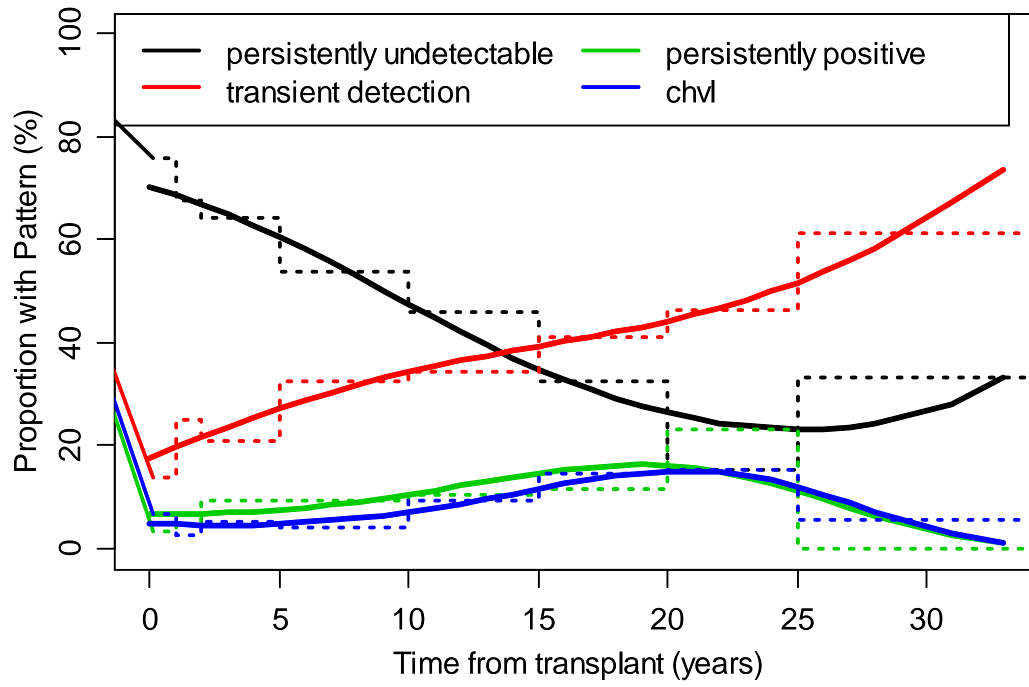
The prevalence of EBV DNAemia increased significantly with time from transplant (Figure 1) from 16% (6/38) in the first year, to 40% (44/111) in the 10th to 14th years, to 66% (19/29) in the 20th-24th years, with an odds ratio of 2.8 (CI 1.9-4.1) for DNAemia for those more than 10 years post-transplant v those <10 years (p=<0.0001). A fall in DNAemia rates was noted in the small number of individuals transplanted for more than 25 years. A similar pattern of increasing prevalence with time was seen in those with transient EBV detection (p=0.001) while proportions of those with persistent detection and chronic high viral load carriage increased over

the first 20 years, but remained less than 15% of all individuals (Figure 4.1.1b). Proportions of those with persistently undetectable EBV levels fell significantly with increasing time from transplant (p=<0.0001).

4.1a



4.1b



4.1C

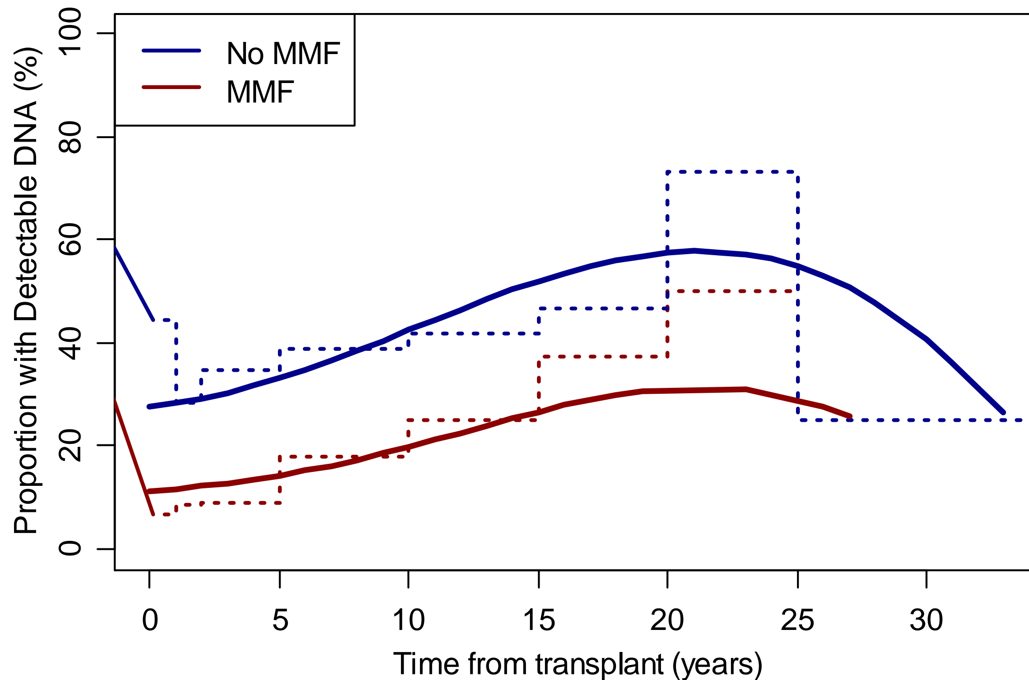


Figure 4.1.1 EBV DNAemia rates with time from transplant

a: detectable EBV DNA in the study population b: patterns of EBV DNAemia during follow up, chvl = chronic high viral load carriage. c: proportion of patients with detectable EBV DNA treated with and without MMF.

Stepped lines show mean values in 5-year intervals and the solid lines a cubic logistic regression fit to the data.

4.1.4.5 Analysis of factors associated with EBV infection

Table 4.1.4 shows the results of a univariate analysis of demographic, treatment, and infection variables in relation to EBV DNAemia at recruitment. Results of adjustment for time from transplant using logistic regression analysis from a cubic model demonstrated that individuals who were EBV seronegative at time of

transplant, those with a diagnosis of PTLD, and those with non-white ethnicity had significantly greater rates of DNAemia. Those with a lymphocyte count <1.0 $x10^9$/L and current MMF had significantly lower rates of DNAemia (p<0.0001) (Figure 4.1.1c), while the effect of other immunosuppressive agents was lost. Other factors, particularly those predicted to be associated with time, including warts and skin cancer, did not remain significantly associated with EBV DNAemia.

In further analysis of lymphocyte counts in relation to immunosuppressive agents, individuals on MMF and azathioprine had significantly lower lymphocyte counts (median 1.27 $x10^9$/L, IQR=0.88-1.66, p<0.0001) than those receiving calcineurin inhibitors. Individuals on MMF were not significantly more lymphopenic than those on azathioprine.

Table 4.1.4 Factors associated with detection of EBV DNA at recruitment: Univariate and time from transplant adjusted analyses

Variable	Detectable DNA n (%)		Univariate analysis		Time from Transplant adjusted analysis*	
	Variable present	Variable absent	OR 95% CI	P	OR 95% CI	P
Age >mean (51 yrs)	79/264 (30)	74/235 (32)	0.9 (0.6-1.4)	0.78	0.8 (0.6-1.2)	0.37
Time from Transplant >10 years	90/207 (44)	63/292 (22)	2.8 (1.9-4.1)	**<0.0001**	1.0 (0.5-2.3)	0.94
Male Gender	88/310 (28)	65/189 (34)	0.8 (0.5-1.1)	0.19	0.8 (0.5-1.2)	0.27
Non-white ethnicity	15/34 (44)	138/465 (30)	1.9 (0.9-4.0)	0.085	2.6 (1.2-5.4)	**0.012**
Non diabetic	123/441 (34)	19/58 (20)	2.0 (1.2-3.5)	**0.014**	1.6 (0.9-2.9)	0.096
EBV seronegative	27/54 (50)	110/397 (28)	2.6 (1.5-4.7)	0.001	2.1 (1.2-3.9)	**0.01**

Transplant no >1	31/94 (33)	122/404 (30)	1.1 (0.7-1.8)	0.62	1.1 (0.7-1.9)	0.63
Treated rejection	40/105 (38)	113/394 (29)	1.5 (1-2.4)	0.074	1.4 (0.9-2.2)	0.18
ATG	11/32 (34)	142/467 (30)	1.2 (0.6-2.6)	0.693	1.5 (0.2-5.6)	0.67
ECOG ≥2	30/89 (34)	120/410 (30)	1.2 (0.7-1.9)	0.525	1.2 (0.7-1.9)	0.58
Karnofsky <80	34/95 (36)	116/404 (29)	1.3 (0.8-2.2)	0.218	1.3 (0.8-1.2)	0.27
Basiliximab induction	41/225 (18)	108/274 (40)	0.33 (0.2-0.5)	**<0.0001**	0.7 (0.3-1.4)	0.31
Monotherapy	45/109 (41)	107/390 (28)	1.9 (1.2-2.9)	**0.0095**	1.5 (0.9-2.3)	0.11
Dual therapy (vTriple)	87/276 (32)	20/110 (18)	2.1 (1.2-3.6)	**0.012**	1.0 (0.6-1.4)	0.82
Triple therapy	20/110 (18)	132/479 (34)	0.3 (0.2-0.4)	**<0.0001**	0.7 (0.4-1.2)	0.15
Current tacrolimus	67/278 (24)	86/421 (39)	0.5(0.3-0.7)	**0.0004**	1.0 (0.6-1.4)	0.98
Current ciclosporin	54/127 (42)	99/372 (27)	2.0 (1.3-3.1)	**0.0012**	1.2 (0.8-2.0)	0.36
Current MMF	31/205 (15)	122/294 (41)	0.3 (0.2-0.4)	**<0.0001**	0.3 (0.2-0.5)	**<0.0001**
Current azathioprine	41/110 (37)	112/389 (29)	1.5 (0.9-2.3)	0.101	1.3 (0.8-2.0)	0.33
Current prednisolone	85/265 (32)	68/234 (29)	1.2 (0.8-1.7)	0.497	1.1 (0.7-1.6)	0.67
Current sirolimus	3/12 (25)	150/487 (31)	0.7 (0.2-2.8)	0.999	0.6 (0.2-2.4)	0.50
PTLD Diagnosis	15/22 (68)	134/477 (28)	5.3 (2.3-12.0)	**<0.0001**	3.7 (1.6-8.7)	**0.002**
CKD 4 or 5	37/100 (30)	113/399 (28)	1.0 (0.6-1.5)	0.999	1.0 (0.6-1.7)	0.90
CKD 1-2	31/105 (32)	119/394 (31)	1.0 (0.7-1.7)	0.903	1.3 (0.8-2.1)	0.32
Hb < 11.5	44/121 (36)	106/378 (20)	1.4 (0.9-2.4)	0.174	1.3 (0.8-2.1)	0.23
Leucopenia	9/24 (38)	141/490 (31)	1.3 (0.6-3.1)	0.503	1.4 (0.6-3.5)	0.43
Raised ALT	4/29 (14)	146/458 (32)	0.3 (0.1-1.0)	**0.04**	0.5 (0.2-1.5)	0.20
Thrombocytopenia	14/42 (33)	136/438 (31)	1.1 (0.6-2.2)	0.73	1.3 (0.6-2.6)	0.48
Lymphopenia $<1.5x10^9$	80/290 (28)	73/209 (35)	0.7 (0.5-1.0)	0.094	0.8 (0.5-1.1)	0.18
Lymphopenia$<1x10^9$	25/110 (23)	128/389 (33)	0.6 (0.4-1.0)	**0.046**	0.6 (0.4-1.0)	**0.045**
Lymphocytosis	1/3 (33)	152/496 (31)	1.1 (0.02-21.9)	0.99	1.3 (0.1-15.9)	0.83
Neutropenia	6/22 (27)	147/477 (31)	0.8 (0.3-2.2)	0.82	1.1 (0.4-3.0)	0.86
Previous PCP	4/10 (40)	149/489 (30)	1.5 (0.5-5.4)	0.50	1.3 (0.3-4.7)	0.74

Previous CMV	18/68 (26)	135/431 (31)	0.8 (0.4-1.4)	0.48	0.9 (0.5-1.7)	0.85
Non mel. skin cancer	32/76 (42)	121/423 (29)	1.8 (1.1-3.0)	**0.03**	1.3 (0.8-2.3)	0.30
Warts	45/115 (39)	108/384 (28)	1.6 (1.1-2.5)	**0.029**	1.2 (0.8-2.0)	0.41
HSV oral	34/113 (30)	119/386 (31)	1.0 (0.6-1.5)	0.908	0.9 (0.5-1.4)	0.52
Shingles	28/73 (38)	125/426 (29)	1.5 (0.9-2.5)	0.132	1.3 (0.8-2.3)	0.30
Primary varicella	5/13 (39)	148/486 (31)	1.4 (0.5-4.4)	0.550	0.8 (0.2-2.8)	0.74
Pulmonary TB	2/4 (50)	151/495 (30)	2.3 (0.3-16.3)	0.590	2.3 (0.3-17.2)	0.42
Extra pulmonary TB	3/3 (100)	150/496 (30)	(0-Inf)	**0.028**	(0-Inf)	0.97
BK viraemia	5/19 (26)	148/480 (31)	0.8 (0.3-2.3)	0.803	1.4 (0.5-4.0)	0.56

** cubic model*

4.1.4.6 Factors associated with high viral load carriage

Factors associated with high viral load carriage versus persistently undetectable EBV DNA levels included: time from transplant >10 years (18/32 v 61/234, OR=3.6, CI 1.7-7.8, p=0.0008), recipient EBV seronegative status at transplantation (11/31 v 19/218, OR=5.8, CI 2.4-13.8, p=0.0002, current ciclosporin use (15/58 v 16/215, OR=4.2, CI 1.9-9.0, p=0.0007), and VCA antibody levels ≥750 U/ml, (22/31 v 68/233, OR=5.9, CI 2.6-13.5, p<0.00001). Low prevalence of high viral load detection was associated with basiliximab induction, (7/136 v 23/125, OR=0.2, CI 0.1-0.6, p=0.001), current tacrolimus, (13/167 v 18/98, OR=0.4, CI 0.2-0.8, p=0.01), current MMF, (2/132 v 29/133, OR=0.06, CI 0.01-0.20, p<0.0001) and lymphopenia <$1x10^9$/L at recruitment (1/48 v 30/217, OR=0.1, CI 0.02-1.0, p=0.02).

4.1.4.7 Patient and graft survival

Three year patient survival is shown in Figure 4.1.2a and was not significantly different between those with detectable EBV at recruitment and those without (HR 1.84, CI 0.76-4.48, p=0.18). Graft loss with subsequent return to dialysis occurred in 11 patients during follow up with no significant association with EBV detection at recruitment (p=0.10). Kidney function (eGFR) declined significantly over the study period for the population, -3.68 ml/min/1.73m^2, p<0.0001, but the rate of decline was not associated with EBV DNA detection status at recruitment, or the pattern of EBV DNAemia.

Figure 4.1.2a

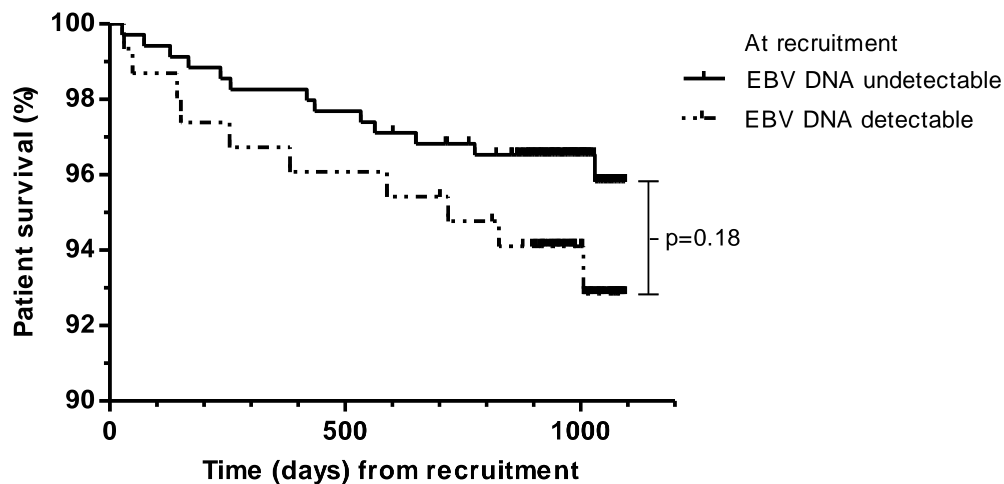


Figure 4.1.2b

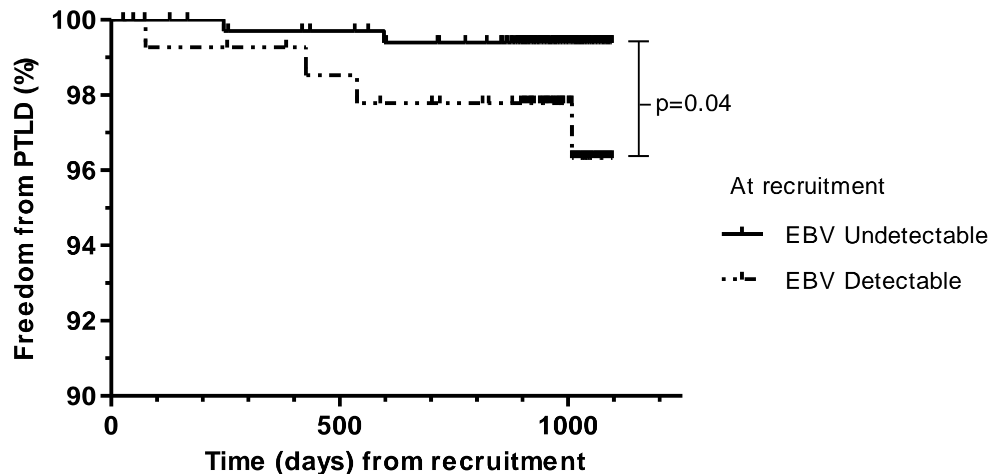


Figure 4.1.2 Overall patient and PTLD free survival during study follow up

Figure 4.1.2a: Overall patient survival during the study follow-up, undetectable EBV DNAemia vs detectable EBV DNAemia at recruitment, HR=1.84, CI=0.76-4.48, p=0.18. Figure 4.1.22b: Freedom from PTLD during the study follow-up, undetectable EBV DNAemia vs detectable EBV DNAemia at recruitment, HR=6.2 CI=1.1-36.0, p=0.04. Kaplan-Meier survival curve and log-rank test

4.1.4.8 Incidence of PTLD

PTLD (first presentation) was diagnosed in 6 individuals during the 3 year follow-up period. Freedom from PTLD in those with detectable EBV at recruitment was 96.6% compared to 99.4% with no detectable EBV (HR=6.2, CI=1.1-36.0, p=0.04) (Figure 2b). Characteristics of the cases are presented in Table 4.1.5. All PTLD cases were EBV seropositive, although EBNA antibody deficiency was seen in 4/6

(67%) compared to 22% of individuals in the study population overall (OR= 6.0, CI 1.0-33.3, p=0.039). Histology was negative for EBV in patients 1 and 2 who had persistent EBV DNAemia during the sampling period, and one of whom also had undetectable EBV in blood at diagnosis.

Table 4.1.5 Characteristics of PTLD cases diagnosed during the study period

Patient No.	Age	Time from Transplant (years)	EBV Serostatus Pre-Tx*	EBV VL$^{\alpha}$ Recruitment	EBV DNAemia pattern	PTLD WHO Class	PTLD EBV$^{\infty}$ status	EBV VL$^{\beta}$ PTLD
1	58	3	Unk	3.4	PDNA	DLBCL	-	Undet
2	74	23	Positive	4.3	PDNA	Mono	-	3.9
3	66	19	Positive	Undet	TDNA	DLBCL	+	3.0
4	37	16	Negative	3.8	CHVL	HL	+	3.7
5	67	15	Negative	Undet	NDNA	Poly	-	Undet
6	59	20	Positive	5.3	TDNA	DLBCL	+	3.4

*EBV recipient pre-transplant serostatus. $^{\alpha}$ EBV viral load at study recruitment log10 copies/ml. $^{\infty}$ EBV status of PTLD tissue at histological examination (In situ hybridisation for EBER)- not detected, + EBER detected. $^{\beta}$ EBV viral load at time of PTLD diagnosis log10 copies/ml.

On univariate analysis, freedom from PTLD during the study period was associated with time from transplant (97.3% ≥10 years v 99.3% ≤10 years, p=0.026), persistent EBV DNAemia (92.4% v 99%, p=0.035), detection of EBV at recruitment (96.6% v 99.4%, HR=6.2, CI 1.1-36.0, p=0.043), previous PCP infection (90% v 98.7%, p=0.015), history of warts (93.5% v 100%, p=<0.0001), history of non-melanoma skin cancer (94.6% v 99.2%, p=0.021), history of gum hypertrophy

(75.5% v 99.1%, p=<0.0001), and EBV EBNA antibody deficiency (96% v 99%, p=0.014).

A previous history of PTLD (65% EBV tissue positive) was recorded in 22 individuals at recruitment of whom 15/22 (68%) had EBV DNAemia. Patterns of EBV DNAemia during the study in 17/22 patients with ≥3 samples included NDNA in 6/17 (35%) (including 4/6 with a history of EBV negative PTLD), PDNA in 5/17 (29%) (all EBV tissue positive disease) and 2 high viral load carriage (both EBV positive disease, 1 polymorphic, 1 infectious mononucleosis early lesion). Relapse of PTLD was seen in three individuals, including a late relapse 6.4 years after diagnosis in one patient with a history of EBV negative diffuse large B cell lymphoma. The recurrent disease was also EBV negative DLBCL with no detectable EBV DNA in blood.

4.1.5 Discussion

Clinical guidelines and recent studies of EBV infection focus predominantly on the incidence and implications of viraemia in the first post-transplant year (208). Screening for EBV DNA during the 1st year is recommended particularly for high risk EBV donor seropositive recipient seronegative patients. There is little data reporting the prevalence, patterns, and outcomes of EBV infection in stable adult renal transplant recipients beyond the first year. Such data is important because most PTLD in this population presents late, and over 50% of late cases are associated with EBV infection (104,210). EBV seronegative recipients appear to have a higher risk of PTLD beyond the first post-transplant year (104,210). In

addition there is conflicting data relating to the associations of EBV DNAemia with PTLD development, graft dysfunction and loss, acute rejection, opportunistic infection, adverse events, risk of death and overall burden of immunosuppression (146,161,164,165,168,211,212).

Recently Bamoulid, Holmes and Holman reported, in adult renal transplant recipients, EBV DNAemia rates in the first year from 30-56% (117,146,206). High DNAemia rates during the first year might be expected as immunosuppression levels are high and seronegative recipients experience primary infection during this period. Falling rates of detection with increasing time from transplant time have been reported in heart and lung transplant recipients (25). We report a prevalence of EBV DNAemia (>1000 copies/ml) at recruitment of 31%, with 46% of individuals having 1 or more samples with detectable DNA during the study year. We show that prevalence and persistence of DNAemia appears to increase, rather than fall, with time from transplant. However, such studies suffer from selection effects, with the prevalence rates being conditional on survival. In the present data the late fall could be due to a survival benefit of low viral loads. Further, DNAemia was associated with factors reflective of prolonged immunosuppression exposure such as warts, non-melanoma skin cancer, and immunosuppressive regimens including ciclosporin and monotherapy, while more recently transplanted individuals receiving basiliximab induction (since 2004) tacrolimus and triple therapy had a lower prevalence of DNAemia. EBV seronegative recipients in our analyses had significantly greater prevalence of DNAemia and chronic high viral load carriage than seropositive recipients.

In analyses adjusted for time from transplant, MMF was the only agent with a significant association with DNAemia rates. Reduced incidence of DNAemia in those receiving MMF has been noted by other studies (117,146,213). It has been suggested that this may be due to an anti-B cell effect, reducing the EBV-carrying B lymphocyte population (117). Treatment with rituximab is associated with transient depletion of B cell numbers with corresponding falls in EBV viral loads, and studies have also reported subsequent rises in viral loads as B cell numbers are later reconstituted (146,214,215). Individuals on MMF in our study had lower total lymphocyte counts than others. However, we found no significant difference in mean lymphocyte counts between MMF and azathioprine treated individuals, though both groups had lower lymphocyte counts than those not on anti-metabolite/proliferatives. Analysis of B cell numbers in relation to EBV viral loads in larger numbers of patients on MMF may be helpful. These observations raise the following questions. Does the observed lower risk of EBV DNAemia in MMF treated patients correspond to a reduced risk of EBV positive PTLD? Should we consider changing asymptomatic individuals with high level EBV DNAemia to MMF, and should low dose MMF with steroids be the maintenance agents of choice in new cases of EBV positive PTLD, rather than low dose calcineurin inhibitors? PTLD risk registry data suggests that MMF treated individuals may have a reduced incidence and risk of PTLD development (123,124,128,216).

In the event of detection of EBV DNAemia in a stable patient, the likelihood of persistence of DNAemia appears to relate to the level of EBV DNA and EBV seronegative recipient status (117,146,168). The higher the viral load at

recruitment in our study the greater the proportion with EBV DNA at 3 months and at the end of the study period, including 54% with >1000 copies/ml, and 84% >10,000 copies/ml, while 88% those with initially undetectable levels remained free of EBV detection.

The implications of EBV DNAemia in an otherwise stable, and often well, adult renal transplant recipient in the late post-transplant period is unclear. EBV is a latent herpes virus and after primary infection it is anticipated that a steady state will be reached, where numbers of EBV infected B cells are controlled by effective EBV specific T cell responses (18). EBV DNA in blood is found in healthy members of the general population, although typically at significantly lower levels than following transplantation (117,217). Detection of viral DNA such as cytomegalovirus and BK polyoma virus, often in the first 1-2 years after transplant, and particularly if >1000 copies/ml, is typically associated with clinical symptoms and end organ damage, and requires medical intervention. Does the detection of late EBV DNAemia identify those with a clinical illness requiring treatment and/or those who are over-immunosuppressed? In our study those with DNAemia did not have significantly greater rates of anaemia, thrombocytopenia or raised liver enzymes (alanine transaminase) (Table 4); nor did they have poorer kidney function, or greater rates of graft function decline or loss. Interestingly, we detected no significant difference in reported clinical symptoms, including those associated with EBV disease, such as B symptoms, sore throat, swollen glands, abdominal pain, or fatigue. Further, triple immunosuppressive therapy was associated with lower rates of DNAemia than dual or monotherapy. No significant difference in

rates of DNAemia was seen in relation to previous ATG use, or ciclosporin or tacrolimus trough concentrations. Neither did we detect any significant difference in rates of lymphopenia, lymphocytosis or neutropenia, or historical or current CMV or BK infection. However, in support of an 'over-immunosuppressed' argument, recent studies have reported associations between EBV DNAemia in the first year after transplant and opportunistic infections, adverse events, and in some studies, greater rates of graft loss and graft dysfunction (145,146,168,179,213). In the Bamoulid report there is also the concern raised that pre-emptive reduction of immunosuppression for DNA levels persistently >10^4 log/ml copies may have influenced subsequent graft loss (146). In our study we show that EBV infection is associated with a history of non-melanoma skin cancer, warts, and extra-pulmonary TB (albeit small numbers). We found no significant association with patient survival after 3 years of follow up between those with DNAemia (93%) at recruitment and those without (96%)(p=0.18), but survival was significantly better amongst those with persistently undetectable EBV DNA levels, although this group represented those more recently transplanted.

Risk of PTLD has been associated with EBV donor positive /recipient negative individuals, those with higher levels of DNA detection, chronic high viral load carriage, detectable DNA in plasma, and those receiving T cell depleting antibodies (115,123,164,170,210,218,219). Pre-emptive strategies to reduce PTLD incidence include reduction of immunosuppression, and more recently the use of rituximab in adult renal transplant populations with persistent high level DNAemia. Reduction in incidence of PTLD is reported in liver transplant and paediatric populations with

such strategies (146,164,172,182,193,220–223). In our series PTLD 1st presentations occurred in 6 individuals during the follow up period, all except one presenting more than 10 years post-transplant, 2/6 (33%) occurring in seronegative recipients and 1/6 only receiving MMF at diagnosis. Individuals with detectable EBV DNA at recruitment had a hazard ratio of 6.2 (CI 1.1-36.0, p=0.043), for subsequent PTLD development over the 3 year follow period compared to those without detectable DNA. An EBV tissue negative patient had persistent EBV DNAemia during the sampling period yet, at the time of presentation of PTLD, the patient had undetectable EBV DNA in blood and EBV negative histology. PTLD development in our series was also associated with time from transplant, and other markers of duration of immunosuppression, including a history of warts, non-melanoma skin cancer, a history of gum hypertrophy and EBV EBNA antibody deficiency. Is duration of immunosuppression as important as intensity? Should screening for EBV DNA be performed in the late post-transplant period, how frequently, and in whom should immunosuppression be reduced pre-emptively? In our study we show late EBV DNAemia is common, 46% ≥1 sample, and 16% with persistent viral loads >1,000 copies/ml, but was not associated with poorer graft function or specific symptoms. EBV seronegative recipients had a greater incidence of DNAemia at recruitment and of chronic high viral load carriage. Screening EBV seronegative recipients for DNAemia, symptoms and lymphadenopathy for the lifetime of their graft is logistically and clinically sensible. These individuals make up <10% adult transplant recipients and have been shown to have a risk of PTLD greater than seropositive recipients that persists into the

late post-transplant period. For seropositive recipients increasing time from transplant is associated with increasing prevalence of EBV DNAemia, and also increasing incidence of PTLD. Attention should perhaps focus as much on screening for symptoms and regular clinical examinations as on EBV DNA screening in blood, particularly as EBV negative histology PTLD accounts for up to 50% of late PTLD cases in adult renal transplant recipients.

4.1.6 Acknowledgements

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4.1.8 Disclosures

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Each author's specific contributions to the work are as follows:

MM, MP, PK, PV, and BC participated in the research design

MM, BC, MP, SR, PK and PV, participated in the writing of the paper

MM, SJ, PK, BC, and KR participated in the performance of the research

MM and SR participated in data analysis

All authors approved the final manuscript

4.2 Analysis of viral, immunological and clinical differences amongst adult renal transplant recipients with undetectable, low level and chronic high level EBV DNAemia

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abbreviations

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*includes abstract and main text up to Acknowledgements)

4.2.1 Abstract

We researched viral, immunological and clinical differences in 60 adult renal transplant recipients with undetectable (n=19), low level (n=20) and chronic high level EBV DNAemia (n=21). We performed analysis of serological responses, compartmental analysis of DNAemia in whole blood and plasma, lymphocyte subset analysis, ultrasound examinations for the detection of occult lymphadenopathy and assessment of infection and malignancy history. Individuals with chronic high level EBV DNAemia had higher levels of anti VCA antibodies than groups with low level or undetectable EBV DNA. Detection of EBV DNA in plasma was seen only in those with whole blood DNAemia levels ≥log 3.47 copies/ml, and was associated with lower CD4:8 ratios (0.95 (0.87-1.181) (detectable) v 1.96 (1.3-4.45) (undetectable), p=0.041). While low CD4 cell number was seen in 41% of all study participants and low CD19 (B cell) numbers in 66% no significant difference between EBV DNAemia groups was detected. Azathioprine treated individuals had the lowest median B cell and NK cell numbers. While MMF can cause B cell depletion and has been linked to low rates of EBV DNAemia we found no direct relationship in MMF treated individuals between B cell number and EBV DNA level. Individuals with chronic high viral load detection had significantly greater numbers of individuals with ≥2 cervical lymph nodes >5mm than other groups (p=0.049). Those with undetectable EBV DNA had lower median tacrolimus and ciclosporin trough levels while significantly greater proportions of individuals in the groups with detectable EBV had “excessive” levels of immunosuppression compared to those with undetectable EBV DNA. Further

work is needed to evaluate the role of lymphocyte subset monitoring in routine kidney transplant care, to assess the risks and benefits of immunosuppressive drug modification in the setting of late EBV DNAemia and in assessing the long term outcome of those with chronic high level EBV DNAemia and in those with DNAemia and ultrasound detectable subclinical lymph nodes in the neck.

4.2.2 Introduction

Epstein-Barr virus infects up to 90% of the adult human population (18). Primary infection is associated with infectious mononucleosis in a small number of individuals but for most the virus establishes a state of latency, persisting lifelong in the human host generally without causing illness (18). In solid organ transplant recipients EBV infection is associated with the development of more serious complications and disease including post-transplant lymphoproliferative disease (152). EBV DNA is detectable in blood even in healthy individuals in the general population (217). In adult kidney transplant recipients up to 40% patients may have detectable DNA, typically at higher levels than that found in the general population and often with persistent high viral load detection (117). The implications of this DNAemia remain poorly defined with the risk of PTLD development difficult to quantify and indications for intervention such as pre-emptive reduction of immunosuppression are not well described. The identification of clinical and laboratory differences that may help stratify individuals with and without DNAemia into high and low risk groups seems sensible. Previous studies have identified groups with EBV DNAemia at higher risk of PTLD as those with detectable EBV

DNA in plasma as opposed to whole blood or PBMC's, those with high viral loads and low EBV specific cytotoxic T lymphocytes numbers and possibly those with impaired T cell function (161,174,201,204,224,225). We aimed to analyse clinical, viral and immunological differences in adult renal transplant recipients, who were EBV seropositive at recruitment, to identify factors to help distinguish high and low risk groups in relation to development of EBV related disease. We performed serological assessment, compartmental analysis of DNAemia in whole blood and plasma, lymphocyte subset analysis, ultrasound examinations for the detection of occult lymphadenopathy and analysis of infection and malignancy history in 60 adult renal transplant recipients. These included 19 with a history of no EBV detection, 20 with low level or transient detection and 21 with persistent high level EBV DNA detection in blood.

4.2.3 Methods

We identified individuals previously recruited to a single-centre cohort study investigating Epstein-Barr virus infection in stable adult renal transplant recipients. This study analysed prevalence and patterns of EBV DNAemia during a 1 year follow up period in 499 participants between 1month and 33 years post-transplantation. Whole blood EDTA samples were analysed for EBV DNA at recruitment and 3 monthly intervals for 1 year. Prevalence of EBV DNAemia was calculated at baseline and then patterns of EBV DNAemia over the following year analysed. Patterns of DNAemia included no detectable DNA (NDNA), transient detection (TDNA)(<75% samples with detectable DNA), persistent DNAemia

(PDNA), ≥75% samples with detectable DNA and chronic high viral load carriage (CHVL), ≥3 samples over >6 months with ≥10,000 copies/ml DNA.

We subsequently invited 60 individuals from this initial study to join a further study investigating viral and immunological differences between individuals with persistent or chronic high viral load detection (n=21) (group 3), low level and transient detection (n=20) (group 2) and those with no detectable EBV DNA in blood during follow up (n=19) (group 3). Group 3 individuals were recruited first and those subsequently recruited to other groups matched to those in group 3 in relation to age and time from transplant.

The study involved a single clinic assessment and recruited patients were stable clinically at time of participation. Blood samples obtained included whole blood (EDTA). The plasma compartment was separated from the sample then extraction performed for EBV DNA from both the blood and plasma compartments. In addition whole blood (EDTA) was obtained for lymphocyte subset analysis using flow cytometry. During the assessment, recruited individuals also underwent an ultrasound examination of the lymph node chains in the neck including anterior and posterior triangles, parotid, submandibular and submental areas.

4.2.3.1 EBV DNA quantification in whole blood and plasma

A quantitative real-time PCR assay as described by Niesters et al was used with the target gene a non-glycosylated tegument protein BNRF-p143 (205). EBV DNA extraction was performed from EDTA whole blood and plasma using the Qiagen BioRobot MDx (Abbott 2007). After extraction, DNA extracts were batched and

stored at -80^{0}c with PCR amplification and detection of EBV DNA subsequently performed using the Applied Biosystems 7500 Fast Real-time PCR (Appendix 9.1).

4.2.3.2 EBV serological analysis

Serum samples at recruitment were analysed for the presence of IgG antibodies to EBV VCA and EBNA-1 antigens using the DiaSorin S.P.A LIAISON indirect chemiluminescence immunoassay (CLIA) performed on the LIAISON analyzer. Quantity of VCA and EBNA antibody is measured as relative light units and reported as units/ml with a maximum concentration of 750 U/ml for VCA and 600 U/ml for EBNA. For pre-transplant serostatus confirmation, stored (-80^{0}c) serum samples taken on the day of transplant were obtained from the Regional Tissue Typing Laboratory and tested for the presence of IgG antibodies to EBV VCA and EBNA-1 antigens.

4.2.3.3 Flow Cytometric analyses

Whole blood (EDTA) samples were analysed for the frequency of CD3, CD4, CD8, CD19 and CD56 staining lymphocytes on a single spot assessment using multicolour flow cytometry (Appendix 9.3). In brief, for labelling of cell surface markers 25 microlitres of whole blood was incubated with 10 microlitres of BD Bioscience 6-color T, B, NK reagent (combined monoclonal antibody) for 30 minutes at room temperature. BD FACS Fix/lysing solution was added with further sample incubation for 10 minutes. Samples were centrifuged and the supernatant removed and the cells resuspended in 0.3ml of 0.5% paraformaldehyde solution

and analysed as soon as possible using a FACS CANTO II flow cytometer and BD FACS DIVA software.

4.2.3.4 Ultrasound

Ultrasound examinations were performed by three NHS sonographers at Manchester Royal Infirmary. Sonographers were blinded to the EBV status of patients and performed a protocol guided examination of the neck including assessment of submental, submandibular, parotid and cervical chains. Position and short-axis diameter of nodes was recorded for all nodes >0.5mm (Appendix 9.2).

Demographic, transplant and immunosuppressive details were obtained from the medical records at time of recruitment.

A history of infectious and malignant complications since time of transplant was obtained from the regional department of clinical virology (virology data), regional microbiology laboratory (bacteriology), medical casenotes and transplant flow charts and patient interview.

Immunosuppressive regimen at time of recruitment was recorded including current agents, dose and drug 12 hour trough levels. Induction therapy and details of immunosuppression used for treatment of rejection were also recorded.

Immunosuppressive burden for individuals was classified as subtherapeutic, adequate and excessive. Classification was based on study physician assessment with consideration for time from transplant and transplant immunological risk, and review of agent types, doses and therapeutic levels. Classification was based on

the recommended immunosuppressive drug target levels and doses in the Symphony-elite study (226), 3 C's study protocol (http://www.3cstudy.org/3C_Protocol_V5.0_19122011.pdf) and Opelz and Dohlers paper on adequate azathioprine dosing (227).

4.2.3.5 Statistics

Groups were compared at median and interquartile ranges. Group comparisons used Fishers exact testing for categorical variables and Kruskal-Wallis for continuous data. Statistical analyses were performed using SPSS with a level of significance of $P<0.05$.

4.2.3.6 Ethical approval

Regional Ethical Committee (REC) review and approval for the study (Greater Manchester NEREC 09/H1013/71) was obtained. Informed consent was obtained from all subjects participating in the study. This was a non-interventional observational study and patients received standard routine care and follow up based on time from transplant and clinical need.

4.2.3.7 Acknowledgements

We are grateful to Dr Susan Martin and Dr Judith Worthington for assistance in obtaining stored serum samples from the regional tissue typing laboratory for analysis of pre-transplant EBV serostatus, to Alan Lord and Ben Brown in the regional Health Protection Laboratory who provided assistance with EBV DNA extraction, PCR and EBV serological analyses, to Victoria Bowman for update and provision of the transplant database at MRI, to Will Hulme and Michael Patrick at

NHS Blood and Transplant and to Lisa Laycock and the transplant clinic team at Manchester Royal for blood sampling assistance.

4.2.4 Results

We recruited 60 individuals to the study. At time of recruitment 35 (58%) individuals had evidence of EBV DNA detection in whole blood and 25 (42%) undetectable levels. We recorded viral loads <1000 copies/ml in 12 individuals, 1000-9999 copies/ml in 17 individuals, and >10000 copies/ml in 6 individuals with range 80-150319 copies/ml (log10 1.9-5.18). All participants were EBV seropositive at recruitment.

Participants were divided into three groups on the basis of serial samples for EBV DNA detection over the preceding year and their level of EBV DNA in whole blood at time of recruitment. The groups included those with a history of no detectable EBV DNA, n=19, those with transient or low level DNA, n=20, and those with persistent high viral load detection, including 8/21 with persistent loads >1000 copies/ml and 13/21 with loads persistently >10,000 copies/ml. A patient with a history of undetectable EBV DNA had low level DNA detection at time of recruitment and was entered into the study group, including those with low level DNAemia leaving only 19 in the group with undetectable DNA. There was no significant difference between groups in relation to age, time from transplant or serum creatinine (μmol/l) at recruitment. Characteristics of the three groups are given below in table 4.2.1.

Table 4.2.1 Characteristics of those with no detectable EBV DNA, transient or low level EBV DNAemia and those with persistent high viral load detection >1000 copies/ml

Characteristic	Undetectable DNA (19)	Detectable DNA (20)	Persistent High Viral load (21)
Mean age (diagnosis) (SD)	56.2 (11.3)	53.4 (11.9)	53.6 (11.2)
Male gender	13	14	15
Ethnicity white	18	19	20
Black	1	1	1
HLA-A*02	15	13	10
HLA-B*07	7	1	6
HLA-B*08	6	8	6
Median time from transplantation (years) (IQR)	8 (6-13)	7.5 (3-11)	7 (4-10)
Median Creatinine (IQR)	123 (91-155)	139 (103-175)	177 (120-234)
Median egfr (IQR)	44.3 (23-65)	41.3 (29-53)	34.9 (21-49)
Cause of ESRD			
Polycystic	3	6	4
GN/Autoimmune	8	6	9
Reflux/CPN/Cong	5	5	4
Other/unknown	3	3	4
Transplant history			
Ist transplant	10	16	17
Transplant number 2,3	9	4	4
Basiliximab induction	6	9	8

Treated rejection	2	2	7
ATG (rejection)	1	2	3
Treated CMV disease	6	5	3
Current Diabetes Mellitus	4	1	1
Immunosuppression at recruitment			
Tacrolimus	8	8	10
Mean tacrolimus level	5.5 (3.3-7)	8 (6.3-9)	8 (6-8.3)
Ciclosporin	8	7	10
Median ciclosporin level	63.5 (53-104)	151 (103-170)	97 (80-122)
CNI containing	16	15	20
Monotherapy	3	3	7
Dual therapy	11	15	10
Triple therapy	5	2	4
Azathioprine	7	6	7
Mycophenolate mofetil	7	8	2
Sirolimus	0	1	0
Prednisolone	10	9	10
Immunosuppressive burden			
Inadequate	6 (32)	1 (5)	4 (19)
Appropriate	13 (68)	13(65)	11 (52)
Excessive	0	6 (30)	6 (29)

4.2.4.1 Serology

All participants had detectable antibodies to EBV Viral capsid antigen at recruitment (EBV seropositive). Those with persistent high viral loads had greater proportions with maximum VCA antibody levels and higher median VCA levels (Table 4.2.4).

Table 4.2.3 Anti VCA and EBNA antibody levels in individuals with no detectable DNA, low viruria and persistent high viral loads

	Undetectable (19)	Low viral load (20)	High viral load (21)	P
VCA level	661 (368-750)	524 (209-750)	750 (750-750)	0.03
Maximum VCA response	8 (42)	8 (40)	17 (81)	0.006
EBNA level	78 (3-194)	66 (22-207)	171(11-416)	0.74
EBNA deficient	6 (32)	5 (25)	7 (33)	0.77
Maximum VCA response pre TX	7	2	7	0.999
Pre transplant VCA undetectable	0/17	2/18	2/21	0.63
Pretransplant EBNA deficient	3/17	5/18	6/21	0.75

(Analysis of variance (Kruskal-Wallis) and Fishers exact, $p<0.05$)

4.2.4.2 Comparison Whole blood v Plasma compartment

All individuals in the study were tested for the presence of EBV DNA in both whole blood and plasma at time of recruitment. EBV DNA was detected in whole blood in 35 individuals of which 7 also had EBV DNA detectable in the plasma compartment (Figure 4.2.2).

Table 4.2.4 Plasma detection of EBV DNA in those with undetectable, low viral load and chronic high EBV viral loads

	Undetectable	Low viral load	High viral load	p
Whole blood detectable	0	14	21	
Plasma detectable	0	1	6	0.005

Individuals with high viral load detection were significantly more likely to have DNA detectable in plasma than those with low or undetectable viral loads (OR 15.6 (1.7-140.7, p=0.005)(Table 3). No patients with undetectable EBV DNA in whole blood had detectable DNA in plasma. The 7 with positive plasma detection included 2/4 individuals in the study who were EBV seronegative pretransplant. At recruitment both of these seronegative individuals had detectable VCA antibodies, both had very high viral loads (log 5.4) and both relatively low CD4:8 ratios, 1.22 and 0.87, and both were many years post-transplantation (17 and 23).

The lowest whole blood sample with detectable EBV DNA had a viral load of log 3.47 and a corresponding plasma viral load of log 2.98. The mean difference in viral load between whole blood and plasma was a log value of -1.47 (0.6), range 0.49-2.07. The higher the whole blood viral load the greater the proportion with detectable EBV DNA in plasma, 66% log >4, 33% log 3.5-3.99, 9% those log 3-3.49 and none with whole blood viral load less then log 3. Low CD4:8 ratios <1 were identified in 12 individuals of which 5 (42%) had a history of detectable EBV DNA including 4 (33%) with persistent and high level DNAemia. All 4 of these had detectable EBV DNA in plasma. Only 1/29 (3%) individuals with a CD4:8 >2 had EBV DNA detectable in plasma although 15/29 (52%) had DNA detectable in whole blood. No significant correlation between whole blood and plasma level of DNA detection was identified (Figure 4.2.1).

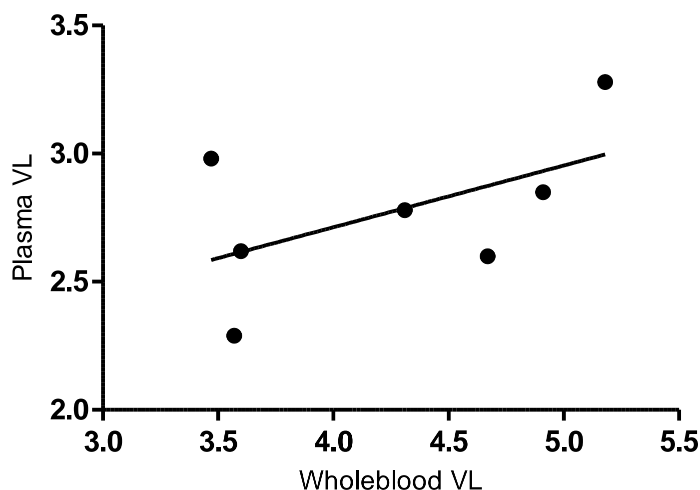

Figure 4.2.1 Scatterplot of Whole blood (X axis) versus Plasma (Y axis) EBV viral loads

(R^2=0.29, slope =0.24+-0.17, p=0.2) showing no significant correlation.

Individuals with detectable EBV DNA in plasma had significantly lower CD4:CD8 ratios (0.95 (0.87-1.81) v 1.96 (1.3-4.45),p=0.041) than those with whole blood detection only. They also had a trend towards higher CD8 counts ($x10^6$/L), 570 (269-736) v 327 (152-4090), P=0.095) and greater proportions with detectable nodes >7mm. 5/7 (71%) v 13/28 (46%) on ultrasound and number of nodes ≥2, 6/7 (80%) v 14/28 (50%), p=0.20). No difference in reported presence or number of B symptoms was seen between those with or without plasma detectable EBV DNA.

4.2.4.3 Lymphocyte subset analysis

Lymphocyte subset analysis was performed using flow cytometric analysis in 59/60 patients with an example given in appendix 9.3. Reference ranges for our population include CD3 622-2402 $x10^6$/L, CD4 500-1500 $x10^6$/L, CD8 109-897 $x10^6$/L, CD19 120-645 $x10^6$/L, CD56 24-406 $x10^6$/L. Overall 12/59 (20%) individuals had low CD3 cell counts (<622), 24/59 (41%) low CD4 cell counts (<500), 7/59 (12%) low CD8 counts (<109), 39/59 (66%) low CD19 (B cell) counts (<120) and 9/59 (15%) low CD56 (NK cell) counts (<24) (Table 6).

Table 4.2.5 Comparison of White cell count differential and lymphocyte subset absolute counts ($x10^6$/L) in relation to EBV DNAemia study group

Variable	Study population	Undetectable	Low level	High viral load	P Kruskal wallis
WCC (median)	6.9 (5.6-8.4)	6.7 (5.5-8.3)	8.2 (5.7-8.3)	6.8 (5.8-8.2)	0.71
Leucopenic	4 (7)	1(5)	1(5)	1(5)	0.99
Lymphocyte	1.4 (1-1.9)	1.4 (1.1-2)	1.5 (0.8-1.8)	1.5 (1-1.9)	0.81

Lymphopenic	32 (54)	9 (47)	9 (47)	10 (48)	0.98
Neutrophil	4.6 (3.4-6.1)	3.7 (3.1-6.2)	4.9 (3.8-6.6)	4.4 (3.3-5.6)	0.64
Neutropenic	6 (10)	2 (11)	3 (15)	1 (5)	0.55
CD3	1061 (730-1544)	1135 (735-1536)	997 (645-1348)	1084 (621-1546)	0.69
CD3 (<622)	12 (20)	2 (11)	4 (20)	5 (24)	0.54
CD4	618 (394-816)	669 (359-979)	492 (300-584)	618 (417-819)	0.58
CD4 (<500)	24 (41)	7 (37)	10 (50)	7 (33)	0.52
CD8	354 (179-529)	299 (99-499)	345 (40-650)	361 (135-587)	0.84
CD8 (<109)	7 (12)	2 (11)	4 (20)	1 (5)	0.31
CD8 Raised	6 (10)	1 (5)	3 (15)	2 (10)	0.60
CD4:8 Ratio	1.8 (1.1-2.8)	2.6 (1.5-3.7)	1.6 (0.7-2.5)	1.8 (0.6-3.0)	0.33
CD4:8 ratio <1	11 (18)	3 (16)	4 (20)	4 (19)	0.94
CD19	70 (41-141)	66 (14-118)	66 (6-126)	98 (44-152)	0.97
CD19 (<120)	39 (66)	12 (63)	14 (70)	13 (62)	0.85
CD56	83 (46-168)	76 (15-132)	91 (44-138)	84 (0-184)	0.39
CD56 (<24)	9 (15)	5 (26)	2 (10)	2 (10)	0.25

No significant difference was seen in total white cell, lymphocyte, T cell subset or B lymphocyte count between study groups.

Analysis was subsequently made of lymphocyte subsets in relation to immunosuppressive agents. Table 4.2.5 presents subset data for regimens that include the agents detailed below. In relation to immunotherapies basiliximab (p=0.006), tacrolimus (p=0.013) and triple therapy (0.036) were associated with higher preserved CD56 levels while monotherapy (p=0.006) and azathioprine (p<0.0001) treated individuals had significantly lower CD56 cell numbers and those on monotherapy also had significantly lower CD19 levels. Azathioprine use was also significantly associated with profoundly low B cell numbers (p=<0.0001), and lymphopenia (p=0.007). Use of mycophenolate was associated with lower mean CD8 counts (p=0.012). Individuals with a history of ATG treatment for rejection did not have lower CD3 counts those other patients.

Table 4.2.6 Immunosuppressive agents and Lymphocyte subset analysis.

Variable	Azathioprine	MMF	Tacrolimus	Ciclosporin	Prednisolone
CD3	901 (410-1436)	949 (634-1339)	1046 (649-1340)	1078 (792-1788)	1275 (780-1763)
CD4	395 (308-795)	465 (404-783)	548 (341-675)	684 (402-884)	645 (358-998)
CD8	324 (111-519)	245*(173-395)	327 (176-529)	371 (172-702)	396 (241-625)
CD4:8 Ratio	2.1 (1.3-2.5)	2.5 (1.1-3.3)	1.9 (0.9-3.1)	1.5 (1.2-2.8)	1.8 (0.9-2.4)

CD19	33 (21-65)	74 (46-178)	86 (49-143)	65 (27-145)	60 (27-111)
CD56	33 (8-61)	98 (59-152)	133*(71-218)	74 (42-180)	67(23-161)
WCC	6.8 (1.7)	7.4 (2.4)	7.7 (2.2)	7.1 (1.9)	7.6 (1.8)
Lymphocyte	1.13 (0.5)	1.44 (0.7)	1.5 (0.8)	1.7 (0.9)	1.6 (0.9)
Neutrophil	4.9 (1.7)	5.1 (2.0)	4.8 (1.9)	4.7 (2)	5.1 (1.8)

Median absolute count and interquartile ranges given. Lymphocyte subset absolute counts are presented as count x10^6/L.

No significant difference was seen between groups in relation to CD4:8 count ratio except for those with DNA detectable in plasma who had significantly lower ratio's than those with DNA in whole blood only (p=0.041)(Table 4.2.5). No significant differences in lymphocyte subset numbers were found in relation to time from transplant or age or those with a history of skin cancer.

4.2.4.4 Immunoglobulins

No significant differences between serum immunoglobulin levels were seen in relation to immunosuppressive agents or viral load groups.

Hypogammaglobulinaemia was rare, low IgG seen in only 3 (6%), low IgA 2 (4%) and low IgM 5 (10%). Raised total IgG was seen in 4 (8%), raised IgA 10 (19%) and raised IgM 5 (10%).

4.2.4.5 Ultrasound findings

Ultrasound detection of lymph nodes >0.5mm is presented below in table 4.2.6. Overall 40/60 (67%) had nodes detectable >0.5mm short axis, 25/60 (42%) >0.7mm and 27/60 (45%) ≥2 nodes >0.5mm.

Table 4.2.7 Ultrasound detection of lymph nodes >0.5mm (short-axis) in patients with undetectable, low level and persistent high EBV DNAemia

	Undetectable (19)	Low level (20)	High viral load (21)	P Fishers
No detectable nodes >0.5mm	8 (42)	7 (35)	5 (24)	0.59
Nodes >0.5mm	11 (58)	13 (65)	16 (76)	0.39
Nodes> 0.7mm	7 (37)	8 (40)	10 (48)	0.59
Total ≥2 nodes >0.5mm	5 (26)	9 (45)	13 (62)	0.049

Table comparing proportions of patients with variable and high viral load carriage against others.(Level of significance $p<0.05$)

Individuals with high viral loads had a higher prevalence of lymph nodes in the neck measuring >0.5mm and >0.7mm (76% and 48%) than those with low viral loads (65% and 40%) and those with undetectable viral loads (58% and 37%). High viral load carriers were significantly more likely to have higher numbers of nodes measuring >0.5mm than those with undetectable viral loads (62% v 26%),

OR 4.6 (95% CI 1.2-17.5) p=0.03. No nodes >1cm were identified. No cases of PTLD have been detected in individuals participating in the study to date.

4.2.5.6 Immunosuppression

Individuals with undetectable viral loads had significantly lower 12 hour drug trough concentrations of tacrolimus compared to low viral load (p=0.029) and high viral load carriers (p=0.03). A similar finding was seen for 12 hour ciclosporin concentrations, low viral load p=0.01, high viral load p=0.05. No significant difference between groups was seen in relation to dose of mycophenolate or azathioprine. Assessment of immunosuppressive burden was performed. In the group with undetectable EBV viral loads no individuals were classified as being in receipt of excessive immunosuppression while 30% low viral load and 29% high viral load were felt to be excessive (p=0.012 Fishers exact). Thirty two percent of undetectable viral load carriers had subtherapeutic immunosuppression compared to 5% low viral load and 19% high viral load (OR 3.3 95% CI 0.9-12.8 p=0.086).

4.2.5.7 Clinical Findings

No significant differences in relation to infectious or malignant complications or hospital admissions during the study period were seen between individuals with undetectable, low level or persistent high EBV viral loads (Table 4.2.7).

Table 4.2.8 Comparison of clinical characteristics, and infectious complications among individuals with undetectable, low level and chronic high EBV viral load detection

	Undetectable	Low viral load	High viral load	P
Platelet count	200 (163-259)	225 (186-267)	240 (207-283)	0.26
Thrombocytopenia	3	1	1	0.36
Haemoglobin (g/l)	124 (120-137)	124 (113-138)	121 (109-127)	0.26
Anaemia	3	6	5	0.58
Alanine transferase	18 (15-21)	18 (12-33)	15.5 (10.5-19.8)	0.41
Any B symptom	6	13	11	0.09
Night sweats	6	9	10	0.55
Weight loss	1	7	3	0.15
Fever	1	0	2	0.38
Fatigue	17	16	17	0.68
Sleep disturbance	10	8	13	0.37
Loss of appetite	3	4	6	0.60
Swollen glands	2	1	3	0.22
Sore throat	4	2	4	0.61
Abdominal pain	3	3	4	0.91
Hospital	1	6	5	0.08

admissions (during study period)				
Non Melanoma skin cancer	4	2	6	0.33
Cancer diagnosis	3	0	5	0.08
Warts	5	2	7	0.20
Shingles	4	1	3	0.33
HSV oral	4	2	6	0.33
CMV disease	1	3	3	0.57
Study period major infection	0	1	2	0.39
Post-transplant Gram + Septicaemia	3	1	3	0.52
Post-transplant Gram - Septicaemia	1	1	1	0.99
Post-transplant Lobar pneumonia	2	0	2	0.34
Post-transplant Diffuse pneumonia	1	2	3	0.67
Complicated UTI	3	0	2	0.20
Gastroenteritis	3	6	3	0.36
Soft tissue infection	7	5	4	0.40
ENT infection	4	0	3	0.11

4.2.6 Discussion

We previously performed a prospective observational study of EBV infection in adult renal transplant recipients. We showed that EBV DNAemia is relatively common, 46% all recruited individuals with ≥1 sample with detectable DNA over a 1 year period. DNAemia prevalence increased with time from transplant and in line with this we found that associated with DNA detection were other factors typically associated with duration of immunosuppression such as non-melanoma skin cancer, warts and ciclosporin and ciclosporin monotherapy. On analyses adjusted for time from transplant we found that MMF use and lymphopenia were associated with low rates of DNAemia. Further in relation to clinical outcomes DNAemia was not associated with anaemia, thrombocytopenia, lymphocytosis, raised liver enzymes such as alanine aminotransferase, level of renal impairment or specific clinical symptoms such as B symptoms. EBV DNAemia was associated with both increased risk of PTLD development and a previous history of PTLD.

This study consequently was performed to investigate viral, immunological and clinical differences between those with and without DNAemia in more detail. We recruited 19 individuals with no previous detectable EBV DNA in blood, 20 with low level or transient DNAemia and 21 with persistent DNAemia >1000 copies/ml including 13/21 with persistent DNAemia >10,000 copies/ml. The groups were not significantly different in relation to age, time from transplant or renal function and all recruited individuals were EBV seropositive at recruitment.

Serological analyses showed a significant increase in VCA antibody levels at recruitment compared to pre transplant levels with high viral load carriers more

likely to have maximal VCA levels compared to low viral load and those with no detectable viral loads (80% v 40%). Such high VCA levels amongst high EBV viral load carriers seems an appropriate response but perhaps reflects the failure to control levels of EBV replication. No such pattern was seen for anti EBNA antibody responses and we even saw lower proportions of individuals with EBNA deficient or equivocal responses at recruitment than pre-transplant (30% v 19%). Further in new PTLD cases diagnosed during the study period 4/4 (100%) were EBNA antibody deficient compared to 30% those in the study overall.

Detection of EBV in plasma as opposed to whole blood has been associated with a higher risk of PTLD. It has been identified as a sensitive marker for both diagnosis and monitoring of PTLD (224). In our series 7/35 individuals with whole blood DNA had viral DNA detectable in plasma also. Those with detectable plasma DNA tended to be those with higher levels of whole blood DNA and no patient with a whole blood level < $\log^{10}$ 3.47 copies/ml had plasma DNA. The 7 included 2/4 seronegative (pre-transplant) recipients. No PTLD has yet developed in these cases. However these 7 individuals had significantly lower CD4:8 ratio's, a trend to higher CD8 levels and also greater proportions (not significant) of individuals with sub-clinical occult lymph node detection >0.5mm but less than 1cm (short axis diameter). The significance of these nodes is unclear but may reflect a poorly controlled benign lymphoproliferation.

To investigate relationships between specific immunosuppressive agents and DNAemia, in particular the association of MMF and lymphopenia with low rates of DNA detection we performed lymphocyte subset testing analysis. We anticipated

that MMF treated individuals would have the lowest levels of CD 19 positive B cells corresponding to low levels of EBV containing B lymphocytes. MMF use has been associated with low incidence of EBV DNA detection post-transplant (129). We found that those on immunosuppressive regimens containing azathioprine had significantly the lowest CD 19 positive numbers and also the lowest lymphocyte counts. Despite this azathioprine was not found in our previous study in itself to be significantly associated with low levels of DNAemia. CD 19 counts were greatest amongst those with high viral load detection but were not significantly different to other groups. Individuals treated with MMF had low but not significantly lower CD 19 counts than others. CD 8 counts were highest in high viral load carriers and CD4:8 ratio's also highest in those with undetectable DNA. Again these were not significant differences but would fit with our knowledge of T cell responses to be EBV infection. We found no significant differences in relation to age, time from transplant or risk of skin cancer for the different lymphocyte subsets in this small number of patients.

In relation to lymphocyte subset testing overall, in general this is not performed as part of routine adult kidney transplant surveillance. Risk of skin cancer and other cancer has been associated with low CD4 and low CD19 cell counts in renal transplant recipients (228) while in pediatric heart and abdominal transplant recipients NK cell depletion and low CD4:8 ratios's may identify individuals at risk of recalcitrant PTLD particularly after previous antilymphocyte therapy (229). A study by Hutchison et al in stable adult renal and Kidney-Pancreas transplant recipients found that up to 85% had B cell lymphopenia, 23% CD4 cell depletion

and 50% NK depletion with no significant association with time from transplant or immunosuppressive agents (230). We found amongst 60 stable transplant recipients recruited from clinic when well that 41% had evidence of CD4 deficiency including 3 with counts <250 and 66% marked B cell (CD19) depletion. Azathioprine was associated with B cell depletion (p=<0.0001) and patients on regimens containing azathioprine also had the lowest CD 56 numbers (p=<0.0001) and the lowest CD4 count numbers (NS). In view of this consideration should be given to screening these individuals routinely with a view to discussing the risks and benefits of prophylaxis for pneumocystis and other opportunistic infections and adjustment of immunosuppression.

4.2.7 Conclusions

Individuals with chronic high level EBV DNAemia have higher levels of anti VCA antibodies and greater proportions with maximal anti VCA responses than groups with low level or undetectable EBV DNA. Detection of EBV DNA in plasma is seen only in those with whole blood DNAemia levels ≥log 3.47 copies/ml, and is associated with lower CD4:8 ratios (0.95 (0.87-1.181) (detectable) v 1.96 (1.3-4.45) (undetectable), p=0.041). While CD4 cell number deficiency was seen in 41% of all study participants and CD19 (B cell) deficiency in 66% no significant difference between EBV DNAemia groups was detected. While MMF is associated with low levels of EBV DNAemia this effect does not appear to relate to depletion of CD 19 positive B cells in our study. Azathioprine treated individuals have the lowest B cell, NK and CD4 positive cell numbers and consideration should be given to routine lymphocyte subset testing particularly in the late post-transplant period to weigh up

risk of opportunistic infection and adverse events. There may be scope to optimise immunosuppressive dosing in the setting of EBV DNAemia as in this small series those with high viral loads had significantly higher tacrolimus and ciclosporin trough levels and individuals on “excessive” Immunosuppression compared to those with no EBV DNA detection. High EBV viral load carriers may have greater numbers and increased size of sub-clinical lymph nodes in the neck than those with low viral loads or those with undetectable viral loads. Further work is needed to assess the longer term outcome of these patients, to screen for changes in the size and number and implication of these nodes, and to evaluate simple ultrasound of the neck as a tool to identify those at higher risk of EBV related disease or PTLD.

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4.3 Epidemiology of Post-Transplant Lymphoproliferative Disorder in Adult Renal Transplant Recipients

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Each author's specific contributions to the work are as follows:
MM, MP, KR, PK, PV, SR and BC participated in the research design
MM, MP, KR, RB, PK, PV, and BC participated in the writing of the paper
MM, RB, PK, BC, and KR participated in the performance of the research
MM and SR participated in data analysis

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Abbreviations:

ATG, anti-thymocyte globulin;

CI, confidence interval;

CTS, collaborative transplant study;

EBNA, Epstein-Barr nuclear antigen;

EBV, Epstein-Barr virus;

HL, Hodgkin's lymphoma;

ICD, international classification of diseases;

LDH, lactate dehydrogenase;

NHL, non-Hodgkin's lymphoma;

PTLD, post-transplant lymphoproliferative disorder;

VCA, viral capsid antigen

4.3.1 Abstract

Background

There is little information in the literature describing the relationship between PTLD incidence and presentation with both recipient EBV serostatus and EBV status of PTLD histology, particularly in the late post-transplant period.

Methods

This study reports the largest UK single centre, single organ analysis of PTLD to date, in a retrospective cohort study of 80 cases occurring in 4189 adult renal transplant recipients.

Results

The incidence rate for PTLD was 2.6 cases per 1000 patient years, (CI 2.1-3.2), non-Hodgkin's lymphoma 1.8, (CI 1.4-2.4), and Hodgkin's lymphoma, 0.2, (CI 0.07-4.2). NHL occurred at a rate 7.6 times that of the adult general population in England and HL at 5.9 times. The incidence of PTLD was highest during the 10th to 14th post-transplant years. Early onset disease was associated with EBV seronegative recipient status, EBV positive histology and involvement of extra-nodal sites. PTLD occurring in EBV seronegative recipients was associated with EBNA antibody deficiency, polymorphic disease and involvement of extra-nodal sites. EBV negative histology occurred in 32% of cases at a median time to presentation of 109 months. PTLD involving the allograft, central nervous system and skin was uncommon and occurred late.

Conclusion

The incidence of PTLD is highest in the late post-transplant period. Close clinical surveillance and education for transplant recipients is required for the duration of time while immunosuppressed. Failure to detect EBV DNA in blood should not reassure, particularly in patients with symptoms such as abdominal pain, oropharyngeal complaints, neck lumps and B-symptoms.

4.3.2 Introduction

PTLD is the second most common malignancy after skin cancer occurring in solid organ transplant recipients (231). It occurs in 1-3% adult renal transplant recipients and is associated with poor survival rates after diagnosis (5,8,105,232). Implicated in the disease pathogenesis is exposure to immunosuppression (105,233), impairment of cellular immunity and the infective agent Epstein-Barr virus (EBV) (199,201,202,234,235). PTLD tissue is EBV positive in up to 75% of cases (104), and EBV seronegative recipients at risk of primary infection have a markedly increased risk of disease (104,236–238). Recent PTLD registry reports for kidney transplant recipients have shown bimodal patterns of incidence with peaks in the first year and then in the later post-transplant period (104,233,237,238).

There is little information in the literature describing relationships between PTLD incidence and presentation with both recipient EBV serostatus and EBV status of histology, particularly in the late post-transplant period. Focus on late PTLD in adult kidney transplant recipients is increasingly important as median graft and patient survival times increase (now over 80% at 5 years).

To address the points above we performed an observational cohort study of our renal transplant population to identify PTLD cases, define incidence rates of PTLD,

and describe the variation in clinical presentation, including organ involvement. We analysed the timing of disease presentation with reference to EBV recipient serostatus and tissue EBV status, comparing those patients with early (<1yr) and late (>1yr) and very late (>10 yrs) onset of disease.

4.3.3 Materials and Methods

4.3.3.1 Identification of PTLD cases

Inclusion criteria for the study were age over 18 years and a functioning renal allograft at the time of diagnosis. All PTLD cases were classified according to the 2008 WHO classification as early, polymorphic, monomorphic or Hodgkin's disease (239). Multiple myeloma was not included. PTLD histological classification, sub-classification and PTLD tumour EBV status based on immunohistochemical staining or in situ hybridisation for EBER was performed by regional specialist Haemato-pathologists.

Data was abstracted from the Regional Tissue Typing Laboratory database and through a comprehensive search of pathology databases. Cases of PTLD occurring from August 1st 2009 onwards were identified prospectively following referral to Regional Haemato-oncology. In the case of individuals who transferred their care to other units outside our region following transplantation (n=175), outcome data including occurrence of graft loss (23/175) and PTLD (n=4) was obtained from the NHS Blood and Transplant Registry. PTLD cases identified were verified with the unit providing follow up care.

4.3.3.2 Incidence of PTLD

Patient years of follow up were calculated for all individuals receiving a kidney transplant between March 22nd 1968 and February 29th 2012. Censorship occurred at the date of disease onset for PTLD cases, and for all others at the onset of graft failure or death or the end of the follow up period. Incidence rates were expressed as number of cases over number of patient years at risk and then as cases per 1000 patient years. Poisson distribution 95% confidence intervals were calculated for the estimated rates.

We calculated the incidence of PTLD in our adult transplant population and compared with age standardised rates of Hodgkin's lymphoma and non-Hodgkin's lymphoma in the general population (aged greater than 19 years) in England (total population n=39,104,000) for the calendar year 2008. Lymphoma incidence in the general population was obtained for ICD 10 code C81 (Hodgkin's) and ICD codes C82-85 and 96 (non-Hodgkin's) from the North West Cancer Intelligence Service (personal communication) and UK Cancer Intelligence Service report (2011) for cases occurring in 2008 and registered by September 2010 (240).

Change in incidence rates of PTLD with increasing time from transplant, and between different eras of immunosuppression was analysed, including 1990-1994 (cyclosporin, azathioprine and prednisolone), 1995-1999 (cyclosporin, mycophenolate and prednisolone), 2000-2004, (tacrolimus, mycophenolate and prednisolone), and since 2004 in line with National Institute for Clinical Excellence guidance, (basiliximab induction followed by calcineurin inhibition with tacrolimus and added mycophenolate, azathioprine and corticosteroids depending on immunological risk (239). Lymphocyte depleting antibodies are not used as

induction agents in our unit and are reserved for steroid resistant and vascular rejection. Acute rejection rates with current immunosuppressive protocols are less than 10% and use of ATG has decreased in recent times. Antiviral prophylaxis is given to individuals at high risk of cytomegalovirus disease (Donor positive recipient negative and those receiving lymphocyte depleting antibodies).

4.3.3.3 Clinical Presentation

Groups were compared using mean and standard deviation for normally distributed variables and median and interquartile ranges for skewed data. Group comparisons between early and late onset presentations and disease presenting in EBV seronegative and seropositive recipients used Chi-square testing for categorical variables and Students 2 sided t-test for continuous variables. Group comparisons and univariate analyses were performed using Graphpad Prism 5 software.

4.3.3.4 EBV serostatus and timing of PTLD

Where EBV serostatus was not known (pre-2008 donors and recipients were not routinely screened for EBV serostatus), stored (-80^{0}c) serum samples taken on the day of transplant were obtained from the Regional Tissue Typing Laboratory and IgG antibody status to EBV VCA and EBNA-1 antigens was determined**.** Kaplan-Meier time to event curves were used to graphically illustrate the cumulative time to presentation for EBV seronegative and seropositive recipients (Fig 4.3.2a) and tissue EBV positive and tissue EBV negative recipients (Fig 4.3.2b). Comparison of the curves used the log-rank test (Graphpad Prism 5 software).

4.3.3.5 Ethical approval

Regional Ethical Committee (REC) approvals for the study (Greater Manchester NWREC II/NW/0001, NEREC 09/H1013/71) and honorary contracts were obtained. Informed consent was obtained from all living subjects.

4.3.4 Results

4.3.4.1 Incidence of PTLD

A summary of the incidence data is shown in Table 4.3.1

Table 4.3.1 Incidence of PTLD, Non-Hodgkin's lymphoma and Hodgkin's lymphoma in adult renal transplant recipients

	MRI adult kidney transplant recipients (n=4189)		**Reference group**	**Rate Ratio (95% CI)**	**p**
	Observed cases/patient years	Rate per 1000 patient years (95% CI)			
PTLD	80/30867	2.6 (2.1-3.2)			
Transplant Organ					
Kidney alone (3990)	78/30106	2.6 (2.1-3.2)			
Simultaneous kidney pancreas (199)	2/762	2.6 (3.2-9.5)	Kidney alone	1.0 (0.1-3.8)	0.99
Gender					
Male	66/19576	3.4 (2.6-4.3)	Female	1.6 (1-2.7)	0.039
Female	24/11577	2.1 (1.3-3.1)			
Age at transplantation					
18-39	37/15451	2.4 (1.7-3.3)			
40-59	33/13003	2.5 (1.8-3.6)	18-39	1.1(0.6-1.7)	0.808
>60	10/2418	4.1 (2-7.6)	18-39	1.7 (0.8-3.5)	0.12
Year of Transplant					
1990-1994	23/5843	3.9 (2.5-5.9)	2005-2009	1.8 (0.8-4.6)	0.15
1995-1999	19/5616	3.4 (2-5.3)	2005-2009	1.4 (0.6-3.7)	0.42
2000-2004	13/4836	2.7 (1.4-4.6)	2005-2009	1.1 (0.4-3.1)	0.81
2005-2009	8/3323	2.4 (1-4.7)			
PTLD WHO class					
Non- Hodgkin's (PTLD)	58/30867	1.9 (1.4-2.4)	Non-Hodgkin's	7.6 (5.7-9.8)	<0.0001

			(General population)		
			Hodgkin's (PTLD)	9.7 (4.2-27.4)	<0.0001
Hodgkin's (PTLD)	6/30867	0.2 (0.07-4.2)	Hodgkin's (General population)	5.9 (2.2-12.9)	<0.0001
Non- Hodgkin's (General population)	9703/39104000	0.248 (0.24-0.25)	Hodgkin's (General population)	7.5 (7.1-8)	<0.0001
Hodgkin's (General population)	1286/39104000	0.03 (0.03-0.04)			

The median follow-up time for all transplant recipients was 2058 days (5.6 years, IQR 676-3983). PTLD occurred in 80 recipients in 30,867 patient years, giving an incidence rate of 2.6 cases per 1000 patient years. Non-Hodgkin's lymphoma was seen more frequently than Hodgkin's, rate ratio = 9.7. Non-Hodgkin's lymphoma occurred at a rate ratio of 7.6 and Hodgkin's lymphoma at 5.9 that of the general population (240). PTLD occurred more frequently in males than females (p=0.039) and there was a trend towards increased rates with age at transplantation. No significant differences in incidence rates were detected between different eras of transplantation (Table 4.3.1 and Table 4.3.2).

Table 4.3.2 PTLD incidence rates (cases per 1000 patient years with 95% Poisson CI) for different eras of immunosuppression in relation to time from transplant

Era of Transplantation and dominant immunosuppressive regimen		Years post- transplant					
		1st	2nd-4th	5th-9th	10th-14th	15th-19th	20th-25th
Complete study period	1968-2012	3.3 (1.7-5.8)	1.2 (0.7-2.1)	2.3 (1.4-3.5)	4.3 (2.6-6.5)	2.2 (0.7-5.0)	3.5 (1-9)
Ciclosporin, Azathioprine, Prednisolone	1990-1994	1.9 (0.05-10.6)	1.1 (0.1-3.9)	3.2 (1.2-7)	11.5 (5.3-21.8)	4.2 (8.6-12.1)	
	1995-1999	7.1 (1.9-18.2)	0.9 (0.1-3.3)	2.0 (0.6-5.2)	6 (2.6-11.9)		
Tacrolimus, MMF[1], Prednisolone	2000-2004	5 (1-14.7)	1.4 (0.3-4.1)	3.2 (1.3-6.7)			
Basiliximab, Tacrolimus, MMF[1], Prednisolone	2005-2009	4.1 (0.9-12)	1.7 (0.5-4.5)				

[1]MMF, mycophenolate mofetil

Incidence rates were associated with time from transplantation. The second to 4th years after transplant were used as a reference group as this period was expected to have the lowest incidence of PTLD. We observed a bimodal pattern of

presentation with rates in the first year after transplant higher than the reference period, rate ratio 2.7 (95% CI 1.1-6.1) (p=0.0098), and peak incidence occurring later between 10 and 14 years after transplant, rate ratio 3.4 (95% CI 1.7-7.3) (p=0.032) (Figure 4.3.1).

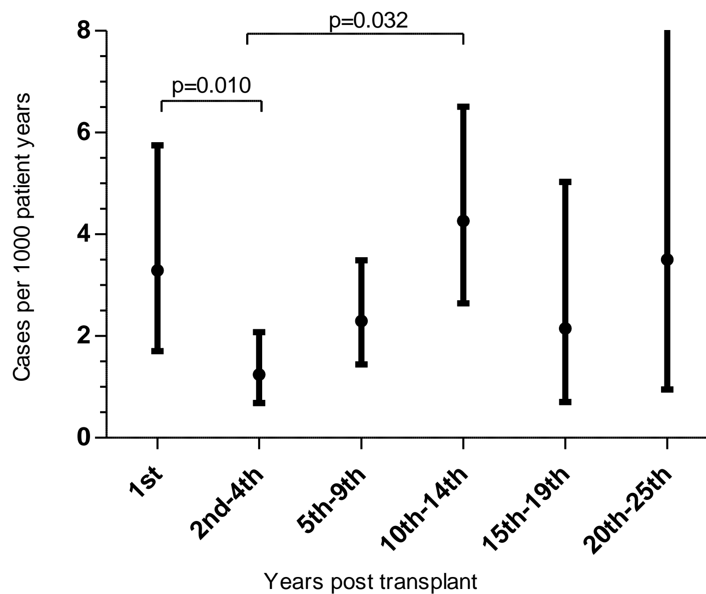

Figure 4.3.1 PTLD incidence rate versus time from transplant for patients transplanted 1968-2010

Incidence rates are presented as cases per 1000 patient years with Poisson 95% confidence intervals. Individuals were censored at diagnosis of PTLD, graft failure, death with a functioning graft or upon reaching the end of the follow up period.

4.3.4.2 PTLD clinical characteristics

Characteristics of the PTLD population are summarised in Table 4.3.3.

Table 4.3.3 Demographics of PTLD cases

Characteristics	**All Cases**	**Early Onset n (%)**	**Late Onset n (%)**	**EBV Sero-ve n (%)**	**EBV Sero+ve n (%)**	**EBV –ve PTLD n (%)**	**EBV+ve PTLD n (%)**
n	80	12 (17)	68 (83)	19/54 (33)	37/54 (67)	18/56 (32)	38/56 (68)
Male	56 (70)	8 (67)	48 (71)	14 (74)	29 (78)	15 (79)	24 (63)
Mean age	49 (14)	52 (14)	49 (14)	48 (17)	51 (11)	53 (15)	50 (13)
Median time to presentation	74 (28-131)	7 (4-7)	89 (48-135)	19 (7-80)	115 (54-140)	106 (55-126)	89 (10-137)
1 year survival	66%	33%	72%	58%	84%	68%	63%
PTLD EBV +ve	38/56 (68)	10/11 (91)	28/45 (62)	13/17 (76)	17/27 (63)		
EBV detectable viral load	30/37 (81)	4/5 (80)	26/32 (81)	8/12 (67)	18/20 (90)	6/11 (55)	22/23 (96)
EBV Viral load log^{10} copies/ml	3.6 (2.3)	4.1 (2.3)	3.5 (2.0)	3.4 (2.7)	3.5 (1.7)	2.3 (2.4)	4.4 (1.5)
EBV VCA+ve EBNA –ve n	19/50 (38)	5/6 (83)	14/44 (32)	13/16 (81)	5/32 (16)	5/15 (33)	11/25 (44)
Treated for acute rejection	28/75 (37)	4/12 (33)	24/63 (38)	7/18 (39)	14/34 (41)	6/18 (33)	13/35 (37)
ATG	10/75 (13)	4/12 (33)	6/63 (10)	5/18 (28)	3/34 (9)	3/18 (17)	5/35 (14)
LDH raised	19/34 (56)	3/3 (100)	16/31 (52)	7/10 (70)	9/20 (45)	4/12 (33)	13/19 (68)
Histology *n(%)*							
Early	5 (6)	1 (8)	4 (6)	1 (5)	4 (11)	1 (5)	4 (11)
Polymorphic	12 (15)	4 (33)	8 (12)	6 (32)	3 (8)	3 (16)	7 (18)

Monomorphic	57 (71)	7 (59)	50 (74)	11 (58)	27 (73)	14 (74)	23 (60)
Hodgkin's	6 (8)	0	6 (9)	1 (5)	3 (8)	1 (5)	4 (11)
***Clinical symptoms at presentation** n(%)*							
Neck swelling	31 (42)	5 (42)	25 (41)	9 (50)	17 (49)	9 (56)	17 (46)
Oropharynx	14 (19)	4 (35)	10 (16)	4 (22)	7 (20)	1 (6)	11 (30)
Abdominal pain	19 (26)	4 (33)	14 (23)	5 (28)	7 (20)	8 (47)	6 (16)
B symptoms	51 (65)	9 (75)	42 (69)	4 (78)	22 (63)	13 (81)	23 (62)
***Disease sites at presentation** n(%)*							
Single site	30 (41)	3 (25)	27 (44)	5 (28)	18 (51)	8 (50)	11 (30)
Extra-nodal	49 (66)	11 (92)	38 (62)	15 (83)	18 (51)	9 (53)	24 (65)
Ann Arbor score ≥3	28 (38)	8 (67)	19 (31)	11 (61)	10 (29)	7 (41)	14 (38)
Neck	31 (42)	6 (50)	25 (41)	8 (44)	18 (51)	9 (56)	17(46)
Oropharynx	15 (20)	3 (25)	12 (20)	4 (22)	8 (23)	2 (13)	11 (30)
Liver	12 (16)	5 (42)	7 (11)	7 (39)	4 (11)	2 (13)	7 (19)
Spleen	10 (14)	6 (50)	4 (7)	7 (39)	3 (9)	3 (19)	5 (14)
GI tract (All)	15 (20)	5 (42)	10 (16)	3 (17)	5 (14)	5 (29)	6 (16)
Bowel	10 (14)	3 (25)	7 (11)	2 (11)	4 (11)	4 (24)	4 (11)
Stomach	5 (7)	2 (17)	3 (5)	1 (6)	1 (3)	1 (6)	2 (5)
Pancreas	2 (3)	0	2 (3)	0	0	1 (6)	0
Groin nodes	6 (8)	0	6 (10)	1 (6)	5 (14)	2(13)	4 (11)
Graft	2 (3)	0 (8)	2 (3)	1 (6)	1 (3)	0	0
Cerebral	4 (5)	0 (0)	4 (6)	0	2 (6)	0	4 (11)
Lung	5 (7)	2 (17)	3 (5)	3 (17)	0	1 (6)	2 (5)
Pericardium	3 (4)	0	3 (5)	2 (11)	0	1 (6)	1 (3)
Bone marrow	6 (8)	1 (8)	5 (8)	3 (17)	1 (3)	1 (6)	3 (8)
Skin	7 (9)	0	7 (11)	0	4 (11)	1 (6)	1 (3)
Soft tissue	2 (3)	0	2 (3)	0	1 (3)	0	0

VCA, viral capsid antigen; Treated for acute rejection, this included clinically suspected and biopsy proven rejection treated with methylprednisolone; ATG, anti thymocyte globulin; LDH, lactate dehydrogenase.

The median time from transplant to presentation of PTLD for the 80 cases was 74 months (6.1 years) (IQR: 28-131). Cases presented early (within 1 year of transplant) in 12/80 (15%) and late (>1 yr) in 68/80 (85%). Very late presentations (>10yrs) occurred in 24/80 (30%) with 3 (4%) beyond the 20th post-transplant year.

4.3.4.3 Early onset disease

Early onset disease (<1year) was associated with EBV positive tissue status (OR:15, 1.8-128, p=0.005), VCA positive, EBNA antibody negative status (OR: 10.7, 1.1-100.5, p=0.02), prior ATG therapy (OR: 4.8, 1.1-20.6, p=0.048), extra-nodal involvement (OR: 7.0, 0.8-57.3, p=0.0497), Ann Arbor staging ≥3 (OR: 4.5, 1.2-16.9, p=0.02), liver (OR: 5.6, 1.4-22.6, p=0.02) spleen (OR: 14.5, 3.1-66.2, p=0.0008) and gastrointestinal tract involvement (OR: 3.7, 0.9-14.2, p=0.059).

4.3.4.4 Late onset disease

Late onset (>1yr) disease involved extra-nodal sites in 62% of cases and was EBV tissue positive PTLD in 29/46 (63%). All cases of disease involving the graft, central nervous system, skin, soft tissue, pericardium and 5/6 cases with bone marrow involvement in our series occurred late.

4.3.4.5 Very late disease

PTLD occurred very late, beyond 10 years, in 24 individuals. Relapses were seen in 6 (25%) of these very late cases compared to 5/44 (11%) cases presenting between 1 and 10 years and 0 in early onset disease. Sites of disease included the neck (10), oropharynx (5), 2 at sites of chronic inflammation including a tooth abscess and hyperplastic gum, groin nodes (5), gastrointestinal tract (2) allograft

(1) and central nervous system (2). Histology was negative for EBV in 5/21 (24%), and 4/19 (24%) of these cases occurred in seronegative recipients.

4.3.4.6 Pre-transplant serostatus and time to presentation

Pre-transplant EBV serostatus was obtained for 70% of the PTLD cases, of which 19/56 (34%) were seronegative and 37/56 (66%) seropositive. The median time to PTLD for seronegative recipients was shorter than for seropositive, 19 months (IQR 7-80) v 115 months (IQR 54-140) (p=0.003) (Figure 4.3.2a), and nearly half of all cases in the seronegative group occurred during the first transplant year. Early onset disease occurred almost entirely in seronegative recipients (90% cases with known serostatus) (OR: 32.4 for seronegative v seropositive, 95% CI 3.7-287, p<0.0001; Figure 2a). PTLD presenting early in seropositive recipients was unusual with only a single case in the first year and 2 cases during the second year, both early type lesions.

PTLD in seronegative recipients was associated with VCA positive EBNA antibody deficiency (p=<0.0001; OR: 23.4, 24.0-161), early onset, (p=0.003), polymorphic histology (OR: 5.2, 1.1-24 p=0.049), extra-nodal involvement (OR: 4.7, 1.2-19.3 p=0.036), Ann Arbor staging ≥3 (OR 3.9, 1.2-13.0 p=0.04) and disease involving liver (OR: 3.9, 0.9-15.4 p=0.08) and spleen (OR: 5.3, 1.2-23.7 p=0.030).

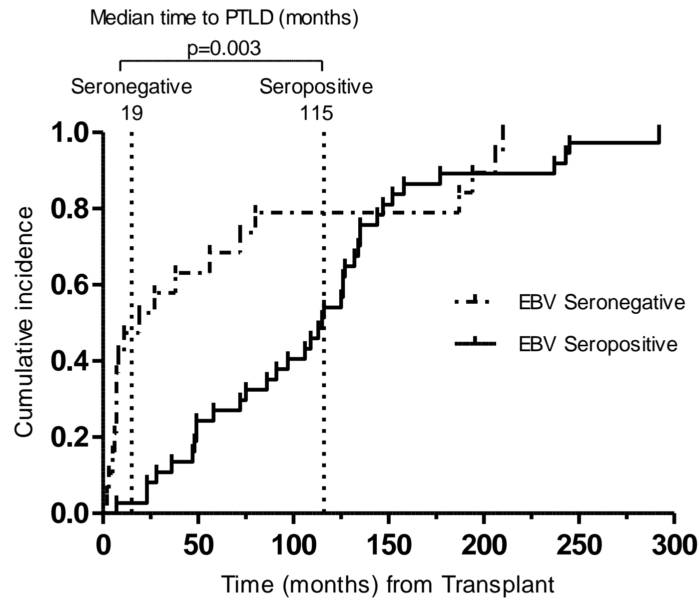

Figure 4.3.2a Pre-transplant recipient EBV serostatus. Time from transplant (months) to PTLD diagnosis

Kaplan-Meier time to event plot for EBV seropositive and EBV seronegative recipients. The median time to presentation of PTLD was significantly shorter in EBV seronegative recipients (Mann-Whitney p=0.003)

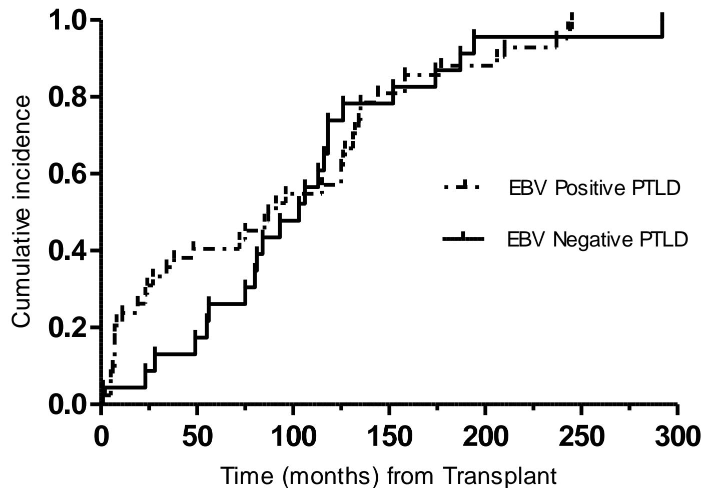

Figure 4.3.2b EBV positive and EBV negative PTLD tissue status at histological examination

Time from transplant (months) to PTLD diagnosis. Kaplan-Meier time to event plot for patients with EBV positive PTLD and EBV negative PTLD (89 months v 109 months respect. ns, Mann-Whitney)

4.3.4.7 EBV negative histology PTLD

EBV status of PTLD histology was available for 56/80 cases of which 18/56 (32%) were EBV negative. EBV negative PTLD was unusual in the early post-transplant period and case numbers increased with time from transplant, 1/11 (9%) in the first year, 3/8 (38%) in the 2nd-4th years, 6/12 (50%) in the 5th-9th years, 9/26 (35%) cases beyond 10 years (Figure 2b). EBV was detectable in blood at diagnosis in 55% of EBV negative histology disease. EBV negative PTLD was seen in 24% of seronegative recipients and 37% of seropositive recipients (p=0.51). Seronegative

recipients who developed EBV negative PTLD all had evidence of sero-conversion (detectable IgG to EBV VCA) at diagnosis.

4.3.4.8 EBV positive histology PTLD

PTLD tissue was EBV positive in 38/56 (68%) of cases including 91% (10/11) of all cases presenting within 1 year of transplantation. Detectable EBV viral loads in blood were seen in 96% of cases. The median time to presentation for patients with EBV positive PTLD was slightly but not significantly earlier than EBV negative, 89 months v 109 months.

4.3.4.9 Presenting symptoms and organ involvement at presentation

Demographics, histology, presenting clinical symptoms and sites of disease at the time of diagnosis are shown in Table 3. Symptoms occurring with the greatest frequency included neck swelling, abdominal pain, oropharyngeal complaints and occurrence of B-symptoms.

4.3.4.10 Survival

Overall patient survival at 1, 5 and 10 years was 66%, 50% and 37% respectively, with a median survival of 1253 days from diagnosis. A complete response to treatment was seen in 46/80 (58%) of patients of whom 11 (24%) subsequently relapsed at a median time from diagnosis of 325 days (IQR = 214-726), and 8 (17%) lost their graft, at a median time of 896 days (IQR = 363-1370) from diagnosis. Graft survival at 1, 5 and 10 years from diagnosis in those who had a complete response was 96%, 82% and 77% respectively.

4.3.5 Discussion

This study reports the largest UK single centre, single organ analysis of PTLD to date. PTLD presented up to 24 years after transplantation in 1.9% of adult kidney transplant recipients. Our incidence rates were similar to those reported recently by Swedish (233), French (104), US (238), and UK (232) studies. Analysis of PTLD timing in relation to EBV serostatus confirmed the increased risk for seronegative recipients in the first year but also highlighted a persistently increased risk for these patients in the late post-transplant period. This study also draws attention to differences in clinical presentation for disease occurring early, late and very late, in EBV seronegative and seropositive recipients and for EBV histology positive and histology negative PTLD.

Compared to the adult general population we found an 8 fold increased risk of non-Hodgkin's lymphoma and 6 fold increased risk of Hodgkin's lymphoma. EBV positivity was found in approximately 40% of Hodgkin's lymphoma cases (241) and in 26% of B cell non-Hodgkin's lymphoma cases (242) in the general population. In our series EBV positivity was more prevalent and seen in 80% of early lesions, 80% of Hodgkin's, 70% of polymorphic and 64% of monomorphic PTLD cases. High rates of lymphoma in solid organ transplant recipients are linked to the use of specific immunosuppressive agents including calcineurin inhibitors (105), and T cell depleting antibodies (5,124,238). Caillard observed a fall in lymphoma incidence more recently in France, which may have been due to reduced use of T-cell depleting antibodies (104). We did not observe any statistically significant changes in recent times in our series, though we have never used T-cell depleting

agents as protocol induction therapy. Compared to the French registry (104) a similar proportion of PTLD cases in our series involved gastrointestinal tract disease. We experienced less early onset disease (15% v 48%), CNS disease (5% v 20%) and graft involving disease than the French series (3% v 24%). Reasons for these differences may be due to a low French rate of recipient seronegative status at transplantation, only 3%, with only 16% of French cases occurring in seronegative recipients compared to 34% in our series. Further, the French had a high rate of ATG induction, 54% amongst controls and 66% in cases, compared to only 13% of our cases.

Our data confirms the first year after transplant as a period of high incidence of PTLD for EBV seronegative recipients (104,218,237,238). French and US registry data has also associated EBV seronegative status with an increased hazard risk of not just early but also late PTLD. In our series 90% of early and a further 22% of all late PTLD occurred in seronegative recipients despite them making up <10% of all adult kidney transplant recipients in our unit. The small number of seronegative recipients and relatively high proportion of cases presenting in this group in the first year and later (>50% of cases) suggests that these individuals should remain under close clinical surveillance for the duration of their transplant life. Disease occurring in our seropositive recipients was unusual in the first few years. Seropositive recipients had a significantly greater likelihood of PTLD in the late follow-up period than in the first 4 years. The aetiology of late PTLD appears complex and the incidence of late disease may increase over the next ten years as transplant rates increase and improved graft survival results in the exposure of

recipients to immunomodulatory medication for longer periods of time. Late disease may relate to impaired immune responses at the time of primary infection, duration of exposure to immunosuppression, age, immunosenescence and perhaps other infective agents. CMV infection has been associated with EBV negative PTLD (104).

Currently UK (2011) (243) and KDIGO (208) guidelines advocate that high risk EBV donor positive / recipient negative patients have EBV viral load monitoring till the end of the first year and that total immunosuppression be reduced when EBV titres rise significantly. However, in the UK, detection of whole blood EBV DNA by PCR in the first 90 days post- transplant was reported in 50% of seronegative recipients and 56% of all adult renal transplant recipients, without the presentation of PTLD during a two year follow up (129). In 20 EBV donor positive, recipient negative transplants receiving ATG induction Martin et al reported detectable EBV DNA in plasma in 74% of patients in the first year, of which 1/20 developed PTLD (244). Screening for detectable EBV viral loads beyond the first year is not yet standard practice. Detection of EBV DNA in whole blood is reported in up to 36% of stable renal transplant recipients at any one time and the prevalence of DNAemia may increase with time from transplant (245). Further 15% of all our late cases of PTLD had no detectable EBV DNA in their blood at the time of diagnosis. Whole blood screening for EBV DNA is sensible and logistically feasible in seronegative recipients in the first year but viral load detection alone does not identify PTLD cases. Close clinical surveillance is also required in the high risk patient for the duration of their transplant life. In our series we identified clinical

features that may help to prompt urgent investigations. Common symptoms included neck pain and swelling, oropharyngeal complaints, abdominal pain, obstructive symptoms and B symptoms. Disease presenting early and in the seronegative patient often involved multiple sites, extra-nodal sites, liver, spleen and the gastrointestinal tract. Disease involving the CNS, skin and soft tissue tended to occur late. Very late disease (>10 years) may have a high rate of relapse and involve neck, oropharynx and sites of chronic inflammation.

4.3.6 Acknowledgements

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4.4 Post-Transplant Lymphoproliferative Disorder in Adult Renal Transplant Recipients: Treatment, Response, Survival, and Prognosis

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Running title: PTLD: treatment, response and survival.   48 characters (max 34)

Key Words: (4-6) kidney, survival, post-transplant lymphoproliferative disorder, transplant, graft, immunosuppression, rituximab, chemotherapy.

4.4.1 Abstract

Post–transplant lymphoproliferative disorder (PTLD) is one of the most common malignancies occurring after skin cancer in solid organ transplant recipients. Optimal treatment strategies and risk stratification guidelines specific to kidney transplantation are lacking. We performed a single centre retrospective analysis of clinical outcomes in 89 adult renal transplant recipients diagnosed with PTLD. We identify factors associated with clinical response and patient survival and analyzed long-term outcomes in compete responders to treatment including relapse rates, change in graft function and graft loss after diagnosis and patterns of immunosuppression. Forty nine (55%) cases presented since the introduction of Rituximab immunotherapy to our unit in 2003. Median overall survival was 5.8 years, with a complete response to treatment in 54/89 (61%) of cases, including 22/54 with immunosuppression reduction alone. Subsequent relapse was seen in 13/54 and graft loss in 11/54. We show that in addition to previously identified prognostic variables including performance status and LDH measures of graft function in particular eGFR <30ml/min/1.73m2 and Acute Kidney injury at diagnosis are highly associated on adjusted analyses with poor survival. Measures of allograft function may need to be incorporated in treatment protocols and risk stratification of PTLD patients in the future. EBV status of histology and serostatus at time of transplant had no significant effect on overall survival or response rates in our series.

4.4.2 Introduction

Post-Transplant Lymphoproliferative Disorder (PTLD) is the second most common malignancy after skin cancer, occurring in 1-3% of adult renal transplant recipients, and is associated with poor survival rates following diagnosis (5,8,103,105,210,231,246). PTLD is a spectrum of disease ranging from an infectious-mononucleosis type early lesion to monomorphic disease including diffuse large B cell lymphoma (108) and is associated with Epstein-Barr virus infection in up to 68% of cases (115,210,233,236). The variety of clinical and histological presentations and underlying disease processes has precluded development of a universal treatment strategy, with existing guidelines generally directed broadly at solid organ transplants, rather than being organ specific (140,151,247). Immunosuppression reduction is the initial intervention for the majority of patients (140) and this strategy may result in disease remission in up to 60% of patients (248,249). In those cases not responding to immunosuppression reduction alone, other therapies may include immunotherapy such as rituximab for CD-20 positive cases, chemotherapy, surgery and radiotherapy (140,189,250,251). Evidence based protocols to guide therapy in renal transplantation are limited, due to the relatively small numbers of cases seen in individual centres (252). Further disease staging and prognostication is difficult, as prognostic scoring models such as the International Prognostic Index (253), designed to stage non-Hodgkin's lymphoma in the general population, and the Choquet model for solid organ transplant recipients with PTLD treated with rituximab (254), were not designed specifically for application in a renal transplant population with variable levels of

kidney function. Most recently Caillard et al reported a new prognostic score following analysis of outcomes of 500 French renal transplant patients with PTLD yet to be validated in another population (103). In our detailed single centre, single organ study we aimed to identify factors associated with survival and response specific to renal transplantation with attention focused on the relevance of graft function and EBV serostatus and tissue status.

4.4.3 Materials and Methods

This is a single centre, single organ analysis of 89 adult kidney transplant recipients, following diagnosis of PTLD. Study inclusion criteria included age at least 18 years at the time of transplant, a functioning allograft at the time of diagnosis, and transplantation or follow up at Manchester Royal Infirmary or our regional referral centres, Salford Royal Foundation Trust and Royal Preston Hospital.

4.4.3.1 PTLD identification and diagnosis

Cases were identified following referral to our regional Haemato-oncology service, from regional pathology databases, and from the NHS Blood and Transplant service records. Histological diagnosis was confirmed by regional haemato-pathologists where tissue blocks were available, and classified according to the 2008 WHO classification as early, polymorphic, monomorphic or Hodgkin's disease (108). Multiple myeloma was excluded. The presence or absence of EBV in tissue was confirmed using LMP-1 immunohistochemistry and / or EBER in situ hybridization. Recipient EBV serostatus was obtained from medical records where

available, or by analysis for IgG antibodies to EBV VCA and EBNA-1 antigens in archived serum samples from the Regional Tissue Typing Laboratory.

4.4.3.2 PTLD treatment and response

The regional specialised service for PTLD includes multi-disciplinary team case review and a standardised protocol for treatment. Treatment strategy in all patients includes reduction of immunosuppression, including withdrawal of anti-proliferative agents, dose reduction or withdrawal of calcineurin inhibitors, and the introduction of 10mg/day of prednisolone (140). Subsequent timing and use of rituximab (since 2003) and chemotherapy is based on risk, stratified by histological classification, CD-20 status, and the clinical status of the patient (140,251).

4.4.3.3 Outcomes and Survival

A complete response to treatment was defined as the clinical and/or radiological disappearance of all signs and symptoms of disease. Overall survival was calculated as the time from diagnosis to death from any cause, or the end of the follow up period, if alive. Graft survival was defined as the time from diagnosis to the end of the follow up period or date of death or commencement of dialysis and graft failure, if occurring first. Individuals who died whilst retaining graft function were classified as 'death with a functioning graft'.

4.4.3.4 Renal transplant function

Serum creatinine values (μmol/l) were identified at 3-6 months pre-diagnosis (baseline), diagnosis, and following diagnosis at 1 month, 6 months, 1 year, and the last follow up appointment with a surviving graft. Estimated glomerular filtration (eGFR) values were calculated using the modification of diet in renal disease

(MDRD) formula and presented as ml/min/1.73m^2 (255). Acute kidney injury (AKI) was defined as a rise in creatinine from baseline ≥1.5 fold (256).

4.4.3.5 Prognostic models

We applied existing prognostic scoring systems retrospectively to our PTLD population, including the International Prognostic Index (253), and those presented by Leblond et al (257), Ghobrial et al (258), Choquet et al, (254), Hourigan et al, (259), Evens et al, (260) and Caillard et al, (103).

4.4.3.6 Statistical methods

Characteristics of the PTLD population were described using the mean with standard deviation for normally distributed variables, and median with interquartile ranges for skewed data. Group comparisons used Chi-square for categorical variables and Student's t-test for continuous variables. Univariate analysis of factors associated with survival with adjustment for performance status and Ann Arbor stage, (since these parameters are known to affect outcomes), was performed using Kaplan-Meier plots and the log-rank test. A p value of <0.05 was accepted as significant. Statistical analyses were performed using SPSS (IBM SPSS Statistics 20.0.1 March 2012).

4.4.3.7 Ethical approval

Ethical approval was obtained for the study (GM West NW REC 9 ref: II/NW/0001, Protocol 'Pathology of PTLD v1'). Informed consent was obtained from all living subjects for review of tissue blocks and analysis of stored serum for EBV antibodies.

4.4.4 Results

4.4.4.1 Patients

Eighty-nine cases of PTLD were identified in adult patients with functioning renal allografts at the time of diagnosis.

4.4.4.2 Presentation

The median time from transplant to presentation was 81 months with an interquartile range of 31-132 months and range from 1 to 292 months. Thirteen percent (12/89) of cases presented early (within 1 year of transplant), and 77/89 (87%) late (>1 year). Late presentations included 27 cases (30%) occurring more than 10 years, 8 (9%) cases more than 15 years, and 3 cases (4%) more than 20 years post-transplantation. PTLD histology was monomorphic in 72% of cases and EBV tissue positive in 65%. Characteristics of those patients with PTLD are presented in table 4.4.1.

Table 4.4.1 Baseline characteristics of patients diagnosed with PTLD

Characteristic	All patients (n=89)
Mean age at diagnosis years (SD)	48 (14)
Male gender n (%)	62 (70)
Median survival (months) n (IQR)	31 (3-92)
Ethnicity white n (%)	77 (87)
Transplant number 2,3	10 (11)
HLA Mismatch (n = 83)	
0 - 2	38 (46)
3 - 6	45 (54)
Era of Transplantation	
Basiliximab Induction/Triple therapy 2004-current	12 (13)
Tacrolimus, Mycophenolate, Prednisolone 2000-2003	13 (15)
Ciclosporin, Azathioprine, Prednisolone 1990-1999	47 (53)
Azathioprine, Prednisolone, Ciclosporin Pre-1990	17 (19)
Cause of ESRD n (%)	
Diabetes Mellitus	5 (6)
Polycystic	10 (11)
GN/Autoimmune	27 (30)
Reflux/CPN/Cong	9 (10)
Hypertension/Renovascular	6 (7)
Other/unknown	32 (36)
Drugs (transplant related) n (%)	
Basiliximab (induction)	13 (15)
Ganciclovir (CMV disease)	10 (16)

Previous treatment for rejection n (%)	
Methylprednisolone pulse	29 (33)
Anti-Thymocyte globulin/T cell depleting agent	10 (12)
WHO Class (n=84) n (%)	
Early	5 (6)
Polymorphic	14 (16)
Monomorphic	64 (72)
Hodgkin Disease	6 (7)
EBV status	
Histology EBV Positive n (%)	42/65 (65)
Histology EBV missing (%)	24/89 (27)
EBV recipient seronegative n (%)	22/64 (34)
EBV recipient serostatus missing (%)	25/89 (28)
Diagnosis EBV viral load copies/ml (IQR)	3.9 (3.0-4.92)
Risk factors n (%)	
ECOG>/=3	24 (28)
LDH Elevated (43)	22 (51)
Hypoalbuminaemia (<35g/l)	22/57(39)
Lymphopenia ($<1.5 \times 10^9$)	32/55 (58)
Renal Function	
Median creatinine µmol/l (IQR)	162 (120-206)
Median eGFR ml/min (IQR)	41 (27-57)
AKI at diagnosis n (%)	11 (16)
Disease stage Ann Arbor stage 3-4 n (%)	30 (37)
Disease site n (%)	
Single site of disease	31(38)
Extra-nodal involvement	56 (68)
Neck	33 (40)
Oropharynx	17 (21)
GI Tract	19 (23)
Liver	13 (16)

Spleen	13 (16)
Cerebral	5 (6)
Graft	3 (4)
Skin	7 (9)
Bone marrow	6 (7)
B symptoms n (%)	56 (68)

4.4.4.3 Treatment and Response

Treatment regimens for all PTLD patients and for those who achieve a complete response to treatment are shown in Table 4.4.2.

Table 4.4.2 Summary of treatment regimens for all PTLD cases (Column 2) and those with complete response (column 3)

Treatment	**All PTLD Cases n (%)**	**Patients achieving CR n (%)**
No of patients	89	54
Palliation	13 (15)	0
Immunosuppression reduction alone	37 (42)	22 (59)
Rituximab (first-line therapy)	10 (11)	6 (60)
Total Rituximab	23 (26)	15 (65)
Rituximab with CHOP	15 (17)	10 (67)

Total Chemotherapy	37 (42)	26 (70)
Total Surgery	21 (24)	14 (67)
Total Radiotherapy	8 (9)	5 (63)
Cytotoxic T Lymphocytes	2 (2)	2 (100)

4.4.4.4 Complete responders

A complete response (CR) to treatment was obtained in 54/89 (61%) of patients overall and included 4/5 (80%) with early lesion histology, 12/14 (86%) with polymorphic disease, 33/64 (52%) with monomorphic disease and 5/6 (83%) with Hodgkin's disease (Table 4.4.3). In addition a CR was seen in 27/42 (64%) cases of EBV histology positive disease, 16/23 (79%) cases of EBV histology negative disease, (24/89 (27%) EBV status unknown), 31/42 (74%) EBV seropositive recipients with disease and 14/22 (64%) EBV seronegative recipients (25/89 (28%) cases serostatus unknown).

4.4.4.5 Complete response with immunosuppressive reduction alone

Immunosuppressive reduction alone (ISRNA) was successful in inducing a CR in 22/89 (25%) of all cases, 4/5 (80%) early lesions, 5/14 (36%) polymorphic, 12/64 (19%) monomorphic and 1/6 (17%) Hodgkin's. A complete response to ISRNA was seen in both EBV tissue positive PTLD, 11/42 cases (26%), and EBV tissue negative disease, 4/23 cases (17%). Similar response rates to ISRNA were seen in EBV seronegative recipients, 7/22 (32%) cases, and EBV seropositive recipients,13/42 (31%). Descriptive characteristics of patients achieving a CR with

ISRNA included Ann Arbor stage 1-2, 19/22 (86%), performance status ECOG <3 21/22 (95%) and late onset presentation in 20/22 (91%).

Table 4.4.3 Comparison of characteristics of those achieving a complete response to treatment and those failing to achieve a complete response

Characteristic	Complete Responders n=54	Non Responders n=34	p
	n (%)	**n (%)**	
Mean age (at diagnosis)	47 (14)	49(15)	0.68
Male gender	37 (69)	24 (71)	>0.99
Median time from transplantation (months)	105 (46-137)	56 (11-89)	0.01
Early onset disease	4 (7)	8 (24)	0.04
Late Onset disease	50 (93)	27 (76)	0.1
Transplant number 2,3	7 (13)	3 (9)	0.74
HLA MM (n=83)			
0-2	25 (50)	13 (41)	0.50
3-6	25 (50)	19 (59)	0.50
Ethnicity white	44 (81)	32 (94)	0.12
Cause of ESRD			
Diabetes Mellitus	2 (4)	3 (9)	0.30
Polycystic	7 (13)	3 (9)	0.41
GN/Autoimmune	21 (39)	5 (18)	0.029
Reflux/CPN/Cong	8 (15)	1 (3)	0.071
Hypertension/Renovascular	4 (8)	2 (6)	0.57
Other/unknown	12 (22)	20 (57)	0.001
EBV recipient seronegative	14 (31)	8 (44)	0.48
EBV recipient seropositive	31 (69)	10 (56)	0.48
EBV serostatus missing	9 (17)	16 (48)	0.25
VCA+EBNA- serology	13/44 (30)	10/13 (77)	0.01
Basiliximab induction	6 (11)	7 (21)	0.36
ATG (rejection)	6/51 (12)	4/32 (12)	>0.99
Ganciclovir (CMV disease)	6/39 (15)	4/21 (19)	0.72
WHO Class (n=84)			
Early	4 (7)	1 (3)	0.64
Polymorphic	12 (22)	2 (6)	0.08
Monomorphic	33 (61)	30 (88)	0.01

Hodgkin Disease	5 (9)	1 (3)	0.39
Histology EBV +ve	27 (63)	15 (68)	0.79
Histology EBV-ve	16 (37)	7 (32)	0.88
Histology EBV missing	11 (20)	12 (35)	0.19
EBV viral load Median (range) copies/ml	5.3 (1.0-48.0)	61.0 (2.6-70.8)	0.09
EBV viral load >log 4.5 copies/ml	6/31(19)	7/13 (54)	0.04
LDH Elevated (n=43)	12/31(39)	10/11 (91)	0.03
ECOG ≥3	6 (11)	18/30 (60)	<0.0001
Hypoalbuminemia (<35g/l)	12/39 (31)	10/17 (59)	0.05
Lymphopenia (<1.5 x 10^9)	20/39 (51)	11/15 (73)	0.07
Median creatinine (μmol/l)	147 (118-192)	167 (139-276)	0.12
Median eGFR (ml/min/1.73m^2)	44 (28-58)	32 (21-50)	0.08
AKI diagnosis	2/48 (4)	9/21 (43)	0.0002
Ann Arbor stage 3-4	14/52 (27)	16/29 (55)	0.02
Single site of disease	23 (44)	7 (24)	0.04
Extranodal involvement	31 (60)	25 (86)	0.03

4.4.4.6 Failure to achieve a complete response to treatment

Thirty-four patients failed to achieve a clinical response to treatment and died due to treatment complications, with either residual disease or progression of PTLD. The median survival time in this group was 30 days (IQR 0.4-9 months, range), compared to 65 months (IQR 34-120 months, range) for complete responders. Compared to responders, non-responders developed PTLD in a significantly shorter time from transplantation, had higher serum LDH, lower albumin levels at diagnosis, and had greater proportions with early onset (< 1 year), monomorphic, extra-nodal, gastrointestinal tract involvement, performance score ≥3, EBV viral loads >log 4.5, Ann Arbor stage 3-4 and VCA positive / EBNA antibody negative serology (Table 4.4.3).

4.4.4.7 Patient survival

Overall patient survival following diagnosis of PTLD was 72% at 6 months, 67% at 1 year, 54% at 3 years, 52% at 5 years, and 40% at 10 years. Median survival for the cohort was 2126 days (5.8 years) (95% CI 86.25-4165.79 days). Deaths during the first year after diagnosis occurred in 29 patients including 18 (62%) during the first month. The cause of death for these initial 18 patients included gastrointestinal tract PTLD with complications such as perforation, peritonitis and obstruction (9), multi organ failure (2), fulminant primary EBV infection (1), inability to create access for dialysis following acute graft failure in a patient with small bowel lymphoma (1), hemorrhage following diagnostic liver biopsy (1), disseminated PTLD (1), and unknown (3).

4.4.4.8 WHO histological class and outcome

Early Lesions

Five patients were diagnosed with early type lesions, including 3 infectious mononucleosis type and 2 plasmacytic hyperplasia. All early type cases were managed with immunosuppression reduction alone and 4/5 had clinical resolution of their disease. One patient with infectious mononucleosis died 8 days after diagnosis of fulminant disease. Three year overall survival was 80% and median survival 2856 days (95% CI 1608-4105).

Polymorphic

Fourteen individuals presented with polymorphic disease. A complete response was achieved in 12; 5 with a reduction of immunosuppression alone, 3 with immunosuppression reduction followed by rituximab, of which 1 required

subsequent R-CHOP, 1 with surgical resection and subsequent R-CHOP, 2 chemotherapy (unspecified) and 1 immunosuppressive reduction and surgical resection. Relapse was seen in 3 cases who had initial disease involving the CNS, small bowel, and neck and groin nodes respectively. Progressive disease was seen in 2 patients, including 1 with early disseminated disease involving liver and spleen who died after immunosuppression reduction alone, and 1 with large bowel and liver involvement who received 1 dose of rituximab. Three year overall survival was 79%, median survival 2913 days (95% CI 1-6712)

Monomorphic

Monomorphic disease was seen in 64 patients who had a 3 year overall survival of 48% with a median survival of 986 days (95% CI 1-2491). A complete response was achieved in 33/64 (52%) cases, 24/64 (38%) with ISRNA. The most common monomorphic subtype was diffuse large B cell lymphoma which accounted for 26/64 (41%) cases and had 3 year overall survival of 57% and median survival 2047 days (95% CI 356-3738).

T cell lymphoma was found in 4/64 cases including 2/4 with skin involvement and was associated with poor outcomes with 3 year survival 25% and median survival 365 days (95% CI 1-780).

Hodgkin's disease

Six cases of Hodgkin's lymphoma were diagnosed including 2 mixed cellularity, 2 nodular sclerosing, 1 lymphocyte-depleted and 1 unspecified. The lymphocyte-depleted case received palliation only and died soon after diagnosis. The other cases had a complete response to treatment; 1 with immunosuppression reduction

alone, 1 with R-ABVD (Rituximab-doxorubicin, bleomycin , vinblastine and dacarbazine), 2 with ABVD, and 1 surgical resection. One of the cases of mixed cellularity relapsed after initial ABVD chemotherapy at 209 days post initial diagnosis, and following discontinuation of immunosuppression the patient survived and returned to dialysis. Three year survival was 44%, median survival 771 days (95%, CI 1-1657) with 2 deaths due to cardiovascular disease and sepsis.

4.4.4.9 EBV positive histology

No significant difference in patient survival was seen between EBV positive and EBV negative PTLD groups with a three year overall survival of 59% seen in both.

4.4.4.10 Impact of Rituximab on survival

Survival rates since 2003 (the Rituximab era) were 60% at 3 years, median survival 2126 days (95% CI 817.7-3434), compared to those presenting before 2003, 46% at 3 years, median survival 771 days (95% CI 1-3631 days) (p=0.377 log-rank).

4.4.4.11 Prognostic Markers

Markers associated with overall patient survival are presented in table 4.4.4. Univariate analysis was performed initially and markers associated with poorer survival included Ann Arbor score ≥3, ECOG performance ≥3, serum creatinine ≥200µmol/l, eGFR <30 ml/min, acute kidney injury at diagnosis, disease involving extranodal, multiple, skin and gastrointestinal sites, raised serum LDH, hypoalbuminaemia, lymphopenia, EBV EBNA antibody deficiency, viral load >50000 copies and early onset disease. Subsequent analysis adjusted for Ann

Arbor stage and performance status showed better survival in those with disease primarily in the neck or oropharynx, and poorer survival in those with creatinine $\geq$150μmol/l, eGFR <30 ml/min, acute kidney injury at diagnosis, early onset disease, treatment with ATG for rejection before diagnosis and T cell lymphoma.

Table 4.4.4 Prognostic markers and patient survival

Variable	n	3 year survival (%)	Median survival (days)	HR (95% CI)	P (log-rank)	HR (adjusted for Ann Arbor and ECOG) (95% CI)	P (log-rank)
PTLD population	89	54	2126				
Ann Arbor ≥3	30	38	746	1.0 (1.1-3.7)	0.03		
ECOG Score 3-4	24	16	32	5.3 (2.9-9.8)	0.0001		
Acute Kidney Injury	11	0	14	11.6 (5.1-26.6)	0.0001	16.2 (5.3-49.4)	0.0001
Creatinine >200 µmol/l	21/74	38	224	2.2 (1.1-4.3)	0.03	3.5 (1.59-7.65)	0.003
Creatinine >150 µmol/l	41	52	1253	1.0 (1.0-4.0)	0.06	2.2 (1-4.9)	0.04
eGFR <30 ml/min	23	39	333	2. (1.3-5.4)	0.01	4.6 (1.9-11.5)	0.001
eGFR<15 ml/min	5/64	20	54	4.30 (1.6-12.8)	0.02	4.6 (1.1-18.2)	0.055
Early onset disease	12	33	26	2.3 (1.1-4.8)	0.04	2.9 (1.3-6.8)	0.02
Raised Serum LDH	22	46	457	4.3 (1.4-13.1)	0.01	2.3 (0.7-7.6)	0.17
Hypoalbuminaemia	22	33	493	2.4 (1.1-5.1)	0.02	2.0 (0.7-5.5)	0.21
Lymphopenia	32	45	986	2.6 (1.1-6.3)	0.02	2.3 (0.8-6.5)	0.11
EBV Positive PTLD	42	59	2126	1.3 (0.6-2.8)	0.44	1.5 (0.6-3.4)	0.356
EBV Negative PTLD	23	59	3629				
EBV seropositive	42	68	3629	0.8 (0.3-1.6)	0.48	0.8 (0.3-2.1)	0.66
EBV seronegative	22	64	3753				
EBNA antibody -ve	24/58	60	2126	2.6 (1.1-6.3)	0.03	2.3 (0.8-6.6)	0.13
Viral load >50000 copies/ml	13/45	39	210	3.3 (1.3-8.1)	0.01	2.03 (0.7-5.9)	0.19
Monomorphic disease	64	48	991	1.9 (0.9-3.9)	0.07	1.1 (0.4-2.4)	0.98
T cell	4	25	462	1.8 (0.6-5.0)	0.31	7.1 (1.7-29.5)	0.02
ATG	10	48	991	1.5 (0.7-3.5)	0.32	2.7 (1.1-6.3)	0.04
Single site	31	74	3629	0.5 (0.3-1.0)	0.04	1.5 (0.2-11.5)	0.70
Multiple site	51	48	1047	2.0 (1.0-4.0)	0.04	0.7 (1.0-5.4	0.70
Extra-nodal disease	56	49	1047	2.5 (1.2-5.5)	0.01	1.3 (0.6-3.2)	0.51
Oropharynx	17	77	2913	0.3 (0.11-0.9)	0.01	0.2 (0.1-0.9)	0.01
Neck	33	68	2913	0.5 (0.2-0.9)	0.02	0.5 (0.2-1.1)	0.01
GI tract	19	36	238	2.4 (1.2-4.6)	0.01	1.1 (0.5-2.6)	0.78
Skin	7	2	553	2.2 (1.0-5.1)	0.01	2.4 (1-5.8)	0.08

Table 4.4.4: Prognostic markers and patient survival (Kaplan-Meier log-rank). Survival estimates (%) and median survival (days) are given for each variable. HR Hazard ratios for the variables, and hazard ratios adjusted for Ann Arbor and ECOG are given.

4.4.4.12 Prognostic scoring models

We applied published prognostic scoring systems retrospectively to our PTLD population, including the International Prognostic Index (IPI) (age, Ann Arbor, LDH, ECOG, extra-nodal sites) (253), Leblond et al (performance status, number of

involved sites) (257), Ghobrial et al (performance status, monomorphic disease, graft involvement) (258), Choquet et al (age, LDH, ECOG) (261), Hourigan et al (LDH, performance status, B symptoms) (259), Evens et al (CNS involvement, bone marrow involvement and hypoalbuminaemia) (260) and Caillard et al (age, serum creatinine, LDH, PTLD localization, and histology) (103). Results of the survival analysis are presented in Table 4.4.5. In our adult renal transplant patients, models that retrospectively predicted poorer survival for higher risk groups included the IPI, Ghobrial, Evens, Choquet and Caillard score.

Table 4.4.5 Retrospective application of published PTLD Prognostic models to our own population

Prognostic model	Model Score	n	3 yr survival	Median survival (days)	HR (95% CI)	P (log rank)
Caillard	0-1	11	91	1327	Ref	Ref
	2-3	20	59	1075	5.7 (0.7-44.9)	0.1
	4-5	12	36	347	10.7 (1.3-86)	0.03
Choquet	0-1	18	40	2126	Ref	Ref
	2-3	25	88	488	8.6 (2.0-37.5)	0.004
Hourigan	0	9	78	78	Ref	Ref
	1	15	86	69	1.2 (0.2-6.6)	0.83
	2	18	61	44	3.8 (0.8-16.9)	0.08
Ghobrial	0	21	84	3753	Ref	Ref
	1	42	59	3089	2.5 (0.9-6.5)	0.07
	2	21	29	99	5.6 (2.1-15.1)	0.001
	3	1	0	92	10.5 (1.2-92.1	0.04
Evens	0	30	76	3753	Ref	Ref
	1	22	39	319	2.6 (1.2-5.9)	0.02
	≥2	3	33	1042	2.0 (0.4-9.5)	0.38
Leblond	0	7	83	3089	Ref	Ref
	1	38	68	3629	1.6 (0.4-6.8)	0.56
	2	40	42	488	2.8 (0.7-11.8)	0.16
IPI	0-2	27	87	1435	Ref	Ref
	3-4	12	32	457	8.1 (2.3-28.0)	0.001

4.4.4.13 Long-term outcomes after diagnosis of PTLD for Complete responders

Long-term follow up is available for the fifty-four cases of PTLD who had initial complete response to treatment. Median follow up time following diagnosis for these cases was 161.8 months (IQR 86-301). Characteristics of these patients at time of diagnosis are seen in table 3 and their treatment regimens seen in table 4.4.2.

4.4.4.14 Relapse

During follow up 13/54 (24%) of patients experienced a relapse of their PTLD at a median time to relapse of 325 days from diagnosis (IQR: 212-841 days, range) (Table 4.4.6). Late relapse, beyond 3 years after diagnosis, occurred in 2 patients at 6.4 and 9.8 years. Following relapse, outcomes were poor, with 4/13 (31%) of patients returning to dialysis, 3 within 1 year, and 7/13 (54%) dying, including 6 within 1 year of relapse.

Individuals successfully treated with ISRNA were less likely to relapse than those requiring other treatments, (1/22 (4.5%) v 12/32 (38%), OR 0.079, CI 0.009-0.608, p=0.008).

Table 4.4.6 Characteristics of PTLD cases with disease relapse (RL)

Patient	Primary disease site*	WHO class	EBV Tissue Status	Initial Treatment (Primary disease)	Time to RL (days)	IS at RL	1yr Outcome Post RL
1	CNS	Poly	Pos	Rituximab	118	unk	GL, RIP
2	Oropharynx	HL	Pos	ABVD	209	P	Func Graft
3	Groin	Plasma	Pos	RCHOP	169	P	GL, RIP
4	Liver, spleen, neck	Mono	Neg	CHOP	693	T, P	DWFG
5	Neck, groin	Poly	Neg	RCHOP	218	P	Func Graft
6	Oropharynx	Plasma	Pos	ISRNA	956	C, P	Func Graft
7	Small bowel	DLBCL	Neg	RCHOP	2343	P	Func Graft
8	Spinal, orbit, prostate	DLBCL	Pos	RCVP	214	C, P	Func Graft
9	Neck, lung, BM	DLBCL	Neg	CHEMO	726	A, P	DWFG
10	unk	T	Pos	CHOP	3592	Dx (6yr)	RIP
11	Skin	Ana L	Unk	CHOP	496	UNK	DWFG
12	Liver, BM	B	Pos	CHOP	274	C	Func Graft
13	Small bowel	Poly	Neg	Surgery	325	T	Func Graft

*Primary site: CNS, central nervous system; BM, Bone marrow. WHO class: Poly, polymorphic; HD, Hodgkin's lymphoma; Mono, monomorphic; Plasma, plasmablastic; DLBCL, Diffuse large B cell Lymphoma; T, T cell; B, B cell; Ana L, Anaplastic large cell. Initial treatment (primary disease), ABVD, adriamycin, bleopmycin, vinblastin, dacarbazine; RCHOP, rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin, prednisolone; ISRNA, immunosuppressive reduction alone; RCVP, rituximab, rituximab, cyclophosphamide, vincristine, prednisolone. IS at RL, Immunosuppression at Relapse, P prednisolone, C ciclosporin, A azathioprine, Dx dialysis, T tacrolimus. 1yr outcome post RL: GL, graft loss; RIP, rest in peace; FG, functioning graft; DWFG, death with functioning graft.

4.4.4.14 PTLD and the kidney allograft

Graft failure and return to dialysis occurred in 11/54 responders during follow up at a median time of 883 days (2.4 years) (IQR: 326-1019) from diagnosis. Four of the 11 cases (36%) experienced a relapse of their PTLD before graft loss, 3/4 within 1 year of relapse. Death censored graft survival rates in patients with a complete

response to treatment were 96% at 1 and 3 years, 93% at 5 years, and 72% at 10 years. Graft failure was seen more frequently in men (10/37 v 1/17, OR 8, CI 0.92-69.24, p=0.039), those with a DR mismatch (5/16 v 3/29, OR 3.94, CI 0.79-19.43, p=0.054), previous CMV disease treated with ganciclovir (3/6 v 4/33, OR 7.3, CI 1.1-49.0, p=0.059) and serum creatinine >200µmol/l at diagnosis of PTLD, (5/11 v 5/39, OR 5.7, CI 1.2-25.7, p=0.030).

In individuals who had complete response to initial treatments we found no significant difference in kidney function between baseline and time of diagnosis. Kidney function improved significantly between diagnosis and 1 month post diagnosis from time of diagnosis to 1 month, +4.7 ml/min/1.73m^2 (0.8-8.6), p=0.02, and no significant deterioration was seen overall at 1 year post diagnosis (table 4.4.7).

Table 4.4.7 Renal function in complete responders (n=54)

Renal function measure	Mean value (SD)	Reference comparison	Mean difference (95% CI)	p
Creatinine*				
baseline	160 (61.9)			
diagnosis	165.1 (72.2)	Baseline	6.1 (-5-17.1)	0.28
1 month	150.7 (64.4)	Diagnosis	-11.9 (-22.1-1.7)	0.02
1 year	152.4 (56.9)	Diagnosis	-0.30 (-13.5-14.1)	0.97
eGFR*				
baseline	46.7 (18.7)			
diagnosis	45.8 (18.8)	Baseline	-1.1 (-4-1.9)	0.48
1 month	51.1 (21.9)	Diagnosis	4.7 (0.8-8.6)	0.02
1 year	49.3 (22.4)	Diagnosis	1.6 (-3.2-6.5)	0.50

*Creatinine measurements are presented in µmol/l and eGFR (estimated glomerular filtration rate) in ml/min/1.73m^2

4.4.4.15 Management of long-term Immunosuppression

Immunosuppression at time of diagnosis (n=89), time of complete response (n=54) and for long-term survivors with complete response and graft survival >3 years after diagnosis (n=34) is given below in table 8. Immunosuppressive data for 4/34 long-term survivors (graft survival >3years) is missing but for the remaining 30, at last follow up, 12/30 were receiving monotherapy and 18/30 dual therapy. Monotherapy including calcineurin inhibitor alone (8) and prednisolone alone (4), and dual therapy, calcineurin inhibitor and prednisolone (7) and antiproliferative and prednisolone (11).

Table 4.4.8 Immunosuppression at diagnosis and after PTLD

Immunosuppression	**Time of Presentation**	**Time of Clinical response**	**Last follow up**[1]
n	89	54	34
Missing	2	4	4
No immunosuppression	0	1 (2)	0
Monotherapy	32 (37)	23 (46)	12 (40)
Dual	30 (34)	26 (52)	18 (60)
Triple	25 (29)	0	0
Ciclosporin cont	52 (60)	16 (32)	9 (30)
Tacrolimus cont	29 (33)	15 (30)	6 (20)
Calcineurin inhibitor	81 (93)	31 (62)	15 (50)
Mycophenolate cont	15 (17)	1 (2)	1 (3)
Azathioprine cont	28 (32)	9 (18)	9 (30)
Antiproliferative	43 (49)	10 (20)	11 (37)
Prednisolone cont	43 (49)	34 (68)	22 (73)
Sirolimus cont	0	0	1 (3)
Ciclosporin dose(μg/l)	250 (175-350)	150 (88-188)	175 (125-200)
Tacrolimus dose (μg/l)	5.5 (4-6.25)	3.5 (2.3-5)	3 (2.5-7)
Mycophenolate (mg)	1500	500	1000

Azathioprine (mg)	100	50	100 (50-125)
Prednisolone (mg)	5	10	7.5 (5-10)

[1]**Graft Survival >3yrs**

4.4.5 Discussion

We performed a retrospective analysis of 89 adult renal transplant recipients diagnosed with PTLD. The study population was predominantly white (87%) with other baseline characteristics similar to recent published registry series for adult renal transplant cohorts with PTLD except only 12% had received T cell depleting antibodies pre-diagnosis compared to 79% in the most recent French series (103,104,251,262,263). Our overall survival rates are very similar to those reported recently by the French registry, 53% at 5 years (103) and the PTLD-1 trial reported by Trappe et al, 5 year survival of 57% overall (251), which including only 29/70 patients with kidney transplants and excluding individuals with severe organ dysfunction or performance status at baseline.

Attention has focused on the remission rates and overall survival in those managed with immunosuppressive reduction alone. Registry data for PTLD in solid organ transplant recipients from Reshef et al and Tsai et al report variable response rates from 37% to 63% with overall median survival rates of 2.8 - 3.7 years and relapse in up to 17%. Those with bulky disease, increasing age and higher stage were less likely to respond to ISRNA. The study also found no difference between EBV positive and negative PTLD in terms of either response to immunosuppression

alone or survival (248,249). A treatment strategy of immunosuppression reduction alone was successful in inducing remission in 25% of all cases in our series. Characteristics of responding patients included those with early lesions, lower Ann Arbor stage and good performance status.

Since 2003 rituximab has been used in our unit with no significant identifiable improvement in overall patient survival as yet. Complete response rates for rituximab alone are reported from 20%-42% and up to 68% remission after rituximab and sequential CHOP chemotherapy most recently (246,248,251,254). While complete response rates with rituximab alone may be relatively low the use of reduction of immunosuppression with rituximab initially may result in improvements in kidney function, reduction in size of the tumour burden and potentially lower rates of subsequent chemotherapy related toxicity and morbidity (251).

Clinical and biochemical prognostic markers identified in renal and solid organ transplant recipients with PTLD are numerous and include age >55 years, raised serum LDH, poor performance status, high Ann Arbor stage, disease involving multiple sites, CNS disease, monomorphic disease, presence of B symptoms, as well as biomarkers such as creatinine >133umol/l, hypoalbuminaemia and lymphopenia (8,103,248,254,257–260,263,264). In our population with initial unadjusted univariate analysis, we also identified early onset disease, EBV VCA positive / EBNA antibody negativity, viral loads >50,000 copies/ml, and those with gastrointestinal tract disease having poorer outcomes. EBV status of histology and EBV recipient serostatus at time of transplant were not significantly associated with

survival in our series. Those with disease in a single site or predominantly involving the neck or oropharynx had better outcomes. A second univariate analysis adjusted for Ann Arbor stage and performance status found a poorer prognosis for T cell disease, previous treatment with ATG, and skin disease, with a better prognosis for those with disease predominantly affecting the neck and oropharynx. An important finding of our study was the importance of renal allograft function at time of PTLD diagnosis to subsequent overall survival. Until recently kidney allograft function had not been identified in PTLD as a prognostic marker (103). In our patients a serum creatinine at diagnosis of >150μmol/l (p=0.004), >200μmol/l (p=0.003), eGFR<30 ml/min/1.73m^2 (p=0.011), and AKI at presentation (p<0.0001), were all highly predictive of poor outcome on both initial analysis, and analyses adjusted for performance status and Ann Arbor score. Poor renal function makes the choice and dosing of chemotherapy problematic and increases the likelihood of treatment related toxicity. In other settings poor kidney function and AKI is associated with higher mortality rates and higher rates of infective complications (256). Understanding the causes of death in these individuals with poor kidney function is necessary to allow the development of strategies to improve survival, including timing and choice of immuno-chemotherapy, preparation for dialysis, aggressive surveillance for sepsis or treatment toxicity, and focus on earlier disease detection, rapid diagnosis and prompt intervention. Exploration of kidney function as a prognostic marker in other renal transplant and solid organ transplant populations would seem sensible.

Identification and adoption of a standardized prognostic scoring system to guide clinical management and use in clinical trials in PTLD seems important. The recent paper from Caillard et al (103) and our own work would suggest that such scores may need to be organ specific and should include measures of kidney allograft function. More work is needed to look at the importance of kidney function in other solid organ populations.

This study has shown that while kidney function at diagnosis is relevant to overall prognosis, graft function in general, at least in the first year of treatment, despite immunosuppressive reduction, is well preserved. Trappe et al previously showed improvement in graft function in renal transplant recipients treated with chemotherapy for PTLD during the first year after diagnosis (262). We found significant improvements in graft function at one month after diagnosis which may relate to weaning or withdrawal of calcineurin inhibitors and an increase in renal perfusion (265) (table 4.4.7). Further graft function in our patients did not significantly deteriorate out to 1 year after diagnosis despite immunosuppressive reduction and adjuvant immuno-chemotherapy. There is little guidance currently concerning optimal long-term immunosuppressive policy to preserve graft function after obtaining remission from PTLD. The risk of disease relapse or progression needs to be balanced against the risks of graft loss. Work looking at choice and dose of immunsuppressive agents is needed. Relapse occurred in 24% of our complete responders most within 1 year of diagnosis, median time of 325 days and other studies report relapse rates of 17-43%, again with most cases occurring in the first year (249,264). Immunosuppressive reduction alone in our patients

resulted in disease remission in 25% of all cases with relapse in only 1 patient and graft loss during the follow up period was seen in only 3/22 (14%) of these patients with a median follow up (overall survival) time of 6.6 years (IQR:4.3-10). In patients receiving adjuvant treatment including rituximab and chemotherapy relapse occurred in 12/32 (38%) and graft loss in 8/32 (25%). Higher rates of graft loss in patients requiring additional treatment may reflect the fact disease in these individuals was more aggressive, perhaps more likely to relapse with subsequent need for further weaning or withdrawal of immunosuppression.

Immunosuppression in 34 individuals with complete response and graft survival > 3 years after PTLD diagnosis, included 27% on CNI monotherapy, 30% on azathioprine and prednisolone, and 13% on prednisolone alone. In our series, we saw no significant differences in rates of graft deterioration at 1 year or during longer term follow up, between CNI monotherapy, CNI and prednisolone, CNI and anti-proliferative, or anti-proliferative and prednisolone. However, decline in renal function was rapid in those on prednisolone alone, though this strategy may well be justified in the setting of aggressive, extensive or refractory disease. Close surveillance for these patients is important, with attention to change in graft function, investigation of cause, and prompt and timely consideration to dialysis access planning.

4.4.6 Conclusions

We show that measures of graft function in particular eGFR <30ml/min/1.73m2 and AKI at diagnosis are highly associated on adjusted analyses for poor survival rates

and provide useful prognostic information in addition to current prognostic models. Further immunosuppressive reduction alone can result in complete remission of disease in many cases and allows significant improvement in graft function in the early period after diagnosis, perhaps due to reduced calcineurin inhibitor activity on renal perfusion. Identification of those who require aggressive treatment including combination rituximab and chemotherapy early on remains difficult and seeking to reduce the high mortality rates in the first month after diagnosis a challenge.

4.4.7 Acknowledgements

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5 Summary and Discussion

PTLD is a serious malignant complication of transplantation and EBV, a human herpes virus associated with PTLD pathogenesis, identified as a marker of over-

immunosuppression requiring intervention. Current guidelines recommend screening for EBV naive individuals pre-transplant with subsequent testing for EBV DNAemia and pre-emptive immunosuppressive reduction to avoid PTLD development and EBV related disease during the first post-transplant year.
The prevalence and and clinical implications of EBV DNAemia in adults, particularly in the late post-transplant period, are not yet well described or studied and the key research question of this thesis was as follows:

What is the clinical significance of EBV infection in adult renal transplant recipients when detected in the late post-transplant period?

A key finding of the studies reported above was the identification of time from transplant as being a factor associated not just with an increasing risk of PTLD development but also EBV DNAemia in otherwise stable transplant recipients. This association of time with EBV and PTLD suggests that duration of exposure to immunosuppression may be more important than total dose of immunosuppression at any one time in contributing to the development of innate and adaptive immune defects that may predispose to chronic viral infection and tumorigenesis. In keeping with this hypothesis was the finding that DNAemia was associated with other factors that identify individuals exposed to immunosuppression for long periods of time including a history of skin cancer, warts and immunosuppressive regimens consistent with older transplant era's including use of azathioprine, ciclosporin and prednisolone. Further DNAemia was not associated with higher ciclosporin or tacrolimus levels and was seen significantly more often in those

receiving anti-rejection monotherapy compared to dual or triple therapy. A late peak in PTLD incidence was identified in our series that was not related only to EBV associated disease as the proportion of EBV tissue negative cases also increased with time from transplant. Further, 15% of all our late cases of PTLD had no detectable EBV DNA in their blood at the time of diagnosis and cases of EBV tissue negative and whole blood EBV DNA negative PTLD developed during follow up in those with a history of high level EBV DNAemia during the study period. Again such findings suggest that while EBV DNAemia may be a marker of long-term immunosuppression it may not directly identify a patient with or developing disease. Not only did the prevalence of DNAemia increase significantly with time from transplant, detected at baseline in 40% of those transplanted for 10-14 years and 66% of those transplanted for between 20-24 years, but it's detection was actually "common". Across the whole recruited population 46% stable individuals had 1 or more sample with detectable EBV DNA in blood in the absence of PTLD and 16% had persistent and high viral load detection over the course of 1 year. At three years of follow up 6 patients from the EBV study cohort developed PTLD, only 1 within 10 years of transplant (mean 16 years) and of these only 1/6 had a history of chronic high viral load detection and 2 were EBV negative PTLD.

In relation to the implications of EBV DNA detection in otherwise stable transplant recipients we found no significant differences in overall patient survival or graft survival at 3 years. Similarly, there was no difference in clinical symptoms including frequency of B symptoms, in clinical findings such as anaemia, thrombocytopenia, lymphocytosis, raised liver enzymes such as alkaline transferase or rate of decline

in renal function between those stable transplant recipients without detectable EBV DNA and those with detectable and even persistent high level DNAemia. The indications for pre-emptive reduction of immunosuppression in late transplant recipients with DNAemia remain unclear.

The incidence of PTLD also increased with time from transplant in our series and was greatest during the 10th-14th post-transplant years. EBV recipient serostatus at time of transplant and EBV status by histology had no statistically significant associations with overall patient survival or complete response rates in our series. On univariate analysis EBV VCA antibody positive EBNA antibody deficient individuals and those with high viral loads at presentation had poorer response rates. It is of interest that EBV negative PTLD responded to immunosuppressive reduction alone as well as EBV positive disease suggesting again that mechanisms behind late post-transplant lymphoma may not be virus specific but more a reflection of immune defects, perhaps T cell exhaustion of a more global nature.

It is clear however that EBV seronegative recipients have an increased risk of lymphoma compared to seropositive recipients. This risk is greatest during the first post-transplant year but studies have also shown an on-going and persistently increased risk for PTLD even in the later post-transplant period. In our PTLD series seronegative recipients accounted for >50% late PTLD cases despite making up only 5-10% of the adult transplant population as a whole. The data presented in this thesis (chapter 4.1) showed that EBV seronegative recipient status at time of transplant was associated with EBV DNAemia and also with chronic high viral load

carriage. In the 6 cases of PTLD that developed during follow up, the single case that was identified in an individual with chronic high viral load carriage during follow up occurred in a patient who was EBV seronegative at transplant. It seems logical perhaps that these seronegative individuals should remain under close clinical surveillance for the duration of their transplant life with education, EBV DNAemia screening and close clinical surveillance. The case for late screening of seronegative recipients, certainly with PCR for EBV DNA detection, is not yet proven.

To further investigate mechanisms and factors associated with EBV DNAemia a sub-study involving 60 patients reported in paper 2 above was performed. To investigate relationships between specific immunosuppressive agents and DNAemia, in particular the association of MMF and lymphopenia with low rates of DNA detection, we performed lymphocyte subset testing analysis. We anticipated that MMF treated individuals would have the lowest levels of CD 19 positive B cells corresponding to low levels of EBV containing B lymphocytes (266)(267). MMF use has been associated with low incidence of EBV DNA detection post-transplant (127,129). We found that those on immunosuppressive regimens containing azathioprine had significantly the lowest CD 19 positive numbers and also the lowest lymphocyte counts. Despite this, azathioprine was not found in data presented in chapter 4.1 to be significantly associated with low levels of DNAemia. CD 19 positive cells were greatest amongst those with high viral load detection but were not significantly different to other groups. Individuals treated with MMF had low but not significantly lower CD 19 positive cells than others. CD 8 positive cells

were highest in high viral load carriers and CD4:8 ratio's were also highest in those with undetectable DNA. Again, these were not significant differences but would fit with our knowledge of T cell responses to EBV infection (65,70,268). We found no significant differences in relation to age, time from transplant or risk of skin cancer for the different lymphocyte subsets in this small number of patients.

The association of low EBV DNAemia rates with use of MMF and the absence of registry data showing increased risk of PTLD with MMF suggest that studies to investigate MMF as the maintenance agent of choice in the setting of EBV tissue positive PTLD or for higher risk EBV seronegative transplant recipients to prevent PTLD or EBV infection may be worthwhile.

Azathioprine treated individuals have low B cell, NK and CD4 positive cells and consideration should be given to routine lymphocyte subset testing particularly in the late post-transplant period to weigh up risk of opportunistic infection and adverse events. There may be scope to reduce or optimise immunosuppressive drug dosing in the setting of EBV DNAemia as, in the data presented in paper 4.2, those with high viral loads had significantly higher tacrolimus and ciclosporin trough levels than those with no EBV DNA detection. High EBV viral load carriers may have greater numbers and increased size of sub-clinical lymph nodes in the neck than those with low viral loads or those with undetectable viral loads. Further work is needed in following up these patients, to re-assess for change in size and number of these nodes and to evaluate simple ultrasound of the neck as a screening tool to identify those at higher risk of EBV related disease or PTLD.

In both the PTLD and EBV prospective studies markers of the high risk patient, and those with poorer survival rates, included those with poor performance status including ECOG and Karnofsky score. Among those with the worst outcomes however were patients with poorer kidney function such as those with eGFR<30 ml/min/1.73m^2 and those with acute kidney injury at time of PTLD diagnosis. Poor renal function makes the choice and dosing of chemotherapy problematic and increases the likelihood of treatment related toxicity. In other settings poor kidney function and AKI is associated with higher mortality rates and higher rates of infective complications (256). Understanding the causes of death in these individuals is important to allow the development of strategies to improve survival, including timing and choice of immuno-chemotherapy, preparation for dialysis, aggressive surveillance for sepsis or treatment toxicity, and focus on earlier disease detection, rapid diagnosis and prompt intervention. Exploration of kidney function as a prognostic marker in other renal transplant and solid organ transplant populations would seem sensible.

5.1 Strengths and weaknesses

Epstein-Barr Virus Infection in Adult Renal Transplant Recipients

This was a large study involving 499 patients. It has a high level of detail in relation to transplant, biochemical and clinical data and benefits from comprehensive and careful follow up of all participants for outcome. A limitation of the study is that

prevalence of EBV DNAemia in stable transplant recipients was analysed over a 1 year snapshot period only. There is no data on EBV DNAemia for individuals before entering the study or following study completion. Outcomes of the study at 3 years are based on DNAemia during the 1 year screening period only. Longer term screening would be helpful but is limited by financial and logistical constraints. The study protocol and findings are still valid as participants were clinically stable at recruitment and for most, once established on maintenance immunosuppression, major changes in regimen would not be anticipated.

Individuals recruited to the study include those transplanted in different eras of immunosuppression with participants at recruitment receiving a large range of different agents and immunosuppressive regimens. This approach is justified as it gives insight into the prevalence of EBV DNAemia in an adult transplant population as a whole, including those transplanted more than 20 years ago, rather than just those transplanted most recently on “modern” immunosuppressive regimens. Further DNAemia prevalence was analysed in relation to current immunosuppression at time of study recruitment, although analysis of previous treatments for rejection including a history of ATG or methylprednisolone pulse was performed. For comparison with other kidney transplanting centres 45% of the EBV study had received basiliximab induction and only 6% ATG for rejection. Our study populations are predominantly of white ethnicity, 93% EBV study and 87% PTLD study.

Recruited individuals did not have a uniform number of samples for EBV DNA detection. In the 53/499 patients with insufficient samples for pattern analysis,

reasons for inadequate sampling included death (n=10), return to base hospital (n=23), return to dialysis (n=2), and missed sampling/lost to follow up (n=18). There is no reason to suspect bias in this population as levels of DNAemia prevalence at recruitment in this group were was not significantly different to those with adequate serial sampling. Pre-transplant recipient EBV serostatus was missing for a small number of individuals 48/499 (10%) and 9/499 (2%) at recruitment.

Analysis of viral, immunological and clinical differences amongst adult renal transplant recipients with undetectable, low level and chronic high level EBV DNAemia

This study recruited from the larger observational EBV prevalence study and allowed patient selection to be informed and guided by the EBV DNAemia patterns of individuals over the previous year. A single centre approach and recruitment by a single clinician with a comprehensive data proforma allowed collection of detailed laboratory and transplant data but also a full history of infective and malignant complications occurring post-transplant in those involved.

Only a single assessment was made of lymphocyte subsets in relation to EBV DNAemia and other clinical markers. Repeated assessments may be sensible in future studies to follow trends and ensure reproducibility.

Epidemiology of Post-Transplant Lymphoproliferative Disorder in Adult Renal Transplant Recipients

This was a comprehensive analysis of the largest reported UK based single centre, single organ series of PTLD cases. The single centre approach allowed detailed data collection and careful follow up of all known recipients. The series included cases transplanted during different era's of immunosuppression and did not just focus on incidence of PTLD in the "modern" era of triple therapy. This is important as we have shown PTLD incidence increases with time from transplant and those many years from transplant may be at the greatest risk. The data is relevant to units caring for adult renal transplant recipients but it is important to note that very few of our patients had received T cell depleting antibodies for induction or rejection.

Individuals whose first presentation of PTLD followed the onset of graft failure and return to dialysis were not included in the study. While most wean or discontinue immunosuppression, a number remain on low level immunosuppression and retain their graft, a potential site of chronic inflammation, and may continue to have an increased risk of lymphoma development. Comparison with rates of lymphoma in dialysis patients and individuals with failed kidney transplants on dialysis would be interesting. Patients aged <18 years at the time of PTLD diagnosis, and/or the time of transplant were also excluded from this study. Data and characteristics of late presenting PTLD in the paediatric group are also lacking.

Statistical analysis of differences in characteristics between patient sub-groups is limited by the small numbers of cases. This is reflected in the wide confidence intervals seen, for example, in comparisons between early and late onset disease. In both the PTLD epidemiology paper (Paper 4.3) and the paper analyzing outcomes of PTLD (Paper 4.4) EBV serostatus at time of transplant and EBV status of histological samples were missing in approximately 28% of cases. EBV data could not be collected if absent from medical records and where stored serum or histology blocks were not available for analysis. In relation to bias attached to the missing data, EBV serostatus and tissue status was more complete where PTLD presentations occurred after the year 2000; for example serostatus 84% complete post 2000 v 67% pre 2000 (Paper 4.4). Results of analyses where missing data was excluded should therefore if anything be biased in favour of more recent experiences than pre 2000. In relation to PTLD outcome data no significant difference in complete response rates or 1 year, 3 year or median survival rates was seen between cases with available EBV tissue status and those with missing data. However survival was worse overall at 1 year and 3 years amongst those with missing EBV serostatus, 40% v 77% at 1 year and 23% v 66% at 3 years (log-rank p=0.001). As the majority (84%) of cases with missing serostatus data presented before the year 2000 this may reflect an improvement in recent times in management of PTLD and survival rates. In the analyses presented in the thesis in paper 4.4 we found no significant improvement in survival following the introduction of rituximab. This may be biased by the missing data or may suggest that if survival has improved improvement in the last ten years or so that this may be due to an

improvement in all aspects of PTLD detection and treatment rather than purely the introduction of rituximab.

Post-Transplant Lymphoproliferative Disorder in Adult Renal Transplant Recipients: Treatment, Response, Survival, and Prognosis

This study, again being single centre, includes high levels of detail and descriptive data and careful follow up of all identified cases for outcome. It complements the previous paper in relation to incidence. Analysis of subgroups in relation to response rates, survival and graft function is limited as these groups may include only small numbers of individuals. Data and bias in relation to missing EBV serostatus at time of transplant and histological EBV status is discussed above.

Implications for Clinical Practice

This study aimed to answer a series of key clinical questions (page16) while focusing on the key research question. These clinical questions are detailed below with answers and recommendations following the completion of the study.

Key clinical questions

1. What is the prevalence of EBV DNAaemia in adult renal transplant populations?

In a population of 499 stable adult renal transplant recipients recruited from clinic, 31% had EBV DNA detectable in blood (>1000 copies/ml) at baseline including 9% with viral loads >10,000 copies/ml. Persistent detection of DNA was seen in 16% while 52% had no detection of DNA over a 1 year screening period.

2. Are rates of EBV DNAemia influenced by time from transplantation?

Prevalence of DNAemia increased significantly with time from transplant from 16% of individuals within 1 year of transplant to 40% of those 10-14 years and 66% of those 20-24 years post-transplant. Chronic high viral load detection was also significantly associated with increased time from transplant.

3. What clinical, viral, and immunological factors are associated with EBV DNAemia?

EBV DNAemia was associated with increasing time from transplant and other markers of long term immunosuppression including skin cancer and warts on univariate analysis. With adjustment for time from transplant, DNAemia was associated with EBV recipient seronegative status at transplant, non-white ethnicity and a previous diagnosis of PTLD while current MMF use and lymphopenia were associated with a low prevalence of DNAemia. Detection of

EBV DNA in plasma was seen only in those with high viral loads in whole blood. While low levels of DNAemia were associated with MMF use and lymphopenia no direct association between T, B or NK lymphocyte subset absolute count and viral load was detected. Individuals with chronic high viral load detection appear to have greater numbers of sub-clinical nodes >5mm on ultrasound examination of the neck than those without DNAemia.

4. What are the clinical outcomes and implications of EBV DNAemia particularly in the late post-transplant period?

No significant difference in overall patient survival, graft survival or graft function decline at 3 years from recruitment was seen between those with and without detectable EBV DNAemia at recruitment. Individuals with DNAemia at recruitment appeared to have an increased risk of PTLD during follow up but only 6 cases occurred. The higher the initial viral load at recruitment the more likely an individual would be to have detectable DNA at the end of the screening period. No significant difference in clinical symptoms, in particular B symptoms, prevalence of haematological abnormalities or liver enzyme abnormalities, occurrence of major or opportunistic infections was seen between individuals with and without DNAemia.

5. What is the incidence of PTLD in UK based adult kidney transplant recipients managed with immunosuppressive regimens following NICE guidelines?

PTLD incidence was 2.6 cases per 1000 patient years in our population with Non-Hodgkin's lymphoma occurring approximately 8 fold higher than that of the general population. Rates increased with time from transplant and were greatest at 10 to 14 years post-transplant.

6. Is EBV infection associated with incidence, timing or outcome of PTLD?

EBV seronegative status at transplant is associated with increased risk of PTLD. This study showed an association of seronegative status with early onset disease. No significant difference in complete response rates or overall patient survival was seen in relation to EBV serostatus at time of transplant or EBV status of PTLD histology at diagnosis in our series. EBV histology negative disease responded to immunosuppressive reduction alone in some cases as well as EBV positive disease. Individuals with EBV VCA antibody positive, EBNA antibody negative status and viral loads $>\log^{10}4.5$ copies/ml at diagnosis had poorer complete response rates to treatment.

5.2 Final Conclusions

EBV DNAemia in stable kidney transplant recipients is common and prevalence increases significantly with time from transplant ($p<0.0001$), from 16% in those within 1 year of transplant to 66% of those between 20-24 years. High baseline DNA levels predicted persistence of DNAemia while time adjusted analyses show significant association of DNAemia with EBV seronegative status and previous PTLD and low DNAemia rates with Mycophenolate Mofetil (MMF) use and lymphopenia. The mechanism did not appear to be directly linked to MMF induced B cell depletion. Chronic high viral load detection was significantly associated with time from transplant, EBV seronegative status at transplant, ciclosporin use and plasma detection of DNA. No significant differences in overall patient survival at 3 years, clinical symptoms or clinical findings such as anaemia, thrombocytopenia or rate of decline in renal function were seen between stable transplant recipients with and without EBV DNAemia. PTLD incidence was also found to increase with time from transplant and was greatest during the 10th-14th post-transplant years. Disease was EBV positive in 68% cases. No statistically significant differences in overall patient survival, or overall disease complete response rates were seen in relation to recipient EBV serostatus or EBV status of PTLD histology.

6.0 Future work

As adult kidney transplant recipients increase in number and survive for longer with functioning grafts strategies to detect, avoid and successfully treat infective and malignant complications of immunosuppression will become increasingly important. In relation to EBV associated early onset PTLD, work to develop a vaccine against the virus and pre-emptive measures in high risk individuals to avoid disease onset and progression will be required. As with the recent French study from Bamoulid et al (146) further assessment of pre-emptive strategies such as IV immunoglobulin at time of transplant and pre-emptive rituximab and or immunosuppressive reduction for high risk individuals with DNAemia during the first post-transplant year is required. A prospective interventional study investigating the risks and benefits of immunosuppressive reduction in healthy individuals with EBV DNAemia in the late post-transplant period may be worthwhile to help determine if a risk of graft loss or graft function decline due to inadequate immunosuppression outweighs the risks of persistent DNAemia in otherwise well individuals. In relation to late EBV DNAemia and late PTLD greater understanding is required of the innate and adaptive immune defects that develop as a consequence of the prolonged administration of immunosuppression. As part of the EBV studies, peripheral blood mononuclear cells were obtained and frozen from participants including those with high viral load carriage. Further assessment and comparison between individuals with PTLD at time of diagnosis, persistent and low grade EBV detection in relation to phenotype, number, function and interaction of T, B and NK cells may prove useful. Investigation of the “exhausted autoimmunity” concept where chronic antigenic stimulation may result in progressive loss of function in CD8 positive T cells should

be explored further in the adult renal transplant population including those with and without evidence of EBV DNAemia (269,270).

A 5 year assessment of outcomes in relation to patient and graft survival and PTLD incidence is planned for those 499 individuals recruited to the observational EBV prevalence study. While these outcomes are based on EBV DNAemia identified in the one-year study period, insight into long-term implications of DNAemia will be obtained. Serum and DNA extract samples from all individuals in the EBV study are frozen and stored. Analysis of prevalence of other herpes virus DNAemia including CMV, HSV-1 and HHV-6 could be performed, again focusing on the late post-transplant period as well as late prevalence of the polyoma virus BK.

More recently attention has focused on viral microRNA expression which may directly contribute to tumour pathogenesis and B cell proliferation (271). Investigation of miRNA function and phenotype in the setting of PTLD and post-transplant EBV infection may be interesting.

7 Academic output

1st author publications

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., et al. (2013). Epidemiology of Posttransplantation Lymphoproliferative Disorder in Adult Renal Transplant Recipients. *Transplantation*, *95*(3), 470–478. doi:10.1097/TP.0b013e318276a237

1st author abstract publications

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., Picton, M. L. Identification of prognostic markers in renal transplant recipients with PTLD. *American Journal of Transplantation*, 2012, vol 12, suppl 3

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., Picton, M. L. Factors Associated with EBV DNAemia in Stable Adult Renal Transplant Recipients. *American Journal of Transplantation*, 2012, vol 12, suppl 3

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., Picton, M. L. Incidence and Timing of PTLD in EBV Seropositive and Seronegative Adult Kidney Transplant Recipients. *American Journal of Transplantation*, 2012, vol 12, suppl 3

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., Picton, M. L. Increasing Prevalence of EBV DNAemia with Time from Transplant in Stable Adult Renal Transplant recipients. *American Journal of Transplantation*. 2011. Vol 11,Suppl 2, 287

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., Picton, M. L. Screening for Specific Symptoms Does Not Usefully Identify

EBV Infection in Stable Adult Renal Transplant Recipients. *American Journal of Transplantation*. 2011. Vol 11,Suppl 2, 287

Oral presentations

Post-transplant Lymphoproliferative Disorder. Renal Transplant Nurses Masterclass, Birmingham 2012.

Presentations and prognostic markers in adult renal transplant recipients. British society of transplantation annual meeting, Glasgow 2012

Identification of prognostic markers in renal transplant recipients with PTLD. American society of transplantation annual congress, Boston 6th June 2012

Early mortality after PTLD. EFTW Edinburgh (ESOT) 25/09/2009

Poster presentations

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., Picton, M. L. Factors Associated with EBV DNAemia in Stable Adult Renal Transplant Recipients. American Society of transplantation Annual Congress 2012. Poster of Distinction

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., Picton, M. L. Incidence and Timing of PTLD in EBV Seropositive and

Seronegative Adult Kidney Transplant Recipients. American society of transplantation Annual Congress 2012. Poster of distinction

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., Picton, M. L. Increasing Prevalence of EBV DNAemia with Time from Transplant in Stable Adult Renal Transplant recipients. American society of transplantation Annual congress. Philadelphia 2011

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., Picton, M. L. Screening for Specific Symptoms Does Not Usefully Identify EBV Infection in Stable Adult Renal Transplant Recipients. American Society of Transplantation Annual Congress. Philadelphia 2011

Morton M, Picton M. L. Renal Allograft Survival After Post-transplant Lymphoproliferative Disorder. American Society of nephrology Annual Congress, San Diego 2009

Morton, M., Coupes, B., Roberts, S. A, Klapper, P. E., Byers, R. J., Vallely, P. J., Ryan, K., Picton, M. L. Prevalence And Implications Of EBV Detection And Viral Load In Stable Adult Renal Transplant Recipients. British Society of transplantation Annual meeting, Bournemouth 2011

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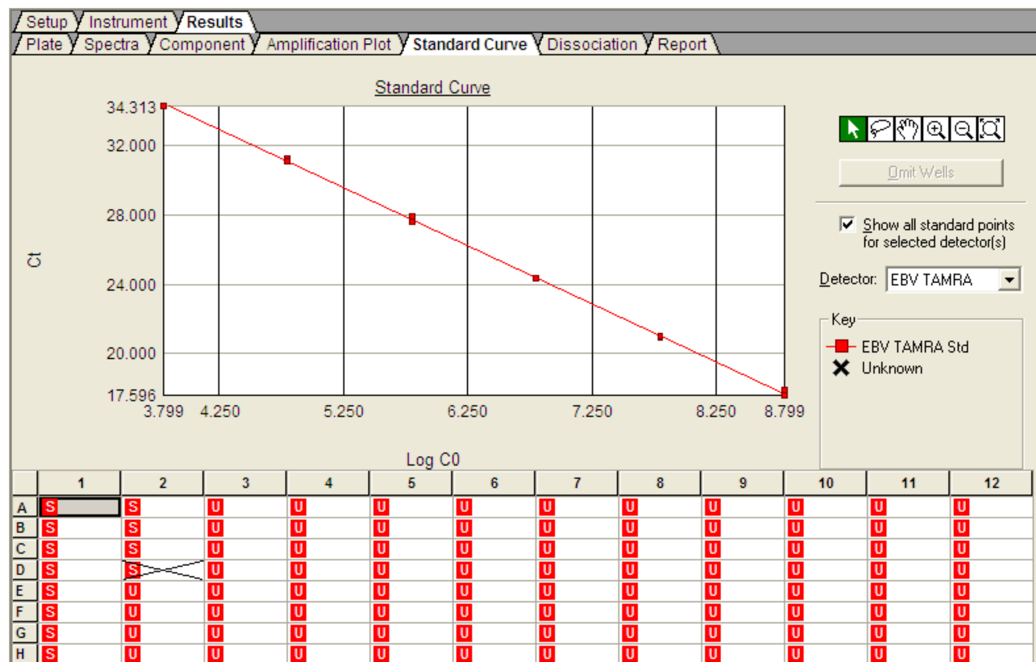
9 Appendices

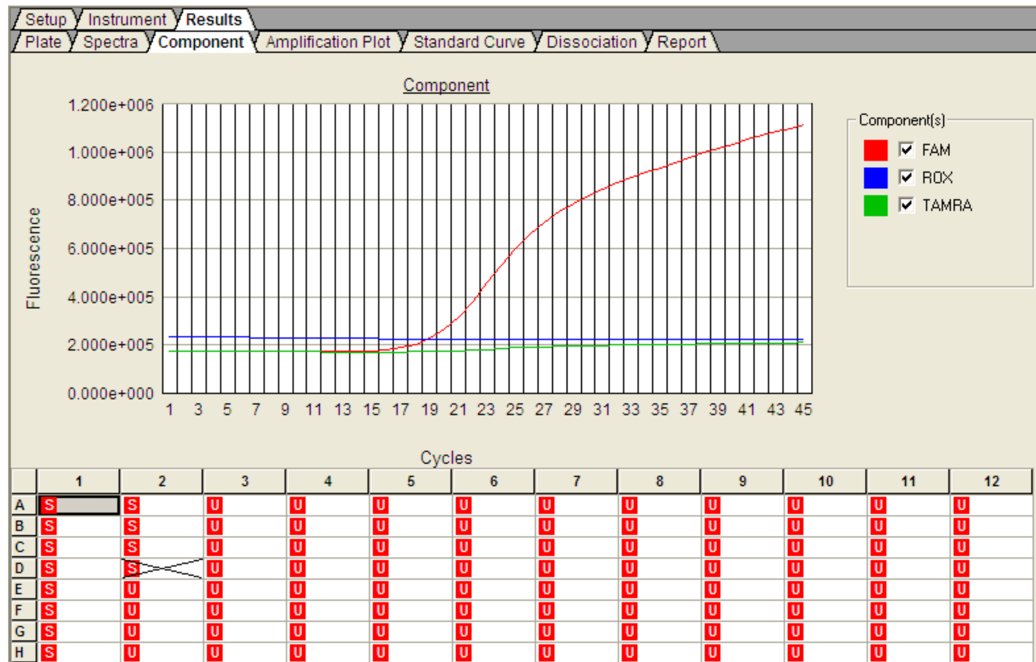
Figure 9.1 Real-time Polymerase chain reaction

A EBV Plasmid dilution series standard curve(6.3E+008-6.3E+003), B Component plot with FAM (red) reporter dye, TAMRA quencher (green) dye and ROX (blue) passive reference dye, C Plasmid dilution series amplification plot, D Standard curve with study sample plots, E Plasmid dilution series and sample amplification plot

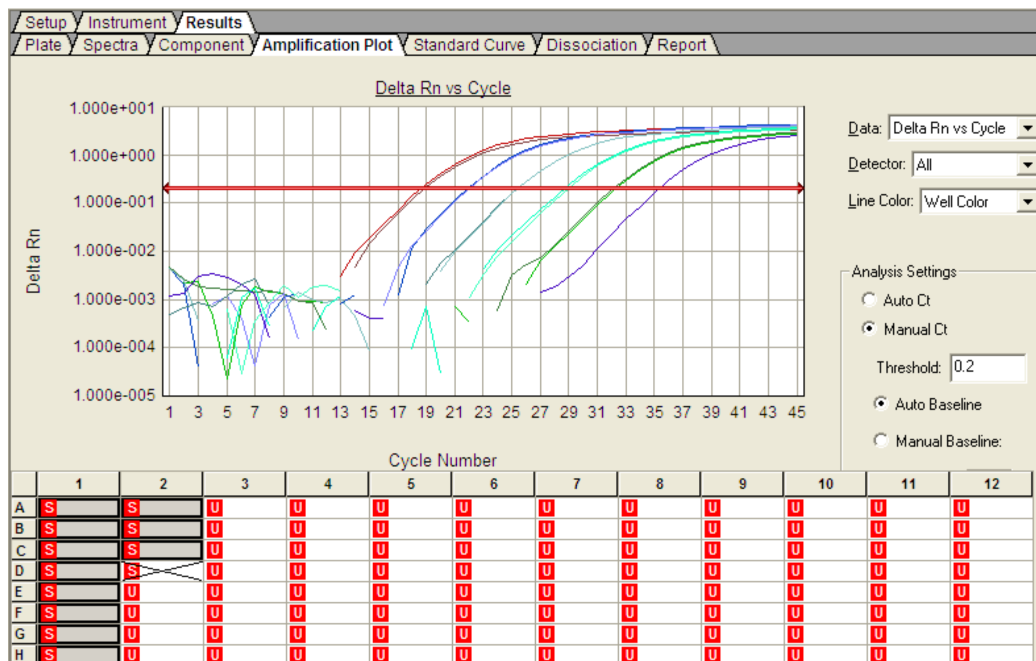
A



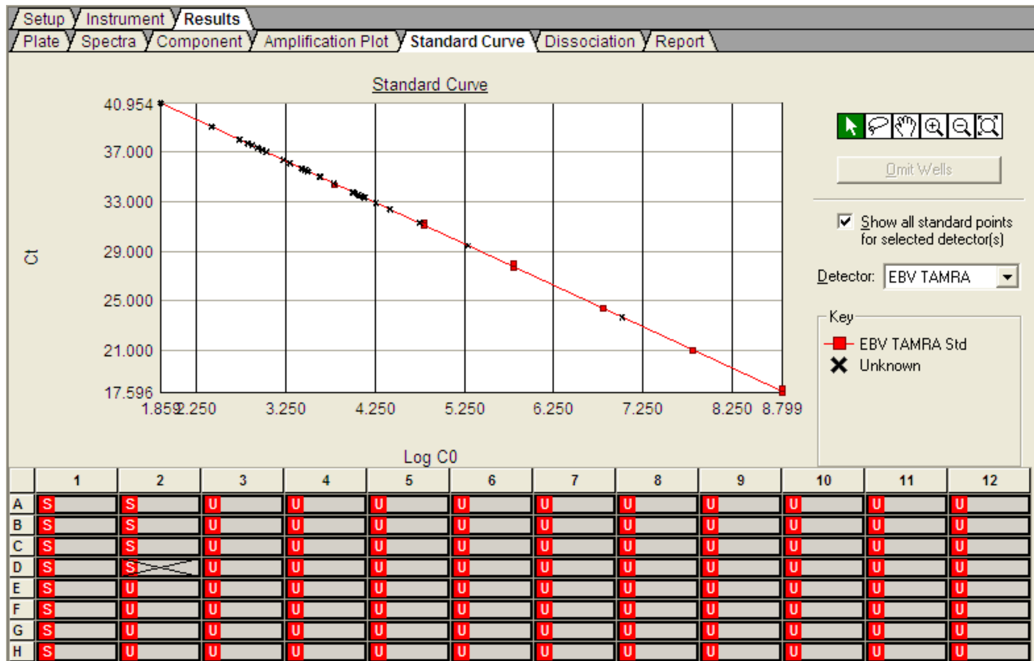
B



C



D



E

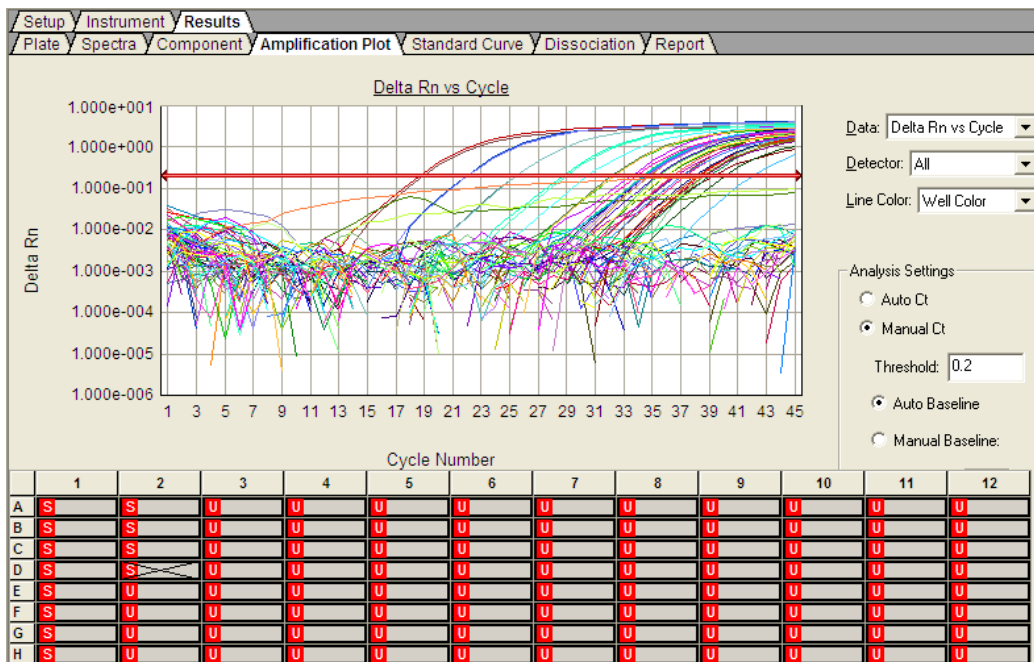
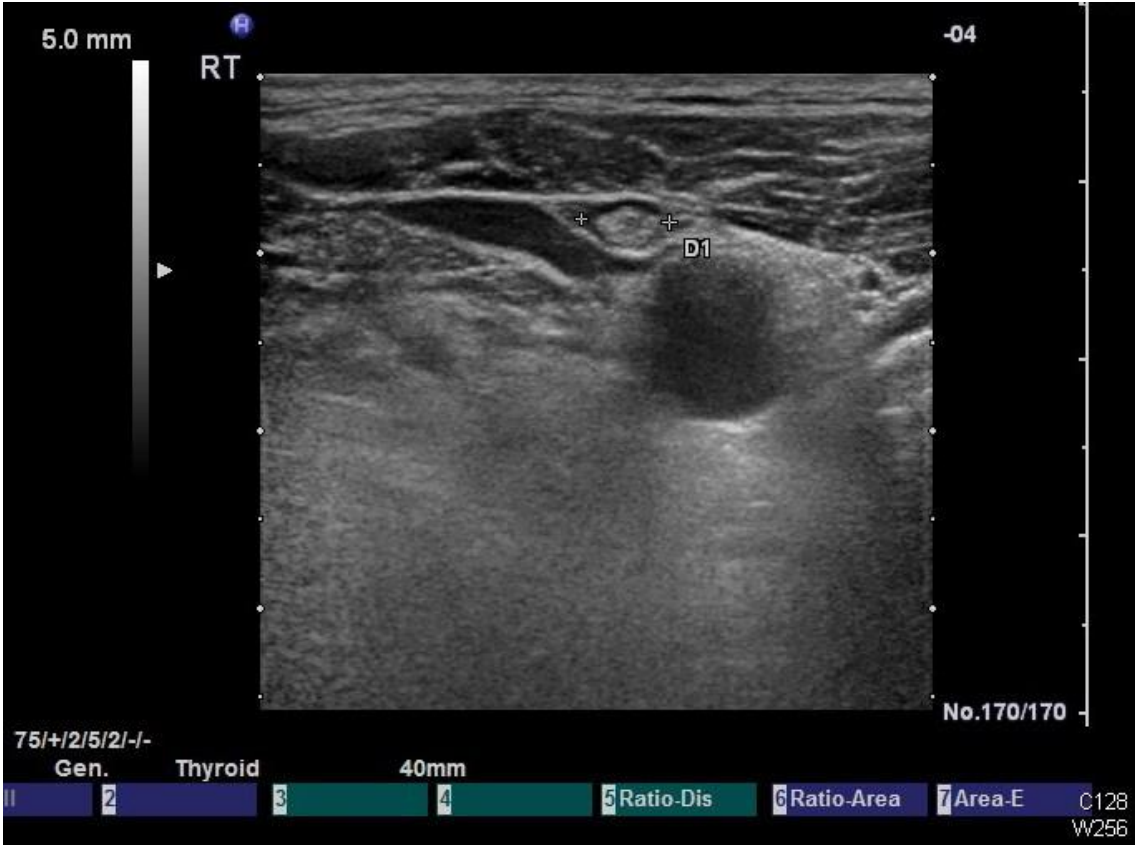


Figure 9.2 Ultrasound images of cervical lymph nodes from participants recruited to the EBV study. A Normal node <5mm short-axis with echo-bright hilum. B Node >5mm lacking echo-bright hilar region. C Multiple small (<5mm) cervical lymph nodes lacking echo-bright hilar regions.

A



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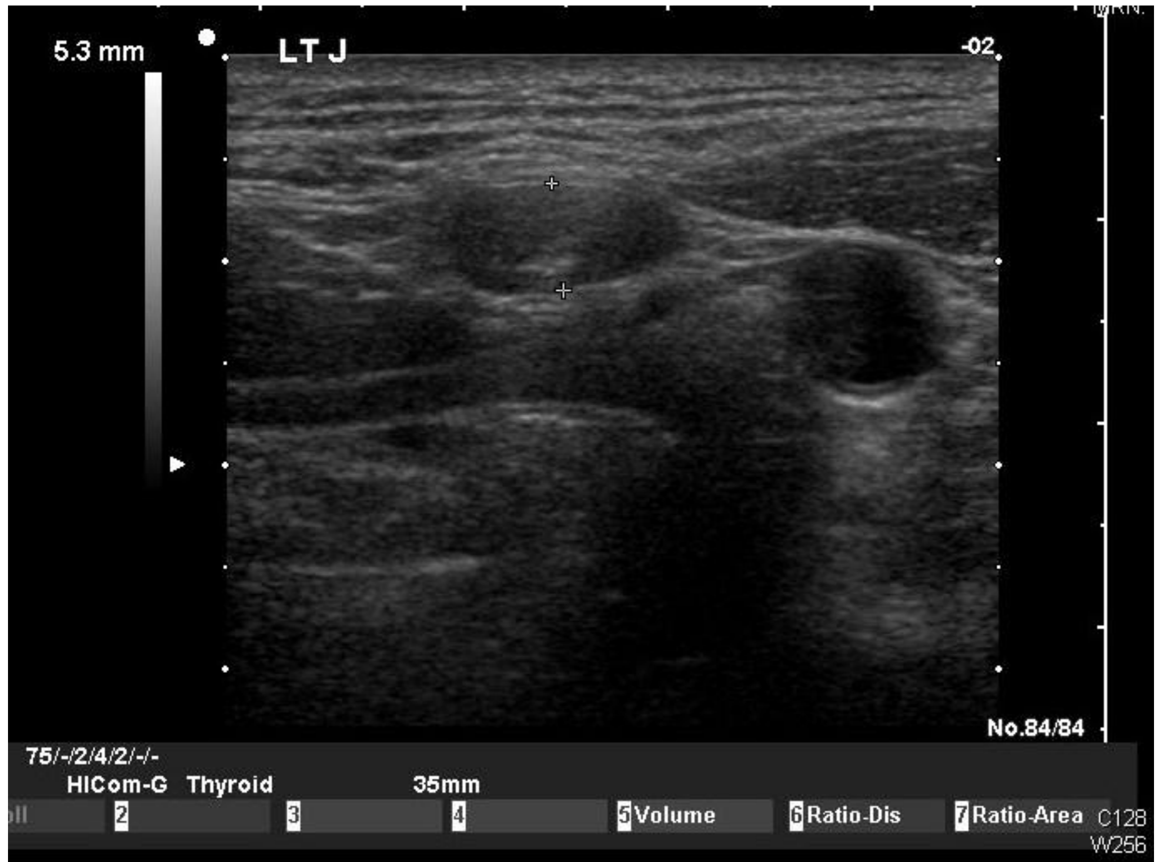

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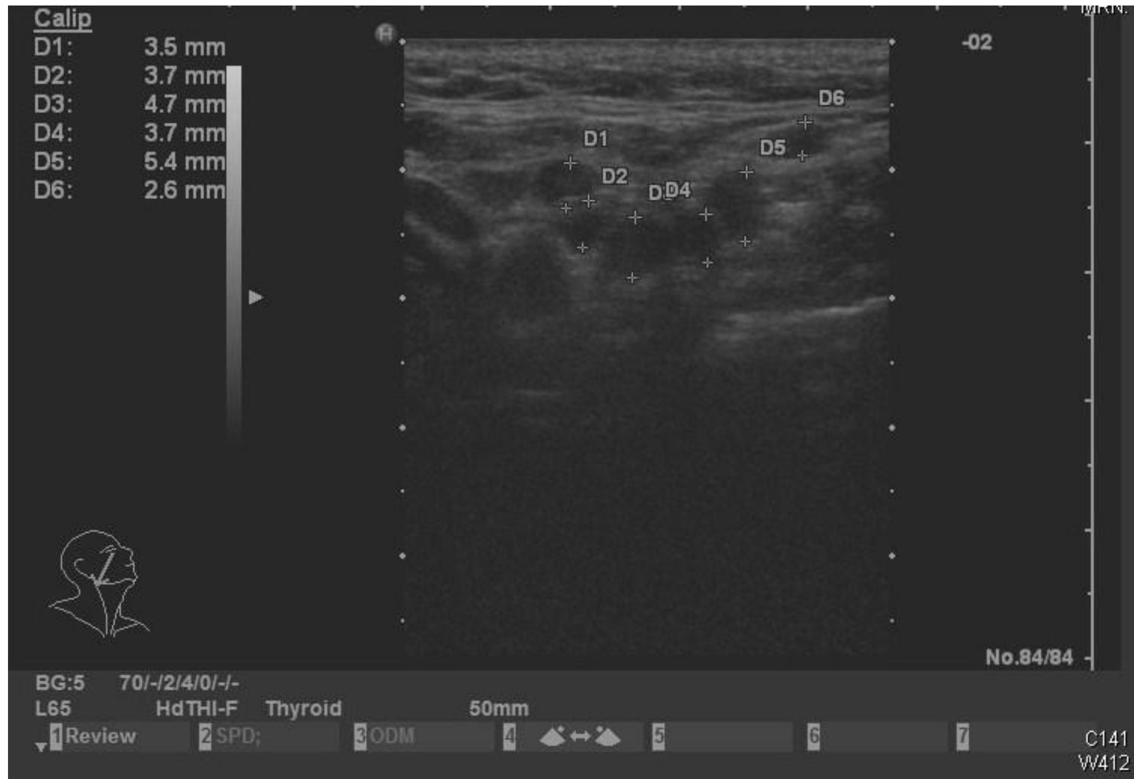
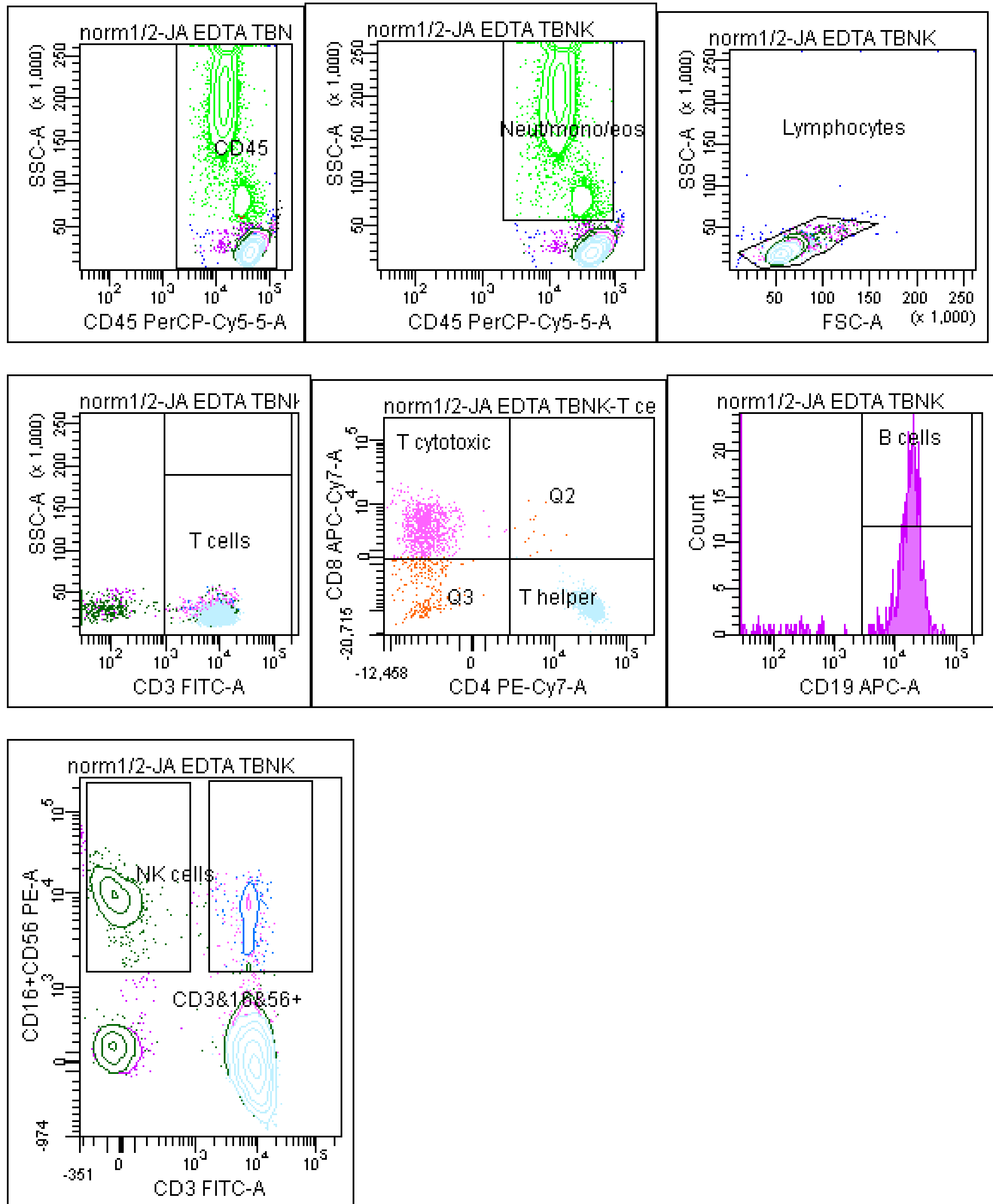


Figure 9.3 T,B,NK analysis of EDTA whole blood sample – using multicolour flow cytometry



Tube: JA EDTA TBNK

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
CD45	9,982	99.8	99.8
Neut/mono/eos	5,443	54.5	54.4
NOT NMEs	4,539	45.5	45.4
Lymphocytes	4,489	98.9	44.9
T cells	3,324	74.0	33.2
T cytotoxic	956	28.8	9.6
Q2	14	0.4	0.1
Q3	252	7.6	2.5
T helper	2,102	63.2	21.0
B cells	451	10.0	4.5
NK cells	612	13.6	6.1
T cells OR B cells OI	4,348	96.9	43.5
CD3&16&56+	288	6.4	2.9