The Role of Regulatory T cells in Primary Infection with Epstein-Barr Virus

Phoebe J Wingate

Doctor of Philosophy
The University of Edinburgh

2008

DECLARATION

I declare that all work included in this thesis is my own, except where otherwise stated. No part of this work has been, or will be submitted, for any other degree or qualification.

Phoebe J Wingate
2007

School of Biomedical Sciences
University of Edinburgh
Summerhall
Ed
EH9 1QH

ABSTRACT

Infection with Epstein-Barr virus (EBV) during adolescence results in an immunopathological disease, Infectious Mononucleosis (IM), in around 25% of cases. A role for Regulatory T cells (T_{reg}) in IM has yet to be established. These suppressive cells may affect the well-characterised cytotoxic T cell (CTL) response to EBV and thus the level of viral persistence and reactivation, potentially creating an environment conducive to the outgrowth of EBV-infected cells and tumour development. The work in this thesis examines the frequency and functional capacity of T_{reg} in primary EBV infection.

The results show that the frequency of T_{reg} within the $CD4^+$ T cell population of IM patients was reduced with borderline significance ($p=0.05$) compared with healthy controls as revealed by fluorescence activated cell sorting. T_{reg} function was confirmed using suppression assays on peripheral blood mononuclear cells (PBMC) from healthy controls but could not be assessed in IM patients due to low cell numbers. EBV-specific T_{reg} function was analysed using Interferon (IFN)-γ ELISPOT assays in which PBMC from IM patients and healthy controls were stimulated with phytohaemagglutinin (PHA) and EBV peptides in the presence or absence of T_{reg}. The IFN-γ response of PBMC to PHA stimulation was significantly reduced in IM patients compared to healthy controls ($p=0.009$) but the IFN-γ response to EBV peptides did not alter, irrespective of the presence or absence of T_{reg}. Investigation of FOXP3 expression by immunohistochemistry provided evidence of T_{reg} presence and preliminary data indicated an increased expression in IM tonsil sections compared with healthy tonsil sections. The proliferative responses and cytokine profiles of healthy controls, as measured by proliferation assays and ELISAs, in response to stimulation with the recall antigen PPD did not significantly alter upon the addition of latent membrane protein (LMP)-1 peptide. In IM patients, the same treatment resulted in a significant reduction in IFN-γ ($p=0.026$) but no significant differences in IL-10 production or cell proliferation.

The significantly reduced frequency of T_{reg} in peripheral blood of IM patients and abundant FOXP3 expression in IM tonsils provides evidence for a T_{reg} role in primary EBV infection. One plausible explanation is the recruitment of T_{reg} to the site of primary infection by an as yet unidentified EBV-specific mechanism. Clarification of T_{reg} activity in IM may expose opportunities for immunomanipulation during early stages of infection.

ACKNOWLEDGEMENTS

Firstly, a massive general thank you to all those who have helped me, in one way or another, to complete this PhD.

I want to thank my excellent supervisor, Prof Dorothy H. Crawford. It was her lectures during my undergraduate studies that first inspired my interest in Epstein-Barr virus and made me contemplate doing a PhD. I made the mistake of mentioning this to her, and 4 weeks later, I was enrolled. Throughout the project she has provided support, understanding and encouragement, I could not have hoped for a better supervisor.

I also want to thank Karen McAulay, whom I am sure I have been a constant burden to; barely a day has passed in the last 3 years when I haven't asked her a question that incidentally, she could always answer. My gratitude also goes to Shonna MacCall, who helped me with flow cytometry, the staff from the UHC where I collected blood samples, and Dr Johannessen for his contributions. I am greatful to Dr Alison Banham, who provided me with the FOXP3 antibodies, and to Dr Bridget Fox who showed me how to use them. Thank you also to the member of the library IS support team, who not only rescued my thesis from my ailing computer but worked tirelessly to fix it when it seemed to be beyond repair after it crashed 3 weeks before my deadline.

My gratitude also goes out to my family, who in the final stages of writing provided much encouragement, support and voices of reason on the phone. Thanks also to my friends who provided much needed bottles of wine and pints of beer throughout the project. I particularly want to thank my PhD companion, Anna Swanson, who forged ahead, meaning that she was subjected to my constant questioning about formatting, binding and creating contents tables, and yet has a bottle of champagne intact for when I finally do submit. Thank you also, to my colleagues in Summerhall, for our frequent 'research lunches' and much fun in the lab, and to all those who contributed insightful remarks during meetings and seminars.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AIDS	Aquired Immunodeficiency Syndrome
APC	Antigen presenting cell
BARTs	Bam A rightward transcripts
BCIP	5-Bromo-4-Chloro-3'Indolyphosphate p-Toluidine salt
BL	Burkitt's lymphoma
BLPD	B lymphoproliferative disease
BrdU	5-Bromo-2'deoxy-uridine
BSA	Bovine serum albumin
cAMP	Cyclic andenosine monophosphate
CD	Cluster of differentiation
CAEBV	Chronic active EBV infection
CMV	Cytomegalovirus
CNS	Central nervous system
Cp	EBV promoter C
CR2	Complement receptor type 2
CSA	Cyclosporin A
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DAB	3,3'-diaminobenzidine
DNA	Deoxyribonucleic acid
E	Early
EAE	Experimental autoimmune encephalomyelitis
EBERs	Epstein-Barr virus-encoded RNAs
EBNA	Epstein-Barr nuclear antigen
EBNA-LP	Epstein-Barr nuclear antigen leader protein
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immunosorbant spot assay
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
FIM	Fatal Infectious Mononucleosis
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box protein 3
GITR	Glucocorticoid-induced TNF receptor family-related gene
GITR-L	GITR ligand
gp	Glycoprotein
HBSS	Hank's Balanced Salt Solution
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HEF	Human embryo fibroblasts
HIV	Human Immunodeficiency Virus

HL	Hodgkin's Lymphoma
HLA	Human leukocyte antigen
HLIL	Hodgkin's lymphoma-infiltrating lymphocyte
HHV	Human Herpesvirus
HRP	Horseradish peroxidase
HRS	Hodgkin Reed-Sternberg
HSV	Herpes simplex virus
ICAM-1	Intercellular adhesion molecule-1
IE	Immediate-early
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin-2 receptor
IM	Infectious Mononucleosis
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IR	Internal repeats
Kbp	Kilobase pairs
KS	Kaposi's Sarcoma
KSHV	Kaposi's Sarcoma herpesvirus
L	Late
LAG-3	Lymphocyte activation gene-3
LCL	Lymphoblastoid cell lines
LD	Lymphocyte depletion
LFA-1	Lymphocyte function-associated antigen-1
LMP	Latent membrane protein
LP	Lymphocyte predominance
LRC	Lymphocyte rich classical
MACS	Magnetic activated cell sorting
MHC	Major histocompatibility complex
mRNA	Messenger Ribonucleic acid
MS	Mixed cellularity/Multiple Sclerosis
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NBT	Nitro blue Tetrazolium Chloride
NF-κB	Nuclear factor κB
NK	Natural Killer cell
NPC	Nasopharyngeal Carcinoma
NS	Nodular sclerosis
ORF	Open reading frame
oriLyt	Origin of lytic replication
*ori*P	Origin of plasmid replication
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PE	Phycoerythrin
PHA	Phytohaemagglutinin
PPD	Purified protein derivative
PTLD	Post-transplant Lymphoproliferative disease
RNA	Ribonucleic acid
RBPJκ	Recombination binding protein Jκ
SAP	SLAM-associated protein
SCID	Severe combined immunodeficiency
SFC	Spot-forming cell
SFIM	Sporadic fatal Infectious Mononucleosis
SLAM	Signalling lymphocytic activation molecule
SNBTS	Scottish National Blood Transfusion Service
TCM	Tissue culture medium
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumour Necrosis Factor
TR	Tandem repeats
Tr1	IL-10-producing T_{reg}
T_{reg}	Regulatory T cell
U_S	Unique sequencing domain short
U_L	Unique sequencing domain long
VCA	Viral capsid antigen
VZV	Varicella zoster virus
Wp	EBV promoter W
XLP	X-linked lymphoproliferative syndrome

1 Introduction

1.1 Herpesviridae

Herpesviruses are one of the most successful virus families known to date, exemplified by their ability to infect species across the animal kingdom from mammals, such as wallabies (Callinan & Kefford, 1981), to reptiles, including a variety of species of tortoise (Une *et al*, 1999). Currently 130 herpesviruses have been identified and sub-classified according to their biological properties.

1.1.1 Structure & Classification

Herpesviruses are identified by the architecture of the virus particle. A herpesvirus virion consists of the core, capsid, tegument and envelope (Roizman & Baines, 1991). The core contains linear double-stranded DNA, the length and base composition of which varies depending on the virus. The core is enclosed by the capsid, a 162-capsomere icosadeltahedral structure of 100-110nm diameter. This structure is surrounded by the protein-rich tegument, which is encased by a lipid bilayer membrane with external glycoprotein spikes, called the envelope (Kieff & Rickinson, 2001). The overall size of the virion varies between 120-300nm according to the thickness of both the tegument and envelope. Figure 1.1 shows an example of a herpes virus particle.

There are four main characteristics which can be ascribed to all herpesvirus species. The first is that all members of this family of viruses encode genes for enzymes and factors

involved in DNA synthesis and protein-processing. The number of these proteins varies between genera. The second common characteristic is that synthesis of viral DNA and capsid assembly occurs in the nucleus and the capsid is enveloped as it transits through the nuclear membrane. Thirdly, infected cells are irreversibly damaged with the production of infectious progeny during lytic replication. The fourth characteristic is that all Herpesviruses have the capacity to remain latent in their natural host, during which the genome circularises and viral gene expression is reduced (Roizmann *et al*, 1992).

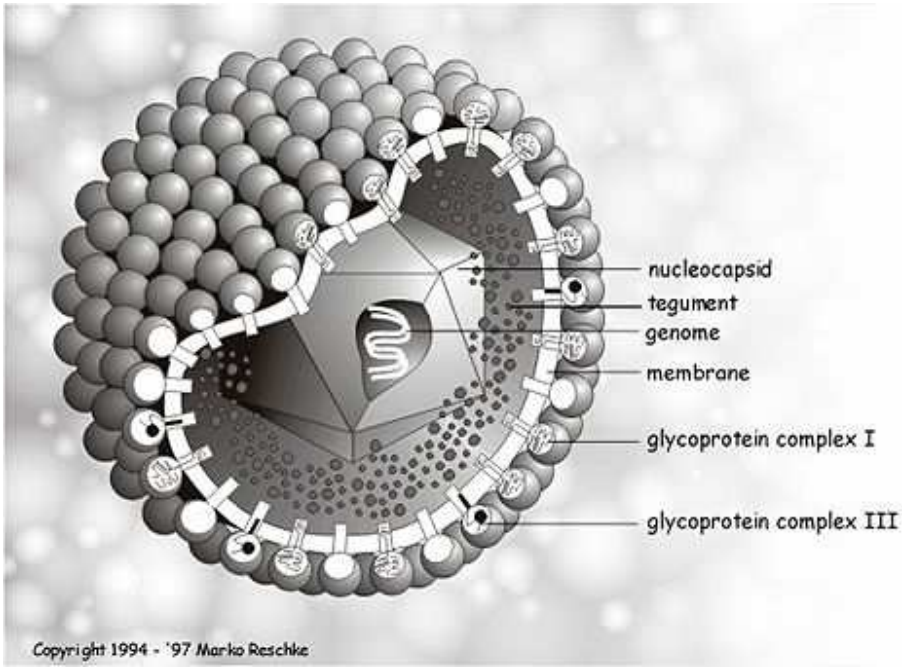

Figure 1.1: Human Cytomegalovirus structure. Reprinted with permission from Dr Marko Reschke in Marburg, Germany, of the Human Cytomegalovirus Group.

Despite these common themes, the herpesvirus family varies with respect to their biological properties. For example, Herpes Simplex (HSV)-1 can infect a wide range of host cells whereas Epstein-Barr virus (EBV) has a more restricted cell host range,

Herpes Simplex (HSV)-2 multiplies rapidly (18-24 hours) whilst Human Cytomegalovirus (HCMV) has a slow replicative cycle (36-48 hours). Factors such as these have allowed further sub-classification into *Alpha*-, *Beta*- or *Gammaherpesviridae.* Table 1.1 summarises the properties of these subfamilies.

Table 1.1: *Biological Properties and characteristics of Herpesvirus subgroups*

subclassification	**biological properties**			
	host animal range	**replicative cycle**	**site of latency**	**virus**
Alpha	broad host range	short replicative cycle	neurons, glial cells	HSV-1, HSV-2
Beta	restricted host range	long replicative cycle	hematopoietic progenitor cells	CMV
Gamma	very restricted host range		lymphocytes	EBV, KSHV

1.1.2 Human Herpesviruses

A viral aetiology had long been suspected for Kaposi's sarcoma (KS), a neoplasm associated with AIDS patients (Vogel *et al*, 1988;Bovenzi *et al*, 1993;Siddiqui, 1983). In an attempt to define the cause of this disease, foreign DNA was sought in KS tissue. The resulting sequences showed homology to the capsid and tegument proteins from *gammaherpesviridae* members, leading to the discovery of the eighth and newest member of human herpesviruses; human herpesvirus (HHV)-8 or Kaposi's sarcoma herpesvirus (KSHV) (Chang *et al*, 1994).

Other members of the family include HHV1-7, the first 5 of which are alternatively named Herpes Simplex Virus (HSV)-1, Herpes Simplex Virus (HSV)-2, Varicella Zoster virus (VZV), Epstein-Barr virus (EBV), Cytomegalovirus (CMV) respectively. These viruses establish lifelong latent infection with periods of reactivation and are responsible for a variety of diseases, see table 1.2 for a summary.

Table 1.2: *Characteristics of Human Herpes viruses and their disease associations*

HHV type	Name	Subfamily	Target cell type	Site of latency	Transmission	Disease association
1	Herpes simplex-1 (HSV-1)	alpha	mucoepithelia	neurons	close contact	oropharyngeal lesions
2	Herpes simplex 2 (HSV-2)	alpha	mucoepithelia	neurons	close contact usually sexual	genital herpes
3	Varicella Zoster (VZV)	alpha	mucoepithelia	neurons	contact or respiratory	chicken pox, shingles
4	Epstein-Barr (EBV)	gamma	B lymphocyte, epithelia	B lymphocyte	Saliva/sexual	infectious mononucleosis*
5	Cytomegalovirus (CMV)	beta	epithelial cells monocytes lymphocytes	monocyte, lymphocytes, possibly others	contact, blood transfusions, congenital	congenital defects, mononucleosis syndrome
6	Human Herpes virus 6 (HHV-6)	beta	T lymphocytes and others	T lymphocytes and others	contact, respiratory	roseola infantum
7	Human herpes virus 7 (HHV-7)	beta	T lymphocytes and others	T lymphocytes and others	unknown	unknown
8	Kaposi's sarcoma- herpes virus (KSHV)	gamma	epithelial cells B lymphocytes	unknown	Blood, sexual	Kaposi's sarcoma

Table created using http://pathmicro.med.sc.edu/virol/herpes.htm
*associated with tumours, see table 1.3

1.2 Epstein-Barr Virus

1.2.1 Introduction

In 1957, a surgeon, Denis Burkitt, working in Kampala, Africa, was called for a consultation on a child with strange jaw lesions. This was the first in a series of events leading to the discovery of what was to become known as Burkitt's Lymphoma (BL). In order to determine the underlying cause of this malignant tumour, Burkitt and his colleagues collected clinical and epidemiological data from all over Africa. The resulting information pointed to a viral aetiology (Burkitt, 1983).

A chance meeting with Dr Anthony Epstein in 1961 resulted in the exchange of ideas and Burkitt agreed to send biopsy samples from BL patients to the UK. When Epstein and his colleagues at the Middlesex Hospital, London, examined cultured BL cell lines under electron microscopes, they discovered virus particles with a similar morphology to the herpes simplex virus (Epstein *et al*, 1965). The new virus was identified as a member of the *herpesviridae* family and named Epstein-Barr Virus (EBV) (Epstein & Barr, 1964;Epstein *et al*, 1964). In subsequent research, the distribution of BL was shown to be coincident with areas of intense malaria infection. Burkitt hypothesised that malaria, with the associated immunosuppression, somehow allowed EBV to act oncogenically.

The oncogenic potential of this virus is now evidenced by a number of EBV-associated malignancies, including Nasopharyngeal Carcinoma (NPC) and Hodgkin's Lymphoma (HL), as well as BL, as summarised in table 1.3. Furthermore, immunosuppression of patients can cause the outgrowth of EBV-infected cells resulting in B Lymphoproliferative Disease (BLPD).

EBV is a highly ubiquitous virus, infecting over 90% of the adult population worldwide (Crawford, 2001c). EBV establishes latency in B lymphocytes and infection is life-long. Primary infection is usually asymptomatic during early childhood although a delay in EBV exposure infection results in Infectious Mononucleosis (IM) in approximately 25% of cases (Crawford *et al*, 2006b).

1.2.2 Genome

In 1984, Baer *et al* sequenced the B95-8 prototype laboratory strain of EBV (Baer *et al*, 1984a), first isolated in 1973 (Miller *et al*, 1973), using a *Bam*HI fragment library. Sequences of interest, such as genes, open-reading frames (ORFs) and promoters are therefore commonly referred to as the corresponding fragment. For example, the ORF (F) encoding an envelope glycoprotein gp110, is the *Bam*HI A (BA) fragment, and its position is fourth leftward, hence BALF4.

Table 1.3: *EBV-associated malignancies*

tumour	cell of origin	approximate EBV association	cofactors/risk factors
Burkitt's Lymphoma	centroblast	African 96% Sporadic 10-70% AIDS 30-40%	malaria, c-myc deregulation, HIV
B Lymphoproliferative disease	B Lymphoblast	90%	immunosuppression, HIV
Hodgkin's Lymphoma	centrocyte	40-80%	Infectious Mononucleosis (IM)
T-cell lymphoma	T lymphocyte	10%	chronic IM, immunosuppression
Nasopharyngeal carcinoma	squamous epithelial cell	100%	genetic & dietary factors
Gastric carcinoma	epithelial cell	10%	unknown

(Table from Crawford, 2001d)

The EBV genome is approximately 172 kb in length and consists of tandem reiterated 0.5 kbp direct repeats (TR) of the same sequence at both termini, 6-12 tandem reiterations of 3 kbp internal direct repeats (IR) and two unique sequencing domains, termed short (U_S) and long (U_L) (Kieff & Rickinson, 2001).

Two types of EBV exist; type 1 is the most commonly occurring whilst type 2 is equally as common as type 1 in equatorial Africa and New Guinea. The difference between the two types comes from slight variation in the genes that encode EBV nuclear proteins (Adldinger *et al*, 1985;Dambaugh *et al*, 1984;Sample *et al*, 1986).

1.2.3 Structure

The general structure of EBV is similar to other herpesviruses in that it consists of linear, double-stranded DNA wrapped around a core, a nucleocapsid, tegument and envelope. Dolyniuk *et al* examined the similarities in structural polyprotein constituents between EBV and HSV (Dolyniuk *et al*, 1976a) and established that whilst parallels existed in nucleocapsid polypeptides, non-nucleocapsid polypeptides, predicted to be glycoproteins, varied considerably between the two viruses (Dolyniuk *et al*, 1976c). Further research revealed the existence of homology between some EBV glycoproteins and glycoproteins found in other HHV, two in particular, termed gp85 and gp110 (Pellett *et al*, 1985;Gong *et al*, 1987;Balachandran *et al*, 1987;McGeoch & Davison, 1986;Heineman *et al*, 1988). However, the major envelope glycoprotein gp350/220, was not only established as exclusive to EBV (Dolyniuk *et al*, 1976b) but also as playing an important role in membrane adhesion allowing the virus entry to host cells.

1.2.4 Latent replication

The *in vitro* infection of resting human B lymphocytes with EBV, which results in the outgrowth of transformed cell lines, known as Lymphoblastoid cell lines (LCL), has been the main tool in understanding key events in EBV infection and replication. Once EBV penetrates the cell, the linear DNA circularises to form an episome (see figure 1.2) and the virus switches to a latent state, expressing only a limited number of genes encoding 6 EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and leader protein), 3 latent membrane proteins (LMPs 1, 2A and 2B), and highly spliced *Bam* A rightward transcripts (*Bam*H1A) (Kieff & Rickinson, 2001). In addition, 2 small

nonpolyadenylated, nonencoding, EB small RNAs (EBERs) are expressed abundantly in LCL. Figure 1.2 shows the episomal form of EBV DNA and the viral genes expressed by LCL and figure 1.3 shows the linear form of the EBV genome.

One of two promoters positioned at the left-hand end of the EBV genome initiates transcription. The usage of the promoters depends on stage of infection; C_p usage occurs during established latency whereas W_p usage is only apparent at early stages of infection. The activity of the promoters is mutually exclusive (Woisetschlaeger *et al*, 1990;Woisetschlaeger *et al*, 1989). Recruitment of host-cell RNA polymerase II initiates transcription of the 6 EBNA genes as a major transcriptional unit from the left-hand end of the genome, which is then spliced, resulting in mRNAs with common 5' exons.

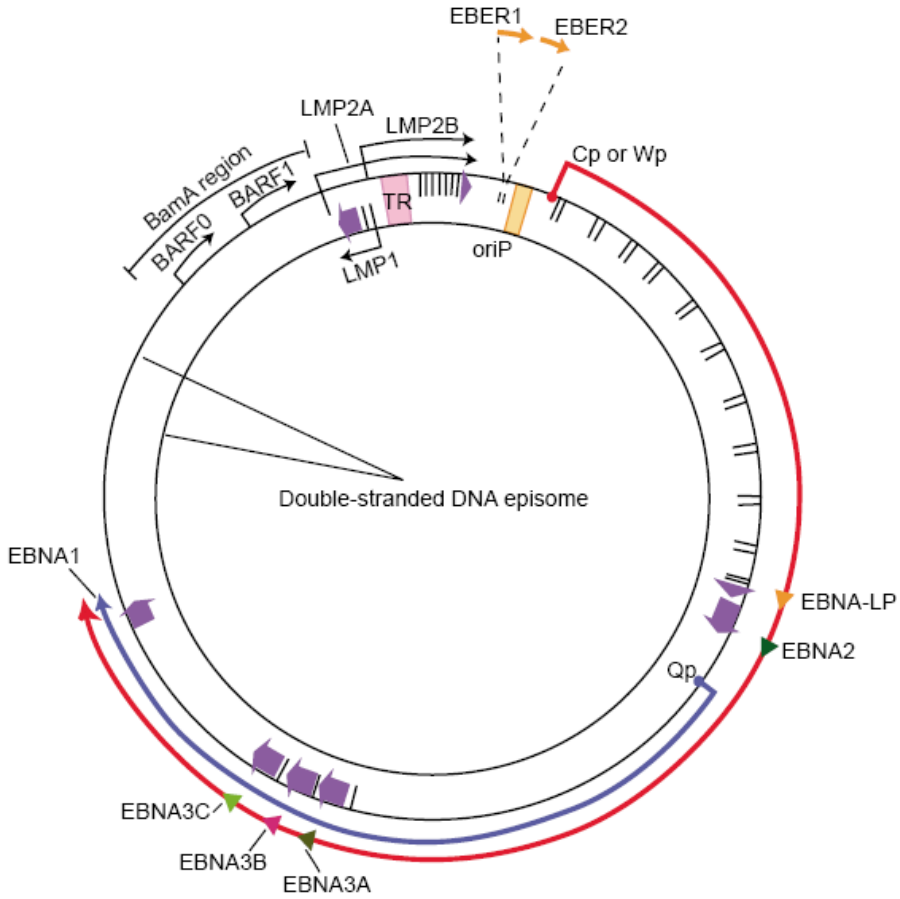

Figure 1.2: EBV episomal genome. The origin of plasmid replication is *oriP* and shown in orange. Latent genes are shown by purple arrows. The promoters from which transcription is initiated are also shown, promoter C (Cp) or Wp and Qp. Diagram from Murray and Young (2001). Adapted by permission from Cambridge Press Publishers Ltd

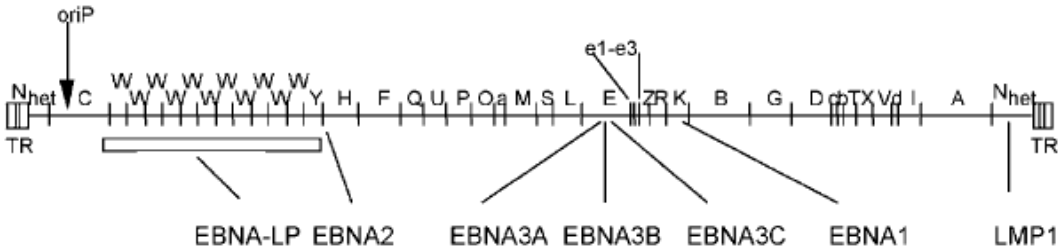

Figure 1.3: BamHI restriction endonuclease map of prototype B95-8 EBV genome. Fragments are named alphabetically according to size, with A being the largest. The origin of replication (*ori*P) is shown as are the two terminal repeats (TR) at both ends of the genome. Reprinted by permission from Macmillan Publishers Ltd [Oncogene] Young & Murray, copyright 2003.

After *in vitro* infection of B cells, EBNA-LP and EBNA-2 are the first EBV genes transcribed under the control of W_p. EBNA-2 plays an important role in up-regulation of gene expression (Abbot *et al*, 1990c;Alfieri *et al*, 1991c) and viral promoter switching (Woisetschlaeger *et al*, 1991). EBNA-2 expression causes the switch from W_p to C_p which is coincident with expression of EBNA-1 and EBNA-3A, -3B and 3C mRNAs. EBNA-1 expression causes a positive feedback loop resulting in increased EBNA transcription. EBNA-2 and EBNA-LP also upregulate expression of LMP1, -2A and -2B promoters (Alfieri *et al*, 1991b). LMP-2A and -2B transcription is upregulated through EBNA-2 response elements upstream of their promoter (Zhang *et al*, 1994;Zimberstrobl *et al*, 1993a). By 32 hours post-infection, all EBNA and LMP mRNAs are expressed (Alfieri *et al*, 1991a). By 48 hours after *in vitro* infection of B cells, all EBNAs and LMP-1 are near levels maintained consistently through latent infection (Kieff & Rickinson, 2001). EBERs expression lags by 24 hours and does not reach substantial levels until 70 hours post-infection (Alfieri *et al*, 1991e).

In addition to viral gene expression *in vitro*, EBV-infection induces LCL to express high levels of B-cell activation markers including cluster of differentiation (CD) 21, CD23, CD30, CD39 and CD40 and a number of cellular adhesion markers including Lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18) and -3 (CD58) and intercellular adhesion molecule (ICAM)-1 (CD54) (Rowe *et al*, 1987). The classic expression of all 9 latent viral antigens and EBERs, each of which are discussed in more detail below, observed in latently infected B lymphocytes *in vitro* is characteristic of the

latency III pattern. *In vivo*, the expression pattern of the viral antigens and host proteins differs depending on the form of latency. Table 1.4 summarises the gene expression pattern found in the 3 forms of latency.

Table 1.4: *EBV latency patterns*

latency type	EBV gene expression	associated tumour type
Latency I	EBNA-1, EBER, BamH1A transcripts	Burkitt's lymphoma
Latency II	EBNA-1, LMP-1, LMP-2A, LMP-2B, EBER, BamH1A transcripts	Hodgkin's lymphoma, Nasopharyngeal carcinoma*
Latency III	EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-LP, LMP-1, LMP-2A, LMP-2B, EBER, BamH1A transcripts	Lymphoproliferative disease

Abbreviations: EBNA – Epstein-Barr viral nuclear antigen; EBER – Epstein-Barr virus encoded small RNAs; LP – leader protein; LMP – latent membrane protein.*LMP-1/-2 only sometimes associated with this tumour type.

EBNA-LP

EBNA-LP is one of the first viral antigens to be expressed, coincident with the expression of EBNA-2 (Alfieri *et al*, 1991d;Allday *et al*, 1989). Although important in efficiency of transformation and cell growth post-infection, EBNA-LP is not essential for this process (Hammerschmidt & Sugden, 1989;Mannick *et al*, 1991;Allan *et al*, 1992). The most critical role for EBNA-LP is in stimulating EBNA-2-mediated transcriptional activation (Harada & Kieff, 1997;Nitsche *et al*, 1997).

EBNA-2

Unlike EBNA-LP, EBNA-2 is absolutely essential for the transformation of primary B lymphocytes (Kieff & Rickinson, 2001). EBNA-2 acts as a transcriptional activator of both cellular genes such as CD23 (Wang *et al*, 1985a;Cordier *et al*, 1990a) and CD21 (Cordier *et al*, 1990b;Wang *et al*, 1990a), and viral genes, including LMP-1 (Kieff & Rickinson, 2001;Abbot *et al*, 1990b;Fahraeus *et al*, 1990;Fahraeus *et al*, 1993;Ghosh & Kieff, 1990;Wang *et al*, 1990d) and LMP-2A and –B (Zimberstrobl *et al*, 1993b). EBNA-2 has 3 essential domains, an acidic domain involved in recruiting cellular proteins, a domain which associates with recombination binding protein (RBP) Jκ, a sequence-specific DNA-binding protein involved in transcriptional activation (Grossman *et al*, 1994;Zimberstrobl *et al*, 1994), and a third domain that mediates homotypic associations (reviewed in Kieff & Rickinson, 2001).

EBNA-3A,-3B,-3C

The EBNA-3A,-3B and -3C genes encode hydrophilic nuclear proteins that share the same gene structure, are tandemly placed in the genome and compete with EBNA-2 (Baer *et al*, 1984b;Bodescot *et al*, 1986;Bodescot & Perricaudet, 1987;Kieff & Rickinson, 2001). Studies have demonstrated that EBNA-3A and EBNA-3C are essential for B cell transformation *in vitro* whereas EBNA-3B is not. All EBNA-3s have been postulated to act as transcriptional activators by virtue of their ability to bind RBPJκ (Marshall & Sample, 1995;Robertson *et al*, 1995;Robertson *et al*, 1996;Zhao *et al*, 1996).

EBNA-1

EBNA-1 binds the viral origin of replication and is absolutely essential for replication and maintenance of viral episome in proliferating cells (Yates *et al*, 1984). In addition, it has also been shown to have transcriptional activator properties by studies which observed an upregulation of Cp and LMP-1 promoter (Kieff & Rickinson, 2001).

LMP-1

LMP-1 is an EBV oncogene and is essential for B cell transformation (Wang *et al*, 1985b;Kaye *et al*, 1993). This integral membrane protein, the expression of which depends on EBNA-2 (Abbot *et al*, 1990a;Wang *et al*, 1990c), alters the cell phenotype, inducing B cell activation and expression of a number of cellular proteins (Henderson *et al*, 1991b;Rowe *et al*, 1995;Wang *et al*, 1990b) via activation of the NF-κB transcription factor pathway (Huen *et al*, 1995). It also induces expression of a cellular oncogene *bcl*-2 which protects the B cell against programmed cell death (Henderson *et al*, 1991a).

LMP-2A,-2B

The expression of these 2 similar viral antigens is not essential for B cell transformation *in vitro* (Longnecker, 2000). The main role of LMP-2A is to maintain latent infection in B cells (Raab-Traub, 2002a). Evidence also indicates a role for LMP-2A in transformation of epithelial cells (Raab-Traub, 2002b). LMP-2B has been implicated in controlling LMP-2A function (Longnecker, 2000).

EBERs

It is likely that these nonpolyadenylated nonencoding RNAs are expressed in all forms of latency (Young & Murray, 2003a) although they are not essential for B cell transformation (Arrand & Rymo, 1982;Swaminathan *et al*, 1991). Evidence from work on BL cell lines indicates EBERs are responsible for inducing the expression of human interleukin (IL)-10, suggesting they have a role in the pathogenesis of EBV-positive BL since blocking IL-10 with an antibody or antisense oligonucleotide resulted in abrogated cell growth (Kitagawa *et al*, 2000).

1.2.5 Lytic Replication

In a latently infected B cell, EBV lytic replication is initiated within the origin of replication, *oriLyt*, and reactivation is concomitant with the expression of 2 immediate-early (IE) lytic genes BZLF1 and BRLF1, viral transactivators that activate promoters of both viral and cellular origin (Young & Murray, 2003b). IE gene expression activates early (E) gene transcription which is followed by the lytic cascade of viral genome replication and late gene (L) expression. During lytic replication EBV is amplified over 100-fold. Late genes encode a number of structural proteins, including the two major glycoproteins involved in cell binding and penetration, gp350 and gp85, and their expression is concordant with cell death and subsequent release of viral progeny.

In the majority of LCL, EBV remains latent with only a small proportion of infected B cells supporting lytic replication. The switch is most likely dependent upon B cell

differentiation into plasma cells (Crawford & Ando, 1986). *In vivo*, there is also little evidence of virus replication (Rickinson *et al*, 1975), despite the fact that EBV has been demonstrated in saliva (Gerber *et al*, 1972;Sixbey *et al*, 1983a). EBV has also been detected in genital secretions of both women (Sixbey *et al*, 1986) and men (Israele *et al*, 1991) albeit at a low level (Thomas *et al*, 2006b). The cellular origin of secreted EBV has been a matter of debate and the topic is still hotly debated (Pegtel *et al*, 2004).

1.2.6 EBV lifecycle

Salivary transmission of EBV results in viral replication in a permissive cell type present in the oropharynx, and high levels of virus shedding. The permissive cell type has yet to be confirmed. However, EBV is known to infect mucosal B lymphocytes, transform them and enter latency. The infected B cells proliferate in the extrafollicular regions of the tonsillar lymphoid tissue and circulate in the peripheral blood. The restricted expression of viral antigens in latently infected B cells allows these cells to evade the immune response. Periodically, by a mechanism not yet fully understood, EBV reactivates and progeny virions are released. Alternatively, epithelial cells may be the primary source of lytic virus (Hislop *et al*, 2007).

1.2.7 B lymphocyte Infection

The main cell type permissive for infection with EBV *in vitro* is the human B lymphocyte (Henle *et al*, 1967) due to their abundant expression of the cell surface marker CD21, which acts as the EBV receptor (Altiok *et al*, 1989;Calender *et al*, 1987;Hansson *et al*, 1983;Marchini *et al*, 1992;Marchini *et al*, 1993;Kieff & Rickinson,

2001). Fully differentiated plasma cells lack CD21 expression and cannot be infected. NK and T cells can also be infected, both *in vitro and in vivo*, albeit with a reduced efficiency than B cells (Shapiro *et al*, 1982;Kanegane *et al*, 1996).

EBV enters B lymphocytes by binding the complement receptor type 2 (CR2), also known as the C3d receptor or CD21, on the cell surface (Fingeroth *et al*, 1984;Frade *et al*, 1985). The main EBV-encoded glycoprotein involved in attachment is gp350/220 (Nemerow *et al*, 1987) as demonstrated in studies where monoclonal antibodies neutralised virus infectivity (Thorley-Lawson & Geilinger, 1980). After binding CD21, the virion fuses to the target cell membrane using a complex of glycoproteins, consisting of gp85, gp25 and gp42 (Li *et al*, 1995;Wang & Hutt-Fletcher, 1998). An interaction is required between gp42 and HLA class II protein, HLA DR (Spriggs *et al*, 1996) which acts as an essential co-receptor in B cell infection *in vitro* (Li *et al*, 1997;Haan *et al*, 2000a). Expression of HLA DP and HLA DQ on B cell lines has been shown to confer susceptibility to EBV infection leading to the suggestion that they may act as substitutes for HLA DR (Haan & Longnecker, 2000;Haan *et al*, 2000b).

1.2.8 Epithelial cell infection

The demonstration of viral DNA in a tumour of epithelial cell origin, nasopharyngeal carcinoma (NPC), provided the first evidence that EBV could infect this cell lineage (Zur Hausen *et al*, 1970). However, initial studies found epithelial cell lines refractory to infection *in vitro* and attributed this finding to lack of epithelial expression of CD21, the EBV receptor (Shapiro & Volsky, 1983;Glaser *et al*, 1980).

In 1980, researchers reported the *in vitro* fusion of EBV-infected lymphoblastoid cells with uninfected human T lymphoblastoid cells (Jurkat cells) and human embryo fibroblasts (HEF cells), both of which are devoid of EBV receptors, resulting in polykaryocytes containing EBV antigens. They hypothesised that this would permit virus entry into cells devoid of CD21 expression, and this might occur *in vivo* between infected B cells and epithelial cells since these two cell types are found in close proximity in Waldeyer's ring (Bayliss & Wolf, 1980).

Meanwhile, the body of evidence suggesting direct *in vitro* EBV infection of, and replication in, epithelial cells grew (Lemon *et al*, 1977;Sixbey *et al*, 1983b;Sixbey *et al*, 1984;Wolf *et al*, 1984). Further fuel for direct epithelial infection came with the discovery of a receptor similar to CR2 on undifferentiated uterine cervical (Sixbey *et al*, 1987) and pharyngeal epithelia (Young *et al*, 1986). Researchers debated whether epithelial cells might provide the major site for EBV replication and that infection of B lymphocytes may be a secondary event in primary EBV infection (Allday & Crawford, 1988).

To date, varied reports have been published on the entry of EBV to epithelial cells. Fingeroth *et al* demonstrated the expression of CD21, albeit at a low level, on an epithelial cell line (Fingeroth *et al*, 1999) providing yet more proof for the direct epithelial infection. However, in support of a CD21-independent mechanism of viral

entry into epithelial cells, 3 theories have been postulated; direct cell-cell contact across the apical cells membranes of infected B lymphocytes and pharyngeal epithelia, basolateral infection with cell-free virions by virtue of interaction between the EBV-encoded BMRF-2 glycoprotein and cell surface β_1 or $\alpha_5\beta_1$ integrins, or lateral spread across the membrane of adjacent cells (Tugizov *et al*, 2003b). Release of progeny virions is thought to occur at both the basolateral and apical membranes (Tugizov *et al*, 2003a).

Reports on the EBV glycoproteins involved in epithelial cell penetration are also varied. A requirement for gp85, as well as gp350, has been demonstrated in a number of studies (Wu *et al*, 2005;Molesworth *et al*, 2000). Borza and Hutt-Fletcher demonstrated enhanced infection of epithelial cells using a recombinant virus lacking gp150 (Borza & Hutt-Fletcher, 1998). Furthermore, Wang and colleagues established that gp42, an essential co-receptor involved in B cell infection, is nonessential, and they speculate that this is largely due to the lack of HLA DR expression on epithelial cells (Wang *et al*, 1998b). The broad spectrum of data on epithelial cell entry by EBV have led researchers to hypothesise that different glycoprotein complexes are required for entry into both cell types (Wang *et al*, 1998a;Borza & Hutt-Fletcher, 2002).

1.3 EBV Disease Associations & Immune Response

1.3.1 Seroepidemiology

Primary infection via salivary transmission of EBV normally occurs asymptomatically at a young age. However, if exposure is delayed until adolescence, viral infection results in Infectious Mononucleosis (IM) in 25% of cases (Crawford *et al*, 2006a). It is thought that virus dose may cause this age-related association, for example small children exposed to a low dose of EBV from family members or due to sucking saliva-contaminated toys whereas high levels of virus will be transmitted during adolescence through kissing (Crawford, 2001b). In addition, EBV has been detected in sexual secretions possibly allowing transmission of the virus via this route (Thomas *et al*, 2006a).

1.3.2 Acute Infectious Mononucleosis

The symptoms of IM, consisting of fatigue, pharyngitis, fever, lymphadenopathy, pharyngitis, with additional clinical findings including splenomegaly and hepatocellular dysfunction, develop after an incubation period of 30-50 days. In addition, IM is accompanied by generalised immunosuppression, demonstrated by the reduced proliferation of PBMC from IM patients in response to stimulation by both PHA and anti-CD3 (Perezblas *et al*, 1992a).

Both humoral and cell-mediated immune responses are induced by EBV infection; B cells produce antibodies with specificities for lytic and latent antigens as well as gp350,

and cytotoxic $CD8^+$ T cells (CTLs) circulate in the blood and infiltrate lymphoid tissue, releasing a plethora of antiviral cytokines including tumour necrosis factor (TNF)-α and -β, IL-1β and IL-6 (Foss *et al*, 1994a). The symptoms of IM are thought to arise as a direct result of the vigorous CTL response to EBV (Williams *et al*, 2005;Foss *et al*, 1994b), although the reason for this exaggerated reaction is unknown. The importance of the CTL response in IM is demonstrated in immunocompromised individuals (see below). However, in healthy individuals the majority of cases IM resolves within 6 months (Crawford, 2001a).

Chronic active EBV infection

Chronic active EBV infection (CAEBV) is characterised by chronic or recurrent IM-like symptoms, resulting in a number of complications including development of lymphoma, myelodysplastic syndrome and virus-associated hemophagocytic lymphohistiocytosis (Rickinson, 1986;Straus, 1988;Kanegane *et al*, 2002). Persistently high levels of EBV-specific antibodies found in patients with CAEBV recognising lytic phase viral antigens suggests a poor control of viral replication. The exact cause of this disease is unclear although it is likely to be a combination of host immune dysregulation and viral factors (Maia & Peace-Brewer, 2000).

Fatal IM

Severe or fatal IM (FIM) is the more aggressive but most rare form of IM, involving early lymphocytosis followed by the invasion on vital organs with EBV-specific

lymphocytes and patients die of opportunistic infections or haemorrhage within a few weeks (Wick *et al*, 2002a). The majority of FIM patients are immunocompromised either by drugs or genetically. For example, patients with X-linked lymphoproliferative syndrome (XLP), caused by a genetic abnormality, fail to control primary EBV infection, resulting in tissue infiltration by $CD8^+$ T cells and widespread damage to the host. Children who survive this go on to develop complications including B cell lymphoma. The gene defective in XLP encodes SLAM-associated protein (SAP), a protein expressed on activated T cells, important in cell proliferation induction (Crawford, 2001). Rarely, FIM arises in a previously healthy individual, in which case it is termed sporadic FIM (SFIM) (Penman, 1970). It is thought that the symptoms and disease progression occurs as a result of the exuberant T cell response, most particularly the cytokines released in response to EBV (Ohga *et al*, 1993).

1.3.3 Immune response

Natural Killer (NK) cells are an essential component of the innate immune response to most pathogens and whilst expansions of activated NK cells have been reported in IM patients (Williams *et al*, 2005), most evidence suggests that this cell type does not feature highly in the response against EBV *in vivo* (Hislop *et al*, 2007).

Due to the lack of symptoms, little is known about primary infection in individuals that undergo clinically silent seroconversion. However, the adaptive immune response to EBV has been extensively researched in IM where the major role played by $CD8^+$ T

cells is well characterised. Clinical primary EBV infection results in a massive expansion of these T cells expressing a broad repertoire of T cell receptors (TCRs) specific for EBV epitopes (White *et al*, 1996b;Steven *et al*, 1996b;Steven *et al*, 1997a). Up to 40% of the total $CD8^+$ T cell population recognise one specific viral epitope, namely from lytic EBV epitopes such as IE and E antigens, whereas the response to latent antigens is somewhat less and focuses on the EBNA 3 family (Hislop *et al*, 2007).

Consistent with activated $CD8^+$ T cells, these cells express perforin, a cytolytic molecule involved in apoptosis of target cells, and the activation markers CD38, and Ki-67 (Callan *et al*, 1998b;Catalina *et al*, 2001b;Hislop *et al*, 2002;Hislop *et al*, 2005b;Dunne *et al*, 2002b). Functional studies revealed that EBV-specific cytotoxic $CD8^+$ T cells were capable of recognising and killing target cells loaded with the relevant peptide epitope *ex vivo* (Steven *et al*, 1996a;Steven *et al*, 1997b;Callan *et al*, 1998a). In addition, a proportion of EBV-specific $CD8^+$ T cells responded to the relevant peptide by producing IFN-γ (Hoshino *et al*, 1999;Catalina *et al*, 2001a;Woodberry *et al*, 2005b;Hislop *et al*, 2005a;Callan *et al*, 2000b;Catalina *et al*, 2002;Precopio *et al*, 2003a). Another characteristic of these cells is the rapidity with which they die upon *ex vivo* culture unless restimulated with antigen (Callan *et al*, 2000a;Dunne *et al*, 2002a;Tamaru *et al*, 1993).

Once the primary $CD8^+$ T cell response to EBV successfully reduces the numbers of circulating infected B cells, the EBV-specific $CD8^+$ T cell population, responding to

both lytic and latent antigens, eventually subsides, concurrent with a downregulation in some activation markers including CD38 (Callan, 2004a). Despite these changes in frequency and phenotype, EBV-specific $CD8^+$ T cells responding to both lytic and latent epitopes can still be detected after the resolution of primary infection (Tan *et al*, 1999b) and account for a significant proportion of the antigen-experienced pool of cells in healthy seropositive individuals, with a frequency of between 0.1-1% in peripheral blood (Callan, 2004b).

The response of $CD4^+$ T cells to primary EBV infection is less well understood and the epitopes to which they respond are not as extensively characterised, partially due to the fact that EBV-specific $CD4^+$ T cells do not expand to the extent of the EBV-specific $CD8^+$ T cells in IM patients, making them more difficult to isolate and study. However, $CD4^+$ T cells responding to both lytic and latent epitopes have been described (Woodberry *et al*, 2005a;Precopio *et al*, 2003b). Precopio *et al*'s study of primary infection in IM patients identified $CD4^+$ T cell responses, by short *in vitro* stimulation followed by intracellular staining for IFN-γ, to lytic antigens BZLF1 and BMLF1 and latent antigen, EBNA-1, with a small minority of $CD4^+$ T cells responding to EBNA-3A (Precopio *et al*, 2003c).

When assayed directly *ex vivo*, Amyes *et al* detected IFN-γ release by $CD4^+$ T cells from acute IM patients when stimulated with EBV-infected B cell lysate. On assaying PBMC from the same patients 4 months post-infection, the response was found to be

significantly reduced (Amyes *et al*, 2003d), furthermore, in healthy EBV carriers, very little response was observed using the same experimental protocol. So although their activity can be demonstrated, $CD4^+$ T cells with EBV-specificity are found at a low frequency in peripheral blood of healthy seropositive individuals.

In general, $CD4^+$ T cells do not phenotypically alter from primary to persistent infection, continuing to express CD45RO, CD27 and CD28, with the exception of CD38, found to be expressed on $CD4^+$ T cells in acute IM patients but not 4 months post-infection and not on healthy EBV carriers (Amyes *et al*, 2003c).

At four months post-primary infection, the frequency of EBV-specific $CD4^+$ T cells has declined to the level detected in the persistent phase of infection (Amyes *et al*, 2003b). The isolation of $CD4^+$ T cells from healthy carriers recognising lytic cycle antigens, such as BZLF1-encoded IE epitopes (Adhikary *et al*, 2006a), BMFL1- and BHRF1-encoded E proteins (White *et al*, 1996a;Amyes *et al*, 2003a;Landais *et al*, 2004) and epitopes from L proteins (Adhikary *et al*, 2006b;Wallace *et al*, 1991), indicates a clear memory response.

To date the majority of EBV-specific T cell targets identified are in certain viral epitopes (Yewdell & Bennink, 1999), a phenomenon observed in most donors irrespective of HLA background, and this hierarchy differs between the $CD4^+$ and $CD8^+$ T cell response

(Hislop *et al*, 2007). The likelihood is that many more epitopes exist and are yet to be discovered.

The generation of EBNA-1 specific T cell lines after *in vitro* stimulation with EBNA-1 peptides resulted in some IFN-γ-producing $CD4^+$ T cell clones with regulatory properties, as evidenced by their inhibition of T cell proliferation in cocultures of naïve $CD4^+$ and $CD8^+$ T cells responding to anti-CD3 stimulation. This, as the authors imply, indicates that some epitopes may induce regulatory T cell (T_{reg}) activity (Voo *et al*, 2005d). Studies have also demonstrated IL-10 release when PBMC are stimulated *in vitro* with LMP-1 protein (Dukers *et al*, 2000b) and a variety of LMP-1 peptides (Marshall *et al*, 2003i). This adds to the concept that some epitopes may induce T_{reg}. Whether this is a mechanism for EBV immune evasion has yet to be established.

1.3.4 Hodgkin's Lymphoma

Hodgkin's lymphoma (HL) is a cancer of the lymphatic system, characterised by the presence of malignant Hodgkin's Reed-Sternberg (HRS) cells. The origin of HRS cells has been identified as germinal centre B cells (Marafioti *et al*, 2000). The frequency and type of non-neoplastic cell populations associated with the HRS cells allow for further sub-classification of HL into nodular sclerosis (NS), mixed cellularity (MS), lymphocyte depletion (LD), lymphocyte rich classical (LRC) and lymphocyte predominance (LP). The first 4 types are strongly associated with EBV and collectively known as classical HL; LP is non-EBV associated and considered a distinct form of lymphoma.

A role for EBV in the development of HL was first suggested in 1966 (MacMahon, 1966) and early evidence linking the virus with HL included the detection of elevated levels of antibodies specific for EBV antigens in HL patients when compared with patients with other lymphomas (Levine *et al*, 1971); this appeared to precede tumour development by a number of years (Mueller *et al*, 1989). In addition it was determined that individuals with a history of IM had a higher risk of developing HL than silent seroconverters (Gutensohn & Cole, 1980). Weiss *et al* detected EBV DNA in a subset of HL (Weiss *et al*, 1987) and Wu *et al* demonstrated the localisation of the EBV genome to the HRS cells (Wu *et al*, 1990). EBV has since been associated with approximately 50% of HL in western societies with a higher incidence in developing countries (Crawford, 2001e).

Where EBV is found in association with HL, HRS cells express a restricted pattern of EBV antigens, termed Latency II, which includes EBNA-1, LMP-1, LMP-2A proteins and EBER and BamH1A transcripts (Pallesen *et al*, 1991). A link between EBV-associated HL and HLA class I microsatellite markers has been established (Diepstra *et al*, 2005). This, in addition to more recent findings that the same HLA class I alleles occur more frequently in IM patients when compared with healthy carriers (McAulay *et al*, 2007), has led researchers to propose that altered EBV peptide presentation to $CD8^+$ T cells may affect the immune response to EBV and hence predispose these individuals to EBV-related diseases (Haque *et al*, 2007b).

1.4 Regulatory T cells

1.4.1 Introduction

Regulatory T cells are characterised by their ability to suppress proliferation of various cell types, including $CD4^+$ and $CD8^+$ T cells, and inhibit cytokine release. Broadly speaking, T_{reg} can be divided into two groups; 'natural' T_{reg} and adaptive, or induced, T_{reg}.

Natural T_{reg} are of thymic origin, developing from $CD4^+CD8^+CD3^{high}$ T cells, and can be detected in the foetus at an early stage of gestation (Darrasse-Jeze *et al*, 2005;Cupedo *et al*, 2005). They comprise 2-5% of $CD4^+$ T cells in human PBMC (Bacchetta *et al*, 2005c). Phenotypically they are defined by their constitutive expression of the high affinity IL-2 receptor α chain CD25 (Shevach, 2002a;Sakaguchi, 2000) and the transcription factor, FOXP3.

In contrast, adaptive T_{reg} arise in the periphery, expressing FOXP3 only when activated by certain stimulatory conditions. For example, type 1 regulatory Tr1 cells develop in an IL-10-rich background. In addition, adaptive T_{reg} can be induced from $CD4^+$ T cells lacking the CD25 activation marker.

Other cell-surface markers associated with regulatory properties have been described including glucocorticoid-induced TNF receptor family-related gene (GITR) (Shimizu *et*

al, 2002d;McHugh *et al*, 2002a) and cytotoxic-T-lymphocyte-associated protein (CTLA)-4 (Read *et al*, 2000c).

FOXP3 is a transcription factor first identified when scientists were investigating the genetic basis for the fatal autoimmune disease immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (Bennett *et al*, 2001b;Wildin *et al*, 2001;Chatila *et al*, 2000). A similar autoimmune disease was observed in mice with the scurfy mutation in the *FOXP3* gene (Brunkow *et al*, 2001b). It was determined that mutations in *FOXP3* caused these diseases by resulting in the loss of T_{reg} and, furthermore, scurfy mice could be rescued from the disease with transgene expression of wild-type *FOXP3* (Brunkow *et al*, 2001a). FOXP3 is now widely accepted as playing a crucial role in T_{reg} development and function (Zheng & Rudensky, 2007;Campbell & Ziegler, 2007b) and to date is the most specific marker for T_{reg} (Hori & Sakaguchi, 2004;Khattri *et al*, 2003).

However, a number of human non-regulatory T cell subsets have been shown to express FOXP3 upon activation under certain conditions, including human $CD4^+CD25^-$ T cells and CD8 T cells (Bacchetta *et al*, 2005b). For example, Sereti *et al* report that a high percentage of $CD4^+CD25^-$ T cells from healthy humans will express high levels of FOXP3 and $CD25^{bright}$ when activated with anti-CD3 and anti-CD28 antibodies (Sereti *et al*, 2005). In a review of the subject, Rouse *et al* suggest that 'in humans, FOXP3

expression is a normal consequence of T cell activation' (Rouse *et al*, 2006), hence an exclusive marker for T_{reg} is still being sought.

Numerous T cell subsets have been ascribed with regulatory activity with roles in suppressing autoimmune disease, controlling infection, allergy and immunopathology. Table 1.5 summarises the characteristics of known T_{reg} subsets. Unless otherwise stated, T_{reg} refers to $CD4^+CD25^+FOXP3^+$ T cells in the following text. Current research indicates a necessity for IL-2, in initiating regulatory activity (Shevach, 2002b;Setoguchi *et al*, 2005), and for survival in the periphery. T_{reg} suppress proliferation and IFN-γ production of both $CD4^+$ and $CD8^+$ T cells (Bacchetta *et al*, 2005a) although the mechanisms by which they achieve this is still the topic of intense research.

A role for GITR has been postulated since antibodies against GITR abrogated suppression in cocultures of T_{reg} and responder T cells (Shimizu *et al*, 2002c;McHugh *et al*, 2002b). Stephens *et al* demonstrated that interaction of GITR ligand (GITR-L), expressed primarily on antigen-presenting cells (APCs), with GITR resulted in a raised suppression threshold on $CD25^-$ cells (Stephens *et al*, 2004). Another marker, cytotoxic T lymphocyte-associated antigen (CTLA)-4, has also been implicated with a role in T_{reg} function since antibodies against this marker result in abrogation of suppression (Perezblas *et al*, 1992b), enhanced antitumour immunity (Leach *et al*, 1996) and exacerbation of autoimmune disease (Karandikar *et al*, 1996;Luhder *et al*, 1998). Furthermore, T_{reg} important in regulating murine colitis have been shown to require

CTLA-4 ligation and secretion of TGF-β in order to carry out this role (Read *et al*, 2000b).

Table 1.5: *Characteristics & Properties of T_{reg} types*

T_{reg} type	**Phenotype**	**Target cells of suppression**	**Mode of action**	**Origin**	**References**
Natural regulatory T cells	$CD4^+CD25^+$ $FOXP3^+$	T cells APCs?	cell-cell contact, cytokines	thymus	(Sakaguchi, 2004) (Sakaguchi *et al*, 2006)
IL-10 secreting Tr1 cells	$CD4^+CD25^{\pm}$ $FOXP3^-$	T cells	cell-cell contact? Secreted IL-10	periphery	(Grazia Roncarolo *et al*, 2006) (Battaglia *et al*, 2006b)
TGF-β-secreting Th3 cells	$CD4^+CD25^{\pm}$ $FOXP3^+$	T cells	secreted TGF-β	periphery	(Weiner, 2001a)
Qa-1-restricted $CD8^+$ T cells		antigen-activated T cells differentially expressing Qa-1-self peptide complexes	cytotoxicity, cell-cell contact, cytokines?		(Lu *et al*, 2006)
$CD8^+CD28^-$ T_{reg}		dendritic cells	upregulation of ILT3 and ILT4 on DCs		(Filaci *et al*, 2004)
$CD8^+CD122^+$ T_{reg}		T cells	?	periphery	(Rifa'i *et al*, 2004)
γ/δ T cells		T cells	cytokines? cytotoxicity? cell-cell contact?	thymus	(Hayday & Tigelaar, 2003)
NKT cells	$Vα24^+Vβ11^+$	tumour cells, pathogen-activated T cells and/or APCs	IL-4, IL-10, TGF-β, IFN-γ, cytotoxicity		(Sonoda *et al*, 2001) (Terabe & Berzofsky, 2004) (Terabe *et al*, 2000)

Abbreviations: APCs – antigen presenting cells; NKT – natural killer cells; TGF-β – transforming growth factor-β; IL-10 – interleukin-10; ILT – immunoglobulin-like transcripts; DCs – dendritic cells; IFN-γ – interferon-γ. (Table created using Rouse & Suvas, 2004a;Lan *et al*, 2005b;O'Garra & Vieira, 2004;Rouse *et al*, 2006).

Natural T_{reg} have been shown to regulate the proliferation, differentiation and effector function of a number of cell types, including $CD4^+CD25^-$ T cells, CD8 T cells and B cells (Lim *et al*, 2005). Determining the mechanisms by which T_{reg} suppress target cell

activity has been a major focal point of research in this field. To date, these fall into one of three categories; cell-cell contact, release of immunomodulatory cytokines and local competition for growth factors.

Roles for membrane TGF-β and CTLA-4 have been shown in cell-cell contact studies although results often conflict and it is likely that slight differences in methodology greatly effect data. A role for cyclic adenosine monophosphate (cAMP), a molecule associated with inhibition of cell proliferation, differentiation and downregulation of genes encoding IL-2 and IFN-γ, has been demonstrated. Minguet *et al*, (2005) showed that T_{reg} increased cAMP levels in target cells, either directly by T_{reg} themselves or indirectly via local generation of adenosine.

Numerous studies have demonstrated that T_{reg} can mediate their activity via secretion of immunomodulatory cytokines such as TGF-β and IL-10, produced either by T_{reg} or T_{reg}-induced production from other cells such as antigen presenting cells (APC). More recently, a novel cytokine, produced by T_{reg} and designated IL-35, has been shown to have an inhibitory effect on T cell proliferation (Collison et al, 2007).

The third mechanism of inhibition employed by T_{reg} is competition for growth factors. *In vitro* studies have shown that T_{reg} compete with naïve CD4 T cells for IL-2 (Barthlott *et al*, 2005). In addition, Pandiyan *et al*, (2007) established both *in vitro* and *in vivo* that

the presence of T_{reg} resulted in cytokine deprivation-induced apoptosis of CD4 effector T cells, whereas activation and proliferation were unaffected.

A recent review on the subject of immunosuppressive mechanisms employed by T_{reg} suggests that the T_{reg} response is likely to be determined by the conditions under which the T_{reg} encounters a target cell (Sojka *et al*, 2008).

1.4.2 History

The late immunologist Richard Gershon was the first to propose that a group of T cells, termed suppressors, were responsible for the tolerance he observed in a mouse model (Gershon, 1975). He postulated that these cells mediated their suppressive role by producing soluble factors, specific for certain antigens and consisted of MHC-encoded I-J determinants and, initially, a number of studies supported this finding (Tada *et al*, 1976;Murphy *et al*, 1976;Okumura *et al*, 1976). These cells were primarily associated with tolerance although Horohov *et al* extended the notion of suppressive cells to infectious disease scenarios (Horohov *et al*, 1985). However, the concept fell into disrepute with the inability of researchers to verify reports of the secreted molecules (Shevach, 2000). With the discovery of the Th1/Th2 lineage of T cells in the late 1980s, suppressive activity of T cells was attributed to counter-production of cytokines by these T cell subsets (Shevach, 2000) and interest in suppressors waned.

Despite the lack of evidence for a regulatory subset of T cells, a minority of groups persisted in their research using murine models of autoimmune disease (Shevach, 2000).

The major break-though came with the discovery in a murine model of autoimmune disease, that $CD4^+$ T cells with suppressive activity expressed the IL-2R α-chain, CD25 (Sakaguchi *et al*, 1995b), finally providing a more definitive marker in this system.

Key observations early in the T_{reg} field were that T cells, distinguishable by high expression of CD25, existed to control the response of autoreactive T cells to self-antigens (Sakaguchi *et al*, 1995a;Thornton & Shevach, 1998a). Furthermore, autoimmune lesions could occur when T_{reg} were absent (Maloy & Powrie, 2001). When such cells were present, the inflammatory effects of autoreactive T cells could be modulated both *in vitro* and *in vivo* (Thornton & Shevach, 1998b;Sakaguchi, 2005).

With the rebirth of T_{reg}, questions were again raised concerning the role of similar T cells in infectious disease. In 2002, the first proof that T_{reg} played a role in infectious disease was provided by Belkaid *et al* in a model of parasitic infection with *Leishmania major* (Belkaid *et al*, 2002a). Since then a number of publications have described a role for T_{reg} in infection with various pathogens (Suvas *et al*, 2003b).

1.4.3 Role of Regulatory T cells in autoimmune disease

The vast number of microorganisms capable of causing disease demands a broad repertoire of TCRs in order for the host to mount an adaptive immune response. Immature T cells are checked for reactivity to self-peptides within the thymus and the majority of cells with high affinity for host-derived proteins are deleted. Peripheral tolerance is maintained through a variety of mechanisms consisting of anergy, deletion

or suppression. Despite these checkpoints, self-reactivity can occur by a variety of mechanisms, such as cross-reactivity or molecular mimicry, resulting in autoimmune disease.

Evidence for a role for T_{reg} in peripheral tolerance came originally from murine models where deletion of $CD4^{+}CD25^{+}$ T_{reg} by thymectomy resulted in spontaneous multiorgan autoimmune disease; adoptive transfer of these regulatory cells prevented the development of the disease (Asano *et al*, 1996;Suri-Payer *et al*, 1999;Asseman *et al*, 1999;Liu *et al*, 2005;Liu *et al*, 2003). Since then T_{reg} have been shown to play a role in many murine models of autoimmunity.

The importance of T_{reg} in human autoimmunity is evidenced by a genetic disorder, IPEX, that manifests as multiple autoimmune disease, severe allergy and inflammatory bowel disease. The symptoms are caused by either a T_{reg} deficiency or their dysfunctional regulation, both as a result of a defective gene encoding the transcription factor, FOXP3 (Bennett *et al*, 2001a). Elucidating the role played by T_{reg} in autoimmune diseases, such as type 1 diabetes and multiple sclerosis (MS), may allow scientists to manipulate or enhance T_{reg} activity thereby having great clinical relevance. Table 1.6 summarises what is currently known about T_{reg} activity in other human autoimmune diseases.

Table 1.6: *Autoimmune diseases & T_{reg}*

autoimmune disease	T_{reg} frequency	suppression of conventional T cell proliferation	cytokine suppression	FOXP3 expression	other abnormalities
Multiple Sclerosis	normal	low	low suppression of IFN-γ	?	low T_{reg} cloning ability in response to IL-2 & PHA
Rheumatoid arthritis	normal	normal	low suppression of IFN-γ & TNF-α	?	inability to convey suppressive phenotype to $CD25^-$ counterparts
Autoimmune polyglandular syndrome type II	normal	none	?	normal	NA
Myasthenia gravis	normal	none	?	low	increased expression of HLA-DR and Fas on T_{reg} and different T_{reg} localisation in thymus
Kawasaki disease	low	?	?	low	low GITR and CTLA-4 mRNA expression
Type 1 diabetes	low	?	?	?	NA
Autoimmune lymphoproliferative syndrome	low	?	?	?	NA
Systemic lupus erythematosus	low	?	?	?	NA

Abbreviations: IFN-γ – Interferon-γ; TNF-α – tumour necrosis factor-α; IL-2 – interleukin-2; PHA – phytohaemagglutin; NA – not applicable; GITR – glucocorticoid-induced TNF receptor family-related gene; CTLA-4 – cytotoxic-T-lymphocyte-associated protein. (Table adapted from Lan *et al*, 2005a).

Experimental autoimmune encephalomyelitis (EAE) is the murine model used to investigate MS since they share many of the same features, in that myelin-specific T cells initiate inflammation of the central nervous system (CNS) resulting in the ultimate paralysis of the host. McGeachy *et al* recovered IL-10 producing $FOXP3^+T_{reg}$ from the CNS that were able to suppress the proliferation of $CD4^+CD25^-$ T cells at ratios as low as 1:30 *in vitro* (McGeachy *et al*, 2005). In an adapted model of EAE, they

demonstrated high rates of T_{reg} proliferation within the CNS but not in the lymph nodes, an indication that the T_{reg} were activated within the CNS rather than homing to the site of inflammation (O'Connor *et al*, 2007). The specific antigen required to initiate T_{reg} activity within the CNS has yet to be determined.

1.4.4 Role of Regulatory T cells in Infection

In autoimmune disease settings, T_{reg} play an important role in controlling the immune response to self. In microbial infection, a balance must be set between an excessive immune response to the infectious agent, resulting in immunopathological damage, and mounting an insufficient response allowing pathogen persistence. The importance of achieving this balance is shown by a murine model in which mice are infected with *Helicobacter pylori*, a bacterium linked to stomach ulcers, gastritis and cancer in humans. Adoptive transfer of lymph node cells, depleted of $CD4^+CD25^+$ T cells, to T cell-deficient mice infected with *H. pylori* drives a more robust immune response to the bacterium than non-depleted lymph node cells but results in enhanced gastric inflammation (Raghavan *et al*, 2003) causing damage to the host. *In vitro* evidence points to a similar role for natural T_{reg} in humans infected with *H. pylori* (Lundgren *et al*, 2003). Similar situations are observed in other infectious diseases including parasitic infection with *Leishmania major* (Belkaid *et al*, 2002b) and viral infection with HSV-1 (Suvas *et al*, 2004c). Table 1.7 summarises the microbial infections with evidence of T_{reg} activity. The consequences of limiting collateral damage are pathogen survival and in some cases persistence. Belkaid & Rouse put it as ‘a compromise between the host and pathogen’ (Belkaid & Rouse, 2005a).

Table 1.7: *Microbial infections in which a role for Natural T_{reg} has been implicated*

Microbe		species	reference
Parasites			
	Leishmania major	mouse	(Belkaid *et al*, 2002c)
	Plasmodium yoelii	mouse	(Hisaeda *et al*, 2004)
	Pneumocystis carinii	mouse	(Hori *et al*, 2002)
Bacteria			
	Listeria monocytogenes	mouse	(Kursar *et al*, 2002)
	Helicobacter hepaticus	mouse & human	(Maloy *et al*, 2003)
	Helicobacter pylori	mouse & human	(Raghavan *et al*, 2003)
	Bordetella pertussis		(McGuirk *et al*, 2002)
Fungi			
	Candida albicans		(Montagnoli *et al*, 2002)
Viruses			
	Friend virus	mouse	(Dittmer *et al*, 2004b)
	Murine AIDS	mouse	(Beilharz *et al*, 2004)
	FIV	cat	(Vahlenkamp *et al*, 2004)
	HIV	human	(Aandahl *et al*, 2004a)
	CMV	human	(Aandahl *et al*, 2004b)
	HSV-1	mouse	(Suvas *et al*, 2003a;Suvas *et al*, 2004b)
	HCV	human	(Sugimoto *et al*, 2003a;Boyer *et al*, 2004)

Abbreviations: AIDS – acquired immunodeficiency syndrome; FIV – feline immunodeficiency virus; HIV – human immunodeficiency virus; CMV – cytomegalovirus; HSV – herpes simplex virus; HCV – hepatitis C virus. Table created using (Rouse & Suvas, 2004c;Belkaid & Rouse, 2005b)

1.4.5 Role of Regulatory T cells in Persistent Viral infection

The majority of viruses possess immune evasion mechanisms, particularly those that cause persistent infection (Rouse & Horohov, 1986;Tortorella et al, 2000). In 2001, Iwashiro *et al* provided the first indication that a virus might subvert the immune response by inducing T_{reg} activity (Iwashiro *et al*, 2001a). The group were studying chronic Friend virus leukaemia infection of mice, characterised by increases in splenic

$CD4^+$ T cells to almost double their normal frequency and loss of ability to reject tumour transplants. Iwashiro *et al* established that the observed immunosuppression of anti-tumour immunity was mediated by $CD4^+$ T cells since adoptive transfer of these cells from infected mice to uninfected animals resulted in a similar effect. Subsequently the group identified T_{reg} as the cause of $CD8^+$ T cell response suppression (Iwashiro *et al*, 2001b), an effect that was only detected in persistently infected animals (Dittmer *et al*, 2004a). Since then much evidence has been collected and several good reviews exist on the role of regulatory T cells in viral infection (Rouse & Suvas, 2004b;Belkaid & Rouse, 2005c;Rouse *et al*, 2006). A role for T_{reg} in Hepatitis C virus (HCV) and HIV infection has been established. Using these infections as the most well studied examples, the role of T_{reg} in human persistent viral infections will be discussed below.

Hepatitis C virus

HCV infects over 170 million people worldwide (Racanelli & Manigold, 2007) and is one of the major causes of chronic hepatitis and commonest cause of liver transplants. The outcome of primary HCV infection is highly variable; ranging from complete erradication to viral persistence, liver cirrhosis and development of hepatocellular carcinoma (Rehermann *et al*, 1996). Robust $CD8^+$ and $CD4^+$ T cell responses are associated with more favourable outcomes (Thimme *et al*, 2001). In 2002, MacDonald *et al* isolated IL-10-producing Tr1 cells from chronically infected patients (MacDonald *et al*, 2002a) implying a role for induced T_{reg} in HCV infection.

Evidence from numerous studies on HCV indicates that an expansion of T_{reg} may account for the suppression of effector $CD8^+$ T cell immunity observed *ex vivo* in chronically infected patients (Sugimoto *et al*, 2003b;Boettler *et al*, 2005g), Cabrera *et al* reported that the suppression observed was dependent on TGF-β and cell-cell contact (Cabrera *et al*, 2004g). In addition, they and others demonstrated specificity by IL-10 release from T_{reg} in response to HCV antigens (Cabrera *et al*, 2004f;MacDonald *et al*, 2002b). However, the dependency on cytokines is disputed by two recent reports in which administration of antibodies to IL-10 or TGF-β did not abrogate suppression (Boettler *et al*, 2005f;Rushbrook *et al*, 2005a). Of interest, both these studies demonstrated suppressed $CD8^+$ T cell responses to other viruses, including Influenza and EBV in chronically infected HCV patients (Boettler *et al*, 2005e;Rushbrook *et al*, 2005g). Currently the majority of studies have been conducted *in vitro* and as Rouse *et al* report in a recent review, preliminary findings of an ongoing chimpanzee model of HCV infection has provided evidence against a role for T_{reg} in viral pathogenesis (Rouse *et al*, 2006). Studies are still required to establish what precise role T_{reg} play in HCV infection if any.

Human Immunodeficiency Virus

Infection with human immunodeficiency virus (HIV) ultimately results in reduced $CD4^+$ T cell numbers, excessive immune activation and eventually HIV patients develop Acquired Immunodeficiency Syndrome (AIDS) and succumb to opportunistic infections (Douek *et al*, 2003).

The investigation of T_{reg} activity in HIV infection is difficult since the phenotype of human T_{reg} is somewhat indistinguishable from highly activated T cells, such as those $CD4^+$ T cells found in HIV^+ patients. In addition, the majority of studies have been undertaken on peripheral blood leukocytes (PBL) which is unlikely to have provided an accurate representation of the immunological events that occur in HIV infection since the critical interactions between HIV and T_{reg} occur in the lymphoid tissue.

An indication that T_{reg} played a role in HIV infection was first demonstrated by Nixon's group by depleting T_{reg} from PBMC and enhancing the HIV-specific T cell response *in vitro* (Aandahl *et al*, 2004c). Several other groups reported similar findings (Kinter *et al*, 2004b;Weiss *et al*, 2004a). The suppression these groups observed appeared to be cell-contact dependent and cytokine independent, although the Weiss team reported that T_{reg} isolated from some HIV^+ patients responded specifically to the HIV antigen, p24, by producing TGF-β and IL-10 (Weiss *et al*, 2004b). These studies indicate a negative role for T_{reg} since they depress the anti-HIV T cell responses.

The question of whether the role of T_{reg} varied with disease progression was addressed by Kinter *et al* in a cohort of HIV-infected individuals at various stages of disease (Kinter *et al*, 2004a). HIV-specific T_{reg} activity was investigated *in vitro* and compared to viral load and the $CD4^+$ T cell count. The results showed that HIV patients with HIV-specific T_{reg} activity had lower viral loads in the plasma and increased $CD4^+$ T cell counts than patients with reduced HIV-specific T_{reg} activity (Kinter *et al*, 2004c). These

findings led researchers to contemplate that T_{reg} may play a beneficial role in HIV infection.

Other research has indicated that T_{reg} may play a positive role in HIV infection by limiting the characteristic immune activation observed with this disease. Oswald-Richter's group observed that the frequency of FOXP3-expressing $CD4^+CD25^{hi}$ T cells was reduced in HIV-infected individuals with low $CD4^+$ T cell counts and high T cell activation in comparison to healthy HIV patients (Oswald-Richter *et al*, 2004b). Eggena *et al* demonstrated a reduced frequency of T_{reg} in HIV^+ patients when compared to uninfected individuals. Furthermore, the reduction in T_{reg} frequency was associated with $CD4^+$ and $CD8^+$ T cell activation (Eggena *et al*, 2005). Both studies imply T_{reg} depletion could hasten progression from HIV to AIDS.

Currently the cause of T_{reg} depletion in HIV infection is unknown although a number of plausible suggestions have been made. HIV has been shown to directly infect T_{reg} and transduction of conventional $CD4^+$ T cells with FOXP3 generates cells that are more permissive to infection, although it is unclear whether this renders the T_{reg} ineffective (Oswald-Richter *et al*, 2004a). This suggests the possibility that T_{reg} are targeted by HIV and infection specifically kills the cell.

Underlying the interest in T_{reg} depletion is the notion that although these cells are reduced in frequency in the blood, they may be redistributed, possibly localising to

lymphoid tissue during in HIV infection, as has been reported in non-HIV systems. Andersson *et al* provided data supporting the latter scenario in a longitudinal study of HIV infected subjects pre- and post-anti-retroviral therapy (Andersson *et al*, 2005b). During periods of HIV viremia, T_{reg} moved from peripheral blood to lymphoid tissue. This was reversed when treated with anti-retroviral therapy. Hence, the reduced frequency of T_{reg} in HIV patients may reflect a redistribution of T_{reg} rather than an overall decrease (Andersson *et al*, 2005a). However, the group only identified T_{reg} phenotypically and no functional studies were carried out.

1.4.6 Evidence for a Role for Regulatory T cells in EBV infection

In order to avoid the host's immune response, persistent viruses have a number of immune evasion tactics. One particular mechanism of immune evasion adopted by EBV has been of interest recently since researchers have not determined a satisfactory answer to the lack of immunogenicity of LMP-1, a latent EBV antigen expressed by infected B cells and other EBV-associated malignancies such as HL and NPC. In light of emerging evidence for T_{reg} activity in persistent viral infections and previous research demonstrating the production of the immunosuppressive cytokine, IL-10, by $CD4^+$ T cells stimulated *in vitro* with autologous LCL (Wilson *et al*, 2001), Vickers and colleagues hypothesised that LMP-1 induced T_{reg} activity and set out to investigate this theory (Marshall *et al*, 2003h).

PBMC isolated from EBV-seropositive donors were stimulated with purified LMP-1, resulting in high levels of IL-10 production by $CD4^+$ T cells. Additional experiments

established that addition of LMP-1 to donor PBMC resulted in the inhibition of both cell proliferation and IFN-γ release to non-EBV antigens, and suppression was dependent on IL-10 since neutralisation of this cytokine abrogated suppressive activity (Marshall *et al*, 2003g). The same group assayed $CD4^+$ T cell function from HL patients and reported high levels of IL-10 secreting regulatory T (Tr1) cells with suppressive activity in HL-infiltrating lymphocytes (HLILs). The T_{reg} they describe were phenotypically identical to induced Tr1 cells, however, the mechanism by which Tr1 cells primarily mediate their suppressive activity is IL-10 alone, whereas this group describe regulatory activity being abrogated by prevention of cell-cell contact as well as by blocking CTLA-4 (Marshall *et al*, 2004a).

Khanna's group took this research one step further and investigated the ability of T_{reg} to suppress responses to EBV latent antigens expressed in HL. They determined that a high level of lymphocyte activation gene (LAG)-3 expression, another marker affiliated with T_{reg} (Huang *et al*, 2004b), on HLILs was associated with the loss of LMP-1/2-specific T cell function. They also demonstrated that $CD4^+LAG\text{-}3^+$ T cells possessed regulatory properties (Gandhi *et al*, 2006a). They showed an association between LAG-3 and EBV gene expression in tumour tissues and established that LAG-3 was expressed more frequently on lymphocytes than FOXP3, and generally lymphocytes did not express both proteins. No association was found between FOXP3 and LAG-3, histology or EBV status. $LAG\text{-}3^+CD4^+$ T cells in the peripheral blood were enriched for $CTLA\text{-}4^{hi}$ and

GITR^{hi} but not FOXP3 and were present at higher frequency in patients with active than inactive disease (Gandhi *et al*, 2006c).

In an attempt to understand the specificity of T_{reg} in EBV infection, Voo *et al* undertook *in vitro* experiments in which they stimulated PBMC with EBNA-1 peptides. They reported that the expanded T cell lines included $CD4^+$ helper T cell clones but also $CD4^+$ T cells with regulatory phenotypes and confirmed suppressive activity by functional assays (Voo *et al*, 2005c). Both types of cell recognised the same EBNA-1 epitopes. The method of suppression was cell-contact dependent although some clones were still capable of suppression even in transwell coculture assays indicating a soluble inhibitory factor which was shown not to be IL-10 or TGF-β (Voo *et al*, 2005b).

Because of the immunosuppressive nature of HL infiltrating lymphocytes and the ability of LMP-1 to stimulate T_{reg} responses in healthy donors, Marshall *et al* hypothesised that LMP-1 was important in the generation of T_{reg} responses in HL. They compared Th1, Th2 and T_{reg} responses to LMP-1 by PBMC and HLILs from EBV-positive and EBV-negative HL patients. EBV-positive HL patients had increased numbers of *ex vivo* IL-10 secreting, CTLA-4-expressing cells compared to EBV-negative HL patients both in the periphery and lymph nodes. PBMC/HLIL responses to LMP-1 in most patients were characterised by IL-10 secretion. These results are consistent with the notion that LMP-1 induces Tr1 cells as shown in previous publications (Dukers *et al*, 2000a;Marshall *et al*, 2003f), and these T_{reg} cells infiltrate the HL tissue (Marshall *et al*, 2007b), but with

no clear evidence of a greater bias toward regulation in EBV-positive HL cases over EBV-negative cases, other mechanisms of T_{reg} cell induction are also likely.

The part played by T_{reg} cells in EBV infection is of interest for two major reasons. Firstly, elucidating the role of T_{reg} cells in primary EBV infection may expose opportunities for immunomanipulation at the initial stage of infection to ameliorate the symptoms of IM or prevent the subsequent risk of HL. Secondly, EBV is an oncogenic virus and infection is associated with the development of a number of malignancies of lymphocytic and epithelial origin (reviewed in Young & Rickinson, 2004). The T_{reg} frequency and functional capacity may affect the level of viral persistence and therefore clarification of the T_{reg} role could provide insight into the development of EBV-related tumours (Marshall *et al*, 2004b). Voo *et al* have described the generation of a $CD4^+$ T cell line via stimulation with EBV peptides. Upon phenotypic analysis, the T cell line was found to contain T_{reg} as well as helper T cells. This highlights the need to determine T_{reg} specificity in EBV-related diseases since a cancer vaccine that causes the development of T_{reg}, as opposed to CD4 helper T cells, could have serious consequences for the recipients (Voo *et al*, 2005a).

An early publication demonstrated suppressor T cells cloned from two IM patients that demonstrated no cytotoxicity, reduced EBV-induced immunoglobulin production and delayed the outgrowth of immortalised cells (Wang *et al*, 1987). Furthermore, the EBV lytic gene BCRF1, that encodes an IL-10 homologue, is thought to be expressed to

downregulate cytotoxic immune responses during viral replication (Suzuki *et al*, 1995). This may provide an environment conducive to T_{reg} induction.

1.5 Project Aims

The aim of this project is to investigate the following hypothesis:

> *'T_{reg} are specifically activated by EBV antigens during primary infection and contribute to the generalised immunosuppression'.*

In order to investigate the hypothesis the following questions need to be answered:

In Healthy donors:

1. Can EBV specific T_{reg} be activated in PBMC from EBV seropositive donors?
2. Do T_{reg} inhibit EBV specific CTL activity?
3. What is the antigenic specificity of T_{reg}?

In IM patients:

1. Is T_{reg} activity detectable in IM PBMCs?
2. Are T_{reg} raised or reduced in IM patients?
3. Does the level of T_{reg} activity correlate with:
    a) Level of generalised immunosuppression?
    b) Level of EBV specific CTL activity?
4. What is the antigenic specificity of T_{reg}?

2 Materials and Methods

2.1 Suppliers, equipment and solutions

2.1.1 Suppliers

AbD Serotec	Endeavour House, Langford Business Park, Langford Lane, Kidlington, Oxford, OX5 1GE, UK.
ATi Atlas	The Grange, Church Road, North Mundham, Chichester, West Sussex, PO20 1JQ, UK.
Becton Dickinson	The Danby Building, Edmund Halley Road, Oxford Science Park, Oxford, OX4 4DQ, UK.
Dako	Denmark House, Angel Drove, Ely, CB7 4ET, UK.
Fisher Scientific	Bishop Meadow Road, Loughborough, LE11 5RG, UK.
GE Healthcare	Amersham Place, Little Chalfont, HP7 9NA, UK.
Invitrogen	Inchinnan Business Park, 3 Fountain Drive, Paisley, PA4 9RF, UK.
Mast Diagnostics	Mast House, Derby Road, Bootle, L20 1EA, UK.
Microgen Bioproducts Ltd	1 Admiralty Way, Camberley, Surrey, GU15 3DT, UK.
Miltenyi Biotec	Almac House, Church lane, Bisley, Surrey GU24 9DR, UK.

Nalgene	Nalge (Europe) Ltd, Unit 1a, Thorn Business Park, Hereford, HR2 6JT, UK.
Novocastra	Balliol Business Park, West Benton Lane, Newcastle Upon Tyne, NE12 8EW, UK.
Nunc Brand	Supplied by Fisher Scientific UK Ltd, Bishop Meadow Rd, Loughborough, Leicestershire LE11 5RG, UK.
Perbio	Unit 9, Atley Way, North Nelson Industrial Estate, Cramlington, NE23 1WA, UK.
Promega	Delta House, Southampton Science Park, Southampton, SO16 7NS, UK.
Roche Diagnostics Ltd	Charles Avenue, Burgess Hill, West Sussex, RH15 9RY, UK.
Sigma Aldrich	The Old Brickyard, New Road, Gillingham, SP8 4XT, UK.
Statens Serum Institut	5 Artillerivej DK-2300, Copenhagen S, Denmark.
Thermo Scientific	Thermo Fisher Scientific Inc. 81 Wyman Street, Waltham, MA 02454, US.
Thistle Research	27 Westbourne Crescent, Glasgow G61 4HB, UK.

Thistle Scientific	DFDS House, Goldie Road, Uddingston, G71 6NZ, UK.
Vector Laboratories	3 Accent Park, Bakewell Road, Peterborough PE2 6XS, UK.
VWR	Hunter Boulevard, Magna Park, Lutterworth, LE17 4XN, UK.

2.1.2 Equipment

Item	**Model and supplier**
AID Elispot Reader version 3.2.3	Autoimmun Diagnostika
Automatic pipette	‘Pipetboy acu’, Integra Biosciences ‘Powerpette plus’, Jencons
Balances	fine: ‘AE163’, Mettler medium: ‘BP310P’, Sartorius gross: ‘EK-200G’, AND
Centrifuges	‘Falcon 6/300’, MSE ‘Micro Centaur’ MSE ‘Mistral 3000E’, MSE ‘Mistral 3000i’, MSE ‘Cytospin 2’, Shandon
Flow cytometers	‘FACScan’, Becton Dickinson ‘FACSCalibur’, Becton Dickinson

Fume hood	Lynwood Installations
Gamma counter	'1480 Wizard', Perkin Elmer
Haemocytometer	Scientific Laboratory Supplies Improved Neubauer Bright
Heating block	'DB.3', Techne
Histology Image Capture	'BX51' Olympus microscope with Polaroid 'PDMC-2' camera and 'DMC le' software
Humidity chamber	Sandrest
Incubators	37°C: Windsor, Sandrest 37°C humidified: Leec, Forma Scientific
Microbiological safety cabinets (Class II)	Medical Air Technology Envair Arrowmight Biosciences
Microscopes	'Laborlux K', Leitz 'TMS', Nikon
Microwave	'SM18', Proline
pH meter	'HI 8521', Hanna Instruments
Pipettes	'Pipetman', Gilson

Plate reader	'MRX II', Dynex Technologies
Refrigeration	4°C: Electrolux
	-20°C: Labcold
	-70°C: 'Ultra 85', Assab
Stirrer	'Magnetic Stirrer Hotplate', Stuart Scientific
Waterbaths	Clifton
	Grant Instruments

2.1.3 Solutions

Tissue Culture Medium 10% (TCM 10%)

Roswell Park Memorial institute (RPMI) 1640	500ml
L-glutamine	2mM
Penicillin	100IU/ml
Streptomycin	100IU/ml
Foetal calf serum (Perbio)	10% v/v

With the exception of foetal calf serum, all the above reagents were from Invitrogen

Tissue Culture Medium 20% (TCM 20%)

RPMI 1640	500ml
L-glutamine	2mM
Penicillin	100U/ml

Streptomycin	100μg/ml
Foetal calf serum	20% v/v

Wash Medium (Wash)

Hank's Balanced Salt Solution (HBSS, Invitrogen)	500ml
Foetal calf serum	2% v/v

Phosphate Buffered Saline (PBS)

PBS tablet (Oxoid)	1 tablet
Sterile distilled water	100ml

Fluorescent Activated Cell Sorting (FACS) buffer

Sterile PBS (pH 7.2)	500ml
Bovine Serum Albumin (Sigma)	1%
Sodium Azide (VWR)	0.1%
Ethylenediamine tetraacetic acid (EDTA, Sigma)	0.02%

Magnetic Activated Cell Sorting (MACS) buffer

Sterile PBS (pH 7.2)	500ml
Bovine Serum Albumin	0.5%
EDTA	2mM

FACS fix

BD CellFix™ 10% (Becton Dickinson)	1ml
Sterile distilled water	9ml

PBS/Tween

Sterile PBS	1l
Tween 20 (VWR)	0.02%

Freezing Medium

Foetal calf serum	45ml
Dimethyl Sulfoxide (DMSO, Sigma)	4.5ml

Antigen Retrieval Solution

Tris (Thistle Scientific)	50mM
EDTA	2mM
pH 9	

Serotec Erythrolyse Buffer

Serotec Erthrolyse buffer (AbD Serotec)	1ml
Sterile distilled water	9ml

2.2 Tissue Culture Techniques

2.2.1 Maintenance of cell lines

All cells, with the exception of CTL, were grown in TCM 10%. They were all incubated in a humidified 5% CO_2 incubator at 37^{o}C. CTL were grown TCM 20%. See table 2.1 for details of cell lines and references. Plastics for cell culture were supplied by Sterilin, BD Falcon, Nunc and Greiner Bio-One.

Table 2.1. *Details of cell lines used.*

Cell lines	Origin	Reference
B95-8	EBV transformed marmoset cell line	(Miller *et al*, 1972)
LCLs	Human B-cell lines transformed *in vitro* with B95-8	(Lam & Crawford, 1991)
P3HR1	Burkitt's Lymphoma	(Hinuma *et al*, 1967)
K562	Erythroleukaemia	(Lozzio & Lozzio, 1975)

2.2.2 Freezing and thawing cells

To store cells long term, viable cells were centrifuged at 180g for 7 minutes and resuspended in freezing medium at between $5x10^6$ and $2x10^7$ cells/ml. Volumes of 1ml were aliquoted into cryovials and placed in a Mr. Frosty Control Freeze container (Nalgene) for overnight slow freezing at -70°C before being transferred to liquid nitrogen. Frozen cell lines were thawed quickly in a 37°C incubator and washed once by centrifugation in wash solution at 180g for 7 min. Pelleted cells were resuspended at the appropriate concentration and media for the cell line

2.2.3 Counting cells

Cell viability and concentration were assessed using trypan blue incorporation. 10µl of 0.4% w/v trypan blue was mixed with 10µl of cell suspension. 10µl of the mix was added to the chamber on a haemocytometer. The unstained viable cells were counted by light microscopy.

2.3 Cell separation by centrifugation

Buffy coat cells were obtained from the Scottish National Blood Transfusion Service (SNBTS). Blood samples were provided by IM patients who were recruited from the Edinburgh University Health Centre. Acute IM was confirmed by immunofluorescence staining for IgG and IgM anti-VCA. Doctors provided further information regarding duration of symptoms. PBMC were isolated from buffy coats or blood samples by density gradient centrifugation with Histopaque-1077 (Sigma). Briefly, blood was over-laid onto the same volume of Histopaque-1077. Tubes were centrifuged at 600g for 20 minutes at 4^{o}C. Plasma was collected and stored either at -80^{o}C or -20^{o}C depending on its later use. PBMC were harvested from the histopaque/plasma interface and washed twice in wash solution, centrifuged at 180g for 7 minutes and finally counted. PBMC were either suspended in an appropriate volume according to use or frozen in freeze mix.

2.4 Virus Techniques

2.4.1 Production of EBV

B95-8 cells were grown up to a volume of 600ml in TCM 10%. Flasks were sealed for 7-10 days and then the contents were centrifuged at 490g for 10 min at 4^{o}C. The remaining suspension was poured into 0.8 micron filters. The filtered suspension was aliquoted into 1ml cryovials and stored at -80^{o}C until required.

2.4.2 Establishing a Lymphoblastoid Cell Line (LCL)

PBMCs were resuspended in an undiluted EBV preparation and incubated at 37°C with occasional agitation. After 1 hour the suspension was centrifuged at 180g for 7 minutes and the PBMC resuspended at 2x10^{6} cells/ml in TCM 10%, supplemented with 1µg/ml cyclosporin A (CSA, Sigma). PBMC were then seeded into a 48-well plate. Culture plates were incubated at 37°C in 5% humidified CO_2 and fed weekly by replacement of half the medium until outgrowth of immortalised cells signalled establishment of a LCL.

2.5 Preparation of therapeutic Cytotoxic T Lymphocyte (CTL) lines

2.5.1 T cell isolation and activation

PBMC were isolated from buffy coats as described in section 1.3. On day zero the PBMC were counted and stimulated with γ-irradiated autologous LCL at a 40:1 ratio. On day 10 the PBMC were re-stimulated, again with γ-irradiated autologous LCL, at a ratio of 4:1. On day 14 and weekly for 8 weeks thereafter the CTL were re-stimulated with LCL at this ratio and IL-2 (Novartis) was added to the culture to give a

concentration of 20U/ml. CTL which were grown without regulatory T cells were depleted of this cells population on day zero (for the method see section 2.9).

2.5.2 Cytotoxicity assay using Chromium

Cytotoxic activity *in vitro* was measured using a standard chromium release assay. Cells from each target cell line were labelled with ^{51}Chromium (Chromium-51 supplied as sodium chromate in sterile sodium chloride solution at 1mCi/ml, GE Healthcare) for 1 hour at 37°C. Target cells were then washed twice by centrifugation at 130g for 5 minutes in wash buffer. Target cells were then plated with effector cells at effector:target ratios of 20:1, 10:1 and 5:1. After incubation at 37°C for 4 hours the release of ^{51}Chromium from lysed cells was measured on a gamma counter. Spontaneous release was calculated by incubating target cells without effector cells, and maximum release calculated by incubating target cells with 1% Triton-X (Sigma). Percent specific lysis was calculated as follows;

$$\% \text{ specific lysis} = \frac{\text{(test release – spontaneous release)}}{\text{(maximum release – spontaneous release)}} \times 100$$

2.6 Animal Model

At the University of Edinburgh there is a breeding colony of CB.17 SCID mice. The colony is kept in a specific pathogen-free environment in microisolator (IVC) cages and handled in microbiological class 2 safety cabinets. The animals are fed autoclaved feed

and water without antibiotics. The animal work in this study was carried out by Dr Ingo Johannessen, under relevant Home Office Project and Personal Licences in accordance with the Home Office 'Animals' (Scientific Procedures) Act 1986', and was monitored by Home Office Inspectors and University Named Veterinary Surgeons.

2.6.1 Inoculation of SCID mice

Unmanipulated PBMC, PBMC with $CD4^+CD25^+$ T cells and PBMC depleted of $CD4^+CD25^+$ T cells, were washed with wash medium and resuspended at $5x10^7$ cells per 500µl in TCM 10%. The cells were then inoculated via subcutaneous injection at 500 µl per animal into the mice. Three mice were used for each cell fraction, and the experiment was repeated using PBMC from 5 healthy seropositive donors.

2.6.2 Monitoring tumour growth

Mice were monitored daily for signs of sickness and tumour development. Measurements were taken when the tumour was palpable using digital callipers. Up to day 100 post-injection, mice were culled by cervical dislocation if they showed signs of illness in accordance with Home Office instructions and then submitted for necropsy. At necropsy, a macroscopic assessment was carried out and the following organs removed: lung, liver, spleen, and any tumour tissue. All remaining animals were culled on day 100.

2.7 Immunohistochemistry

2.7.1 Cell Preparation

PBMC, CD4-depleted cells, $CD4^+CD25^-$ T cells or $CD4^+CD25^+$ T cells were separated as described later (see section 2.9) and resuspended in $1x10^6$ cells per ml in sterile PBS. In some experiments, cell fractions were incubated for 3 days with the Dynabeads®

CD3/CD28 T cell expander kit (Invitrogen), at a ratio of 1 bead to 1 cell, and 500IU/ml IL-2 prior to suspension in sterile PBS and cytospin preparation.

2.7.2 Cytospin Preparation

Double cytofunnels (Thermo Scientific) were prepared by dampening the filters with the addition of 50µl PBS and centrifugation at 250g for 3 minutes at room temperature. PBMC (100µl) were added to double funnel chambers and centrifuged onto polysine slides (72-Polysine microscope slides, VWR) so each spot consisted of $1x10^5$ cells. Slides were centrifuged as described above, left to air dry overnight and were not fixed. For long term storage slides were wrapped in silver foil and stored at -20^oC.

2.7.3 Immunohistochemistry for FOXP3

Paraffin embedded sections were dewaxed by dipping serially into Xylene (VWR), 100% Ethanol, 50% Ethanol and finally distilled water. Slides were microwaved in 50mM Tris/2mM EDTA pH 9 antigen retrieval solution for 4 minutes, then topped up and microwaved for a further 4 minutes. The slides were then transferred into PBS. The slides were developed with the EnVision®+ System-HRP Kit (DakoCytomation) as per manufacturer's instructions. Briefly, the slides were removed from PBS, tissue sections were circumscribed with a hydrophobic barrier pen (ImmEdge™ Pen, Vector Laboratories) and peroxidase block was added for 5 minutes. The slides were then washed in PBS/T then the primary antibody, anti-FOXP3 E7 (kindly provided by Dr Alison Banham's laboratory, Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Level 4 Academic Block, John Radcliffe Hospital, Headington,

Oxford, Oxon, OX3 9DU, UK), was applied undiluted to cover the sections. The slides were incubated with the primary antibody for 30 minutes, and then washed with PBS/T and a polymer anti-human mouse secondary antibody was added for a further 30 minutes. All incubations were carried out in a humid chamber. Slides were developed using 3,3'-diaminobenzidine (DAB), counterstained with hematoxylin (Vector Labs) and mounted with Faramount aqueous mountant (Dako). Controls performed using PBS as a primary antibody, then PBS or the polymer antibody as the secondary antibody ensured that positives seen were due to specific staining. An antibody which binds the B cell membrane, L26, also kindly provided by Dr Alison Banham, was used as a positive control.

2.7.4 Immunocytochemistry for FOXP3

With the exception of increased incubation periods (40-50 minutes) with both the primary and secondary antibodies, cytospins were stained using the same method outlined in section 2.7.3, starting with the peroxidase block step.

2.8 Enzyme-linked Immunosorbent Assays (ELISAs)

2.8.1 Sample Collection

Plasma from healthy controls and IM patients was obtained from whole blood after PBMC isolation and aliquots of 1ml were centrifuged at 1000g for 15 minutes. Samples were then stored at -20^{o}C until required. Plasma collected in this manner was used to measure levels of cytokines in plasma *ex vivo*. Cell culture supernatant from PBMC

stimulated with 15μg/ml LMP (PRG) peptide (Thistle Research), 10μg/ml Tuberculin PPD (Statens Serum Institut), 10μg/ml Phytohaemagglutinin (PHA, Sigma), or a combination of LMP peptide and PPD antigen, was collected after 5 days of incubation. Details of the LMP peptide can be found in table 2.2, section 2.10.3. Samples were centrifuged at 11000g for 7 minutes to remove particulates. These samples were used to determine levels of cytokines in cell culture supernatant after incubation of PBMC with a variety of stimulants.

2.8.2 Interleukin (IL)-10 ELISA

This ELISA was carried out as per manufacturer's instructions (R&D Systems). Briefly, 200μl of an IL-10 standard dilution, healthy seropositive plasma or IM plasma were incubated in duplicate wells for 2 hours at room temperature. The wells were washed and IL-10 conjugate was added and incubated for 2 hours at room temperature. The wells were washed again and the substrate was added and incubated for 30 min at room temperature in the dark. Finally stop solution was added. The optical density for each well was read at 450nm with the reference wave length set to 540nm using a microplate reader. IL-10 concentration was established using a standard curve. Since EBV is known to produce an IL-10 homologue (Hsu *et al*, 1990b), an ELISA was selected that recognised human IL-10 only.

2.8.3 Transforming growth factor (TGF)-β_1 ELISA

This ELISA was carried out as per manufacturer's instructions (R&D Systems). TGF-β_1 is generally secreted in an inactive form. The conjugate supplied in the kit only

recognises active TGF-β_1; therefore an activation step was incorporated into the protocol. Briefly, samples were incubated with 1 N HCl for 10 minutes at room temperature, followed by the addition of 1.2 N NaOH/0.5 M HEPES (Sigma). Samples were then diluted 20-fold in Calibrator Diluent. TGF-β_1 standard dilutions, healthy seropositive plasma and IM plasma were added to duplicate wells (wherever possible) in 50µl aliquots and then incubated for 2 hours at room temperature. The wells were washed and TGF-β_1 conjugate was added and incubated for 2 hours at room temperature. The wells were washed again and the substrate was added and incubated for 30 min at room temperature in the dark. Finally stop solution was added. The optical density for each well was read at 450nm with the reference wave length set to 540nm using a microplate reader. TGF-β_1 concentration was established using a standard curve.

2.8.4 Interferon (IFN)-γ ELISA

This ELISA was carried out as per manufacturer's instructions (R&D Systems). Briefly, 100µl of IFN-γ standard dilution, healthy seropositive plasma or IM plasma were incubated in duplicate wells for 2 hours at room temperature. The wells were washed and IFN-γ conjugate was added and incubated for 2 hours at room temperature. The wells were washed again and the substrate was added and incubated for 30 min at room temperature in the dark. Finally stop solution was added and the optical density for each well was read at 450nm with the reference wave length set to 540nm using a microplate reader.

2.9 Cell Separation

Regulatory T cells were enriched by negative selection followed by positive selection as per manufacturer's instructions ($CD4^+CD25^+$ T cell isolation Kit, Miltenyi Biotec). PBMC were isolated as previously described in section 2.3 and counted. Up to $1x10^8$ PBMC were centrifuged at 180g for 10 minutes with MACS buffer. The supernatant was removed entirely and the cells were resuspended in 90µl of MACS buffer per 10^7 cells. Biotin-Antibody Cocktail (containing monoclonal anti-human antibodies against CD8. CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and glycophorin A) was added at 10µl per 10^7 cells and incubated at 4-8^{o}C for 10 minutes. Anti-biotin microbeads were then added at 20µl per 10^7 cells and incubated at 4-8^{o}C for 15 minutes. The cells were then washed by centrifugation at 180g for 10 minutes in 2ml of MACS buffer, resuspended in 500µl buffer and applied to a prepared LD (designed particularly for stringent depletion of unwanted cells) column (Miltenyi Biotec). Flow-through was collected ($CD4^+$ enriched population) and the column was washed with MACS buffer twice. The column was then removed from the magnet and the remaining cells were eluted ($CD4^+$ depleted population). Both cell populations were counted. The $CD4^+$ enriched population was then washed by centrifugation at 180g for 10 minutes, resuspended in 90µl MACS buffer per 10^7 cells and 20µl of anti-CD25 microbeads added per 10^7 cells. The cells were again incubated at 4-8^{o}C for 15 minutes. The cells were washed by centrifugation at 180g for 10 minutes, resuspended in 500µl MACS buffer and applied to a prepared MS (specifically designed for positive selection of cells) column (Miltenyi Biotec). Flow-through was collected (CD25 depleted population) and

the column was washed with MACS buffer twice. The column was then removed from the magnet and the remaining cells eluted (CD25 enriched population). Cell subsets were then counted as described in section 2.2.3.

2.10 Enzyme-linked Immunosorbent Spot (ELISPOT) assays

2.10.1 General Protocol

The ELISPOT assay was carried out as per manufacturer's instructions (R&D Systems). Briefly, PBMC were set up at a variety of densities and with various stimulants. TCM 10% was added to each well of an ELISPOT plate for 20 minutes. The medium was then removed and the plate blotted dry. Cells were added to appropriate wells according to the particular assay and incubated for 48 hours at 37^{o}C. Excess medium was removed by flicking and the plate was rinsed four times with wash solution. Anti-IFN-γ detection antibody was then added and the plate was incubated overnight at 4^{o}C. The antibody was removed by flicking, the plate was rinsed four times with wash solution and Streptavidin AP was added and incubated for 2 hours at room temperature. The plate was then washed four times and BCIP/NBT (5-Bromo-4-Chloro-3'Indolyphosphate p-Toluidine salt/ Nitro blue Tetrazolium Chloride, IFN-γ ELISPOT kit, R&D Systems) was added for 1 hour at room temperature. Finally the plate was washed with deionized water and allowed to dry at room temperature. Initially spots per well were counted using a dissection microscope and then again using an AID ELISPOT reader (Autoimmun Diagnostika). A background level of IFN-γ was obtained from

unstimulated PBMC which was subtracted from the IFN-γ produced in response to stimulation.

2.10.2 LCL Stimulation

LCL were used to stimulate PBMC at the following ratios: 1:1, 1:2, 1:5 and 1:10. Cells were incubated for 48 hours before continuing with the ELISPOT protocol. Stimulation with 5μg/ml PHA was used as a positive control, PBMC alone were used as negative controls. Tests were run in duplicate.

2.10.3 Peptide Stimulation

PBMC were resuspended in TCM 10% and added to wells of the ELISPOT plate at a density of $2x10^5$ cells/well. Peptides (see table 2.2 for details) were added individually at 10μg/ml to the PBMC and incubated for 48 hours before continuing with the ELISPOT protocol. Stimulation with 5μg/ml PHA was used as a positive control, PBMC alone and PBMC stimulated with a non-A2 restricted peptide were used as negative controls. Tests were run in triplicate.

Table 2.2. *Details of peptides used in ELISPOT and ELISA assays*

Peptide sequence	Abbreviated sequence	HLA restriction	EBV protein	Peptide Number
GLCTLVAML	GLC	A2	BMFL1	P1
SVRDRLARL	SVR	A2	EBNA 3A	P2
LLDFVRFMGV	LLD	A2	EBNA 3C	P3
QAKWRLQTL	QAK	B8	EBNA 3A	P4
PRGPPLSSSLGLALLLLLLA	PRG	unknown	LMP 1	LMP

Abbreviations used: HLA, Human Leukocyte Antigen; EBNA, Epstein-Barr nuclear antigen; LMP, latent membrane protein.

2.11 Flow Cytometry

2.11.1 Frequency of Regulatory T cells

Cells were counted and centrifuged at 180g for 7 minutes then resuspended in FACS buffer at $5x10^6$ cells/ml. FACS buffer was added to polystyrene round bottom tubes (BD Falcon) at 50µl/tube. Either no antibody, isotype control, CD4 FITC, CD25 PE or a combination thereof was applied to the appropriate FACS tube. Cell suspensions were added to give a density of $5x10^5$ cells/tube. The cells and antibodies were then incubated for 20 min at 4^oC. Cells were then washed twice with FACS buffer by centrifugation at 120g for 5 minutes. Cells were fixed with 200µl CellFix™ (Becton Dickinson) on a shaker for 10 minutes. Finally 300µl FACS buffer was added. Cells were stored at 4°C in the dark for up to one week until analysed on either a FACScan (Becton Dickinson) or FACSCalibur (Becton Dickinson) flow cytometer in conjunction with CellQuest software.

2.11.2 HLA A2 Typing

Either unstained, isotype control or HLA A2 antibodies were added to 100µl of neat blood in polstyrene round bottom tubes (BD Falcon) and incubated at room temperature for 30 minutes. Serotec Erythrolyse buffer (AbD Serotec) was then added and incubated at room temperature for 10 minutes. The cells were washed by centrifugation at 120g for 5 minutes and the supernatant discarded. FACS buffer was added and the cells were washed by repeat centrifugation. Cells were then suspended in CellFix™ (Becton Dickinson) and incubated on a shaker for 10 minutes. Finally 300µl FACS buffer was

added to the cells. Cells were immediately analysed on FACScan in conjunction with CellQuest software (Becton Dickinson).

2.12 Proliferation Assay

2.12.1 MTT Proliferation Assay

This proliferation assay works on the principle that proliferating cells reduce the tetrazolium compound, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), so it forms insoluble purple formazan dye crystals. When a detergent is added, the crystals dissolve and the absorbance can be read using a plate reader. The absorbance, i.e. rate of tetrazolium reduction, is proportional to the rate of cell proliferation. The MTT proliferation assay was carried out as per manufacturer's instructions (TACS^{TM} MTT assay, R&D Systems). Briefly, PBMC were suspended at a density of $5x10^6$ cells/ml in TCM 10% with 1μg/ml PHA, 10μg/ml PHA or no stimulant. Doubling dilutions were made and plated out in a flat-bottomed 96-well plate at 100μl/well and incubated overnight at 37^{o}C, 5% CO_2. After 16 hours 10μl of MTT was added to each well and the plate was incubated for a further 2-4 hours until the appearance of intracellular precipitate. Detergent reagent was then added at 100μl per well and PBMC were incubated at 37^{o}C, 5% CO_2 overnight. The plate was read at 540nm with the reference wave length set to 650nm. Cell proliferation assays were carried out in triplicate.

2.12.2 BrdU Proliferation Assay

This proliferation assay works on the principle that 5-Bromo-2'deoxy-uridine (BrdU) is incorporated into freshly synthesized DNA. Cells are then fixed and the DNA is partially digested which allows the binding of peroxidase-labelled anti-BrdU antibodies. Finally peroxidase substrate is added, which is cleaved by the peroxidase enzyme, the resulting product is coloured and hence absorbance of the sample can be established using a plate reader. Absorbance is proportional to the amount of BrdU incorporated into cellular DNA. The BrdU assay was carried out as per manufacturer's instructions (BrdU Labelling and detection Kit, Roche Diagnostics). Briefly, cells were set up in U-bottomed 96-well plates at various densities and incubated for various time periods ranging from 24 to 120 hours with a variety of stimulants (see sections 2.10.2 and 2.10.3). The outermost wells were filled with PBS to humidify the cells. BrdU was added at 10µg/ml and the plates were incubated overnight at 37^{o}C and 5% CO_2. Plates were centrifuged at 180g for 7 min at 4^{o}C and the supernatant removed by blotting. The plates were then dried at 60^{o}C for an hour. Plates were kept for up to a week at 4^{o}C. The DNA in the cells was denatured by incubation with FixDenat for 30 minutes at 15-25^{o}C. FixDenat was removed and anti-BrdU-POD was added for 90 minutes and plates were incubated at 15-25^{o}C. The plates were then washed and incubated with substrate solution for 20 minutes at room temperature. The reaction was stopped with the addition of 25µl/well 1M H_2SO_4 and the plates were read with an ELISA plate reader at 340nm with reference wavelength set at 370nm.

2.12.3 Stimulation using anti-CD3

In some of the proliferation assays, plate-bound anti-CD3 (Clone UCHT1, BD Pharmingen) was used as a stimulant. To optimize the concentration of antibody, anti-CD3 was used at 0.5, 1, 2.5, 5, 10 or 20µg/ml. The dilutions were made up in sterile PBS (sPBS). The appropriate antibody dilution was added to wells of a 96-well U-bottomed plate. The plates were covered with parafilm and incubated at 37^{o}C for 2 hours. Excess antibody was removed by tapping and the plates were washed twice with 200µl sPBS before the cells were applied. Cells were then incubated for 5 days and the BrdU proliferation was continued as described in section 2.12.2.

2.12.4 Stimulation with Tuberculin PPD and peptide

PBMC were counted and resuspended at 1.25x10^{6} cells/ml in TCM 10% and 1ml aliquots were added to 15 wells of a 48-well plate. Either PRG peptide (15µg/ml, see table 2 in section 2.10.3 for peptide details), control recall *Mycobacterium tuberculosis* PPD antigen (RT50; Statens Seruminstitut, Copenhagen, Denmark) at 10µg/ml, PHA (10µg/ml) or PPD in combination with PRG peptide were used to stimulate PBMC. All stimulants were added to triplicate wells. PBMC were incubated for 5 days whereupon 400µl tissue culture supernatant was carefully removed to avoid disturbing the cells and stored as described in section 2.8. The remaining cells were resuspended and 400µl from each well was applied to a 96-well U-bottomed plate at 200µl per well. BrdU was added at 10µg/ml and the proliferation assay was continued as described in section 2.12.2.

2.12.5 Suppression Assay

The ability of $CD4^+CD25^+$ cells to suppress proliferative responses of other cell populations was assessed in a suppression assay. Un-separated PBMC, $CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells and recombined cocultures of $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells were cultured at $1x10^5$ cells per well in 200µl of TCM 10%. The cell cultures were incubated for 5 days with 10µg/ml PHA, 5µg/ml plate-bound anti-CD3 or no stimulation. After 5 days proliferation was assessed with BrdU proliferation assay (details in section 2.12.2).

2.13 Serology

2.13.1 Monospot

A Microgen Bioproducts IM kit was used as per manufacturer's instructions to detect serum heterophile antibodies indicative of acute IM. Heterophile antibodies comprise two types; Forssman and non-Forssman antibodies, IM causes the production of the latter which agglutinate horse red blood cells. Briefly, 25µl of positive control or test serum was placed in each of two ovals of a white glass slide. A drop of guinea pig antigen was added to one oval (binding and thus removing Forssman antibodies) and a drop of ox antigen (binding and thus removing nonForssman antibodies) was added to the other. A drop of horse cell suspension was added to both ovals and the slide was rocked gently. Agglutination occurring in an oval containing guinea pig antigen but not ox antigen was indicative of a positive result.

2.13.2 Immunofluorescence for EBV IgG and IgM anti-VCA

Immunofluorescence for EBV antibodies was carried out for diagnosis of acute IM. All sera were heat-treated prior to testing by incubating for 20 minutes at 56^{o}C. The test serum was diluted with PBS to give the following dilutions: 1:5, 1:10, 1:40, 1:160, 1:640 and 1:2560. Dilutions were added to IgG anti-VCA test slides comprising 12 wells of $2x10^{4}$ fixed P3HR1 cells. In addition, 1:10 dilutions were applied to IgM anti-VCA slides (ATi Atlas). The slides were incubated for 1 hour in a humidity chamber at 37^{o}C. The slides were then washed twice with PBS for 5 minutes and then 15µl of FITC conjugated rabbit anti-human IgG (Dako), diluted 1:50 in PBS, was added per well. The IgM slides were overlayed with 15µl of FITC conjugated rabbit anti-human IgM (Dako), diluted 1:50 in PBS. The slides were then further incubated for 1 hour at room temperature in a humidity chamber. The slides were then washed in PBS twice and mounted in PBS:Glycerol. The slides were read using an epifluorescent microscope. On the IgM anti-VCA a reading at 1:10 was a positive result. On the IgG anti-VCA, the most dilute sample showing positive cells was taken to be the antibody titre.

2.13.3 Rheumatoid Test

Serum samples that tested positive for IgM antibody were tested for rheumatoid factor activity since this can cross-react with IgM antibodies leading to false positive results The Mastalex^{TM} RA80 kit was used as per manufacturer's instructions (Mast Diagnostics). One drop of undiluted serum was applied to the reaction card provided in the kit. One drop of latex reagent was added to the undiluted serum and the card was gently rocked until agglutination was observed in the positive control. Agglutination of

the undiluted serum sample indicated a positive rheumatoid factor reaction, indicating a possible false positive result in the IgM anti-VCA immunofluorescence test.

2.14 Statistical Analysis

The Mann-Whitney U test was used to test for differences in the medians of quantitative variables. Survival analysis was carried out using the Logrank test for trend. All tests were two-tailed and a *P*-values < 0.05 were considered significant. All statistical analysis was carried out using graphpad PRISM software.

3 Results

3.1 Samples & Donors

Details of the donors used in this study are shown in tables 3.1 and 3.2.

3.1.1 Healthy donors

Table 3.1: *Donor details*

Donor Number	Sex	Age	EBV status
D1	F	37	seropositive
D2	M	24	seropositive
D3	M	27	seropositive
D4	F	28	seropositive
D5-53*	unknown	unknown	seropositive

* Donors recruited from SNBTS and anonymised

3.1.2 IM Patients

Table 3.2: *IM patient information*

Number	Sex	Age	Monospot	IgM anti VCA	Symptom duration
IM1	F	22	negative	positive	2/52
IM2	M	22	positive	positive	2/52
IM3	M	23	positive	positive	N/A
IM4	M	21	positive	positive	N/A
IM5	F	25	positive	positive	N/A
IM6	F	25	positive	positive	2/52
IM7	M	26	positive	positive	N/A
IM8	M	26	positive	positive	N/A
IM9	F	24	positive	positive	1/52
IM10	M	24	positive	positive	1/52
IM11	M	23	positive	positive	1/52
IM12	M	23	positive	positive	1/52
IM13	F	21	negative	positive	N/A
IM14	F	22	positive	positive	1/7
IM15	F	22	positive	positive	3/52
IM16	M	22	positive	positive	1/52
IM17	F	19	positive	positive	3/7
IM18	F	23	positive	positive	3/52
IM19	M	32	positive	positive	2/52
IM20	M	24	positive	positive	1/52

Number	Sex	Age	Monospot	IgM anti VCA	Symptom duration
IM21	M	22	positive	positive	N/A*
IM22	F	21	positive	positive	N/A*
IM23	M	21	positive	positive	1/52
IM24	F	22	positive	positive	1/52
IM25	M	22	positive	positive	4/7
IM26	M	27	positive	positive	N/A*
IM27	M	24	positive	positive	2/52
IM28	F	20	positive	positive	1/52
IM29	F	23	positive	positive	2/52
IM30	F	21	positive	positive	4/7
IM31	F	22	positive	positive	3/52
IM32	F	24	positive	positive	1/12
IM33	F	24	positive	positive	1/52
IM34	F	25	negative	positive	1/52
IM35	M	27	positive	positive	1/52
IM36	M	23	positive	positive	5/7
IM37	F	N/A	negative	negative	Non IM**
IM38	M	25	positive	positive	N/A*
IM39	M	22	positive	positive	N/A*
IM40	M	21	positive	positive	10/7
IM41	M	20	positive	positive	3/7
IM42	F	26	positive	positive	3/52
IM43	M	20	negative	weak positive	N/A*
IM44	F	17	positive	positive	2/52
IM45	M	23	weak positive	negative	Non IM**
IM46	M	23	positive	positive	2/7
IM47	F	19	positive	positive	2/52
IM48	M	22	positive	positive	N/A*
IM49	M	21	positive	positive	N/A*
IM50	M	18	positive	positive	4/7
IM51	M	19	positive	positive	1/12
IM52	F	24	negative	positive	N/A*
IM53	M	19	positive	positive	2/52
IM54	F	26	positive	positive	1/52
IM55	M	24	positive	positive	1/52
IM56	F	19	positive	positive	N/A*
IM57	F	22	positive	positive	1/52
IM58	M	25	positive	positive	5/7
IM59[†]	N/A	N/A	N/A	N/A	N/A*
IM60[†]	N/A	N/A	N/A	N/A	N/A*
IM61	M	24	positive	positive	5/7

Number	Sex	Age	Monospot	IgM anti VCA	Symptom duration
IM62	F	21	positive	positive	1/52
IM63	F	24	positive	positive	4/7
IM64	M	32	positive	positive	6/7
IM65	M	22	positive	positive	6/7
IM66	M	24	positive	positive	6/7
IM67	F	22	positive	positive	2/7
IM68	M	20	positive	positive	4/7
IM69	M	19	positive	positive	2/52
IM70	M	20	positive	positive	3/52
IM71	F	21	positive	positive	1/52
IM72	M	22	positive	weak positive	4/52
IM73	F	23	positive	positive	2/52
IM74	F	21	negative	weak positive	2/52
IM75	M	20	positive	positive	2/52
IM76	F	21	positive	positive	2/52
IM77	F	22	positive	positive	3/52
IM78	F	21	negative	positive	3/52

Abbreviations: N/A – not available. * Samples from IM patients with unknown symptom duration were not used in cytokine analysis. ** Samples not used in experimental analysis. †only tonsil sections used from these patients.

3.2 Role of Regulatory T cells

3.2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Introduction

Regulatory T cell subgroups have been shown to potentiate their activity via cytokine release, in particular IL-10 (Grazia Roncarolo *et al*, 2006;Battaglia *et al*, 2006a) and TGF-β (Weiner, 2001b). Analysis of these cytokines has featured highly in T_{reg} research, providing indirect evidence for the presence of various subsets of regulatory T cells. Assessing the levels of IL-10 and TGF-β_1 in plasma collected from IM patients and normal healthy controls was the logical starting point for research investigating

whether T_{reg} cells played a role in primary infection with EBV. Plasma samples were grouped as IM patients who had experienced symptoms for 1 week or less, IM patients who had experienced symptoms for over a week and healthy seropositive controls.

IL-10 and TGF-β_1 levels in plasma from healthy controls and IM patients

Median levels of IL-10 (shown in figure 3.1) were found to be significantly higher ($p<0.001$) in IM patients who had experienced symptoms for one week or less (median IL-10 71.8pg/ml, range 0 to 290pg/ml) in comparison to healthy seropositive controls (median IL-10 11.3pg/ml, range 0 to 50pg/ml) and IM patients who had experienced symptoms for over 1 week ($p=0.0112$). IL-10 levels were lower when symptoms had been prolonged for over a week (median IL-10 48.43pg/ml, range 0 to 97.2pg/ml), although this was still significantly higher than IL-10 levels found in healthy seropositive controls ($p=0.0028$).

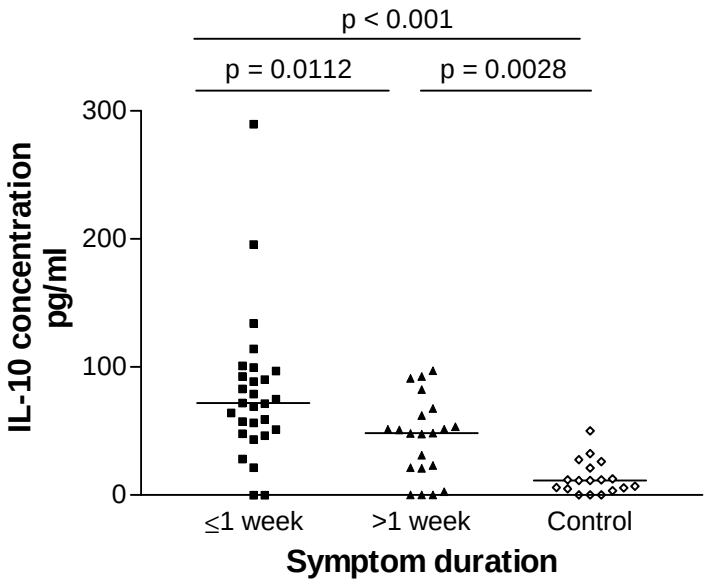

Figure 3.1: IL-10 concentration (pg/ml) in plasma from IM patients who had experienced symptoms for 1 week or less (closed squares), IM patients who had experienced symptoms for over 1 week (closed triangles) and healthy seropositive controls (open diamonds). Median indicated by horizontal line.

Levels of TGF-β_1 (figure 3.2) were compared in the same groups of patients. In the first week of IM symptoms median TGF-β_1 levels (median 18.0ng/ml, range 4.4 to 28.6ng/ml) were significantly higher (p=0.0185) than those found in healthy seropositive controls (median 9.3ng/ml, range 0 to 50.8ng/ml). An even greater statistically significant increase in median TGF-β_1 level was observed when symptoms had been endured for over a week (median 26.9ng/ml, range 9.8 to 41.1ng/ml) in comparison with both controls (p=0.0001) and IM patients with symptoms experienced for less than a week (p = 0.004).

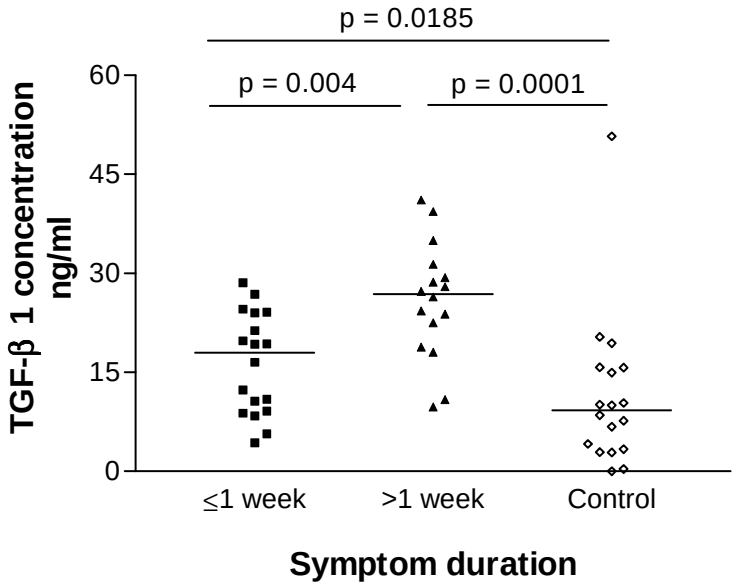

Figure 3.2: TGF-β1 concentration (ng/ml) in plasma from IM patients who had experienced symptoms for 1 week or less (closed squares), IM patients who had experienced symptoms for over 1 week (closed triangles) and healthy seropositive controls (open diamonds). Median indicated by horizontal line.

Conclusions

The significantly higher median level of IL-10 seen in IM patients during the first week of symptoms, in comparison with healthy seropositive controls, suggests that T_{reg} producing this cytokine as their mediator are present early in IM. A significantly high median level of TGF-β_1 is also observed early in IM, which increases further as the symptoms persist for over a week, suggesting a role for another subset of regulatory T cells, possibly with more emphasis later in infection.

3.2.2 FACS analysis

Introduction

A strict phenotype has yet to be established for regulatory T cells. To date, the most widely accepted cell-surface markers distinguishing human T_{reg} are CD4 and high levels of the IL-2β receptor, CD25 (Baecher-Allan *et al*, 2001a). Previous studies looking at roles for human T_{reg} in persistent viral infections have found differences in the frequency of these regulatory cells in the peripheral blood of infected individuals when compared with healthy, uninfected controls (Cabrera *et al*, 2004e;Weiss *et al*, 2004c;Boettler *et al*, 2005d). The frequency of T_{reg} in the peripheral blood of IM patients was investigated and compared to healthy controls.

Frequency of regulatory T cells in healthy controls and IM patients

PBMC from IM patients and healthy seropositive controls were stained with a panel of antibodies, shown in table 3.3. Tubes 1-5 were used as controls to set up the FACS analysis.

Table 3.3: *Antibody staining panel used to analyse frequency of regulatory T cells in PBMC*

Tube Number	FITC-conjugated antibody	PE-conjugated antibody
1	unstained	unstained
2	IgG_{2a} isotype control	-
3	-	IgG_{2a} isotype control
4	-	CD25
5	CD4	-
6	CD4	CD25

Representative plots from a healthy seropositive control and an IM patient are shown in figure 3.3 and figure 3.4, respectively. Based on their size (forward scatter) and granularity (side scatter), PBMC were gated on the lymphocyte population (panel A). Dead cells were excluded since they exhibit lower forward and side scatter than live cells. A further gating strategy was applied (panel B), to allow collection of data from three populations of T cells; $CD4^{+}CD25^{-}$, $CD4^{+}CD25^{low}$ and $CD4^{+}CD25^{high}$. Figure 3.5 shows representative results for PBMC stained with CD4 FITC and CD25 PE from a healthy seropositive control (**A**) and an IM patient (**B**).

Forward and side scatter plots of PBMC from the healthy seropositive control show that the PBMC are small with low levels of granularity (figure 3.3, panel A). In contrast, PBMC from IM patients show an altered distribution of cells in the FACS plot with increased levels of forward scatter and side scatter (figure 3.4, panel A). As a consequence, gating on the lymphocytes in IM patients was less stringent.

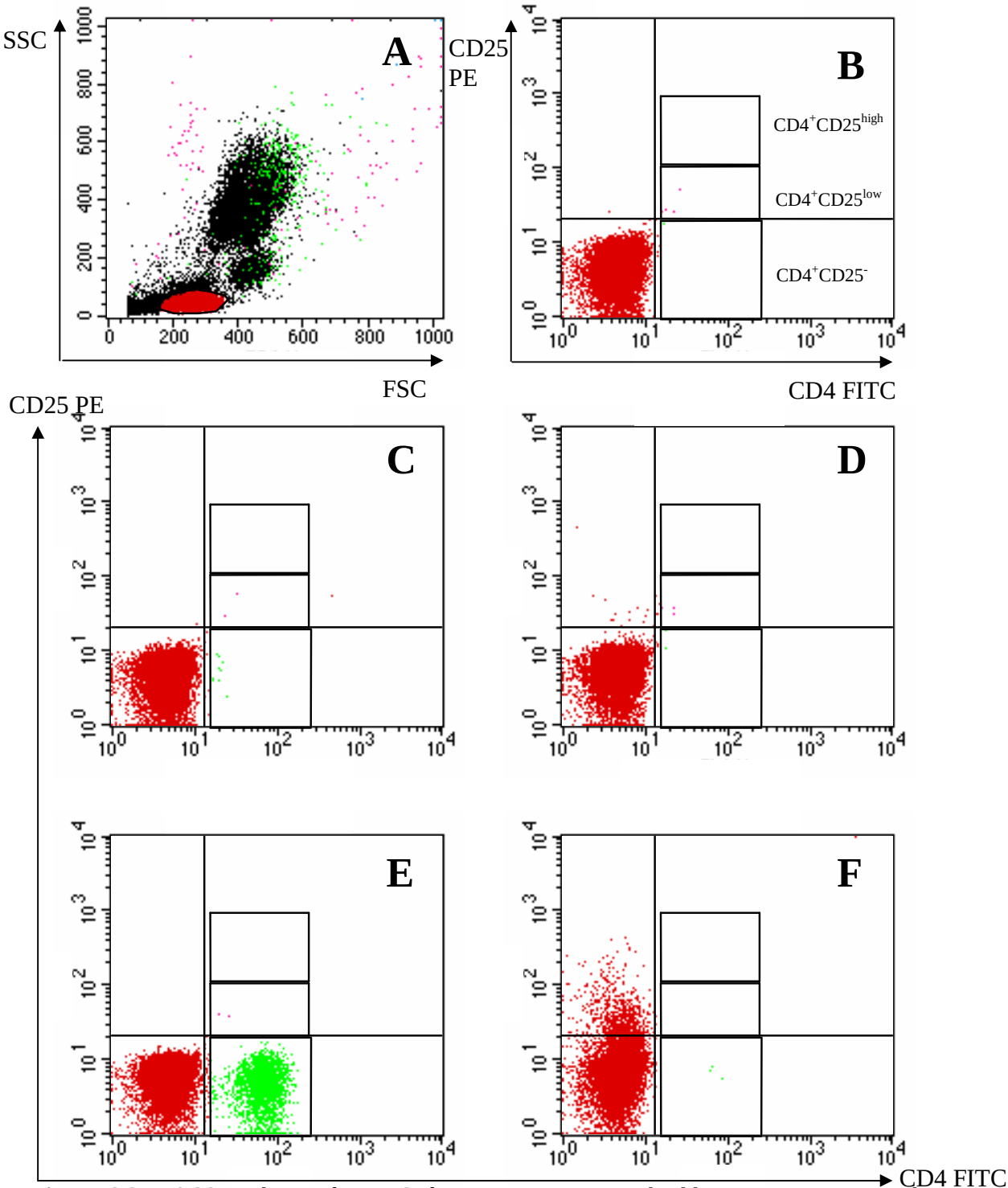

Figure 3.3: FACS analysis of PBMC from a representative healthy seropositive control. Lymphocyte gate (**A)**. Further gating was based on expression of CD25 (**B).** PBMC were stained with a panel of antibodies; unstained (**B**), IgG_{2a} FITC isotype (**C**), IgG_{2a} PE isotype (**D**), CD4 FITC (in green, **E**), CD25 PE (**F**).

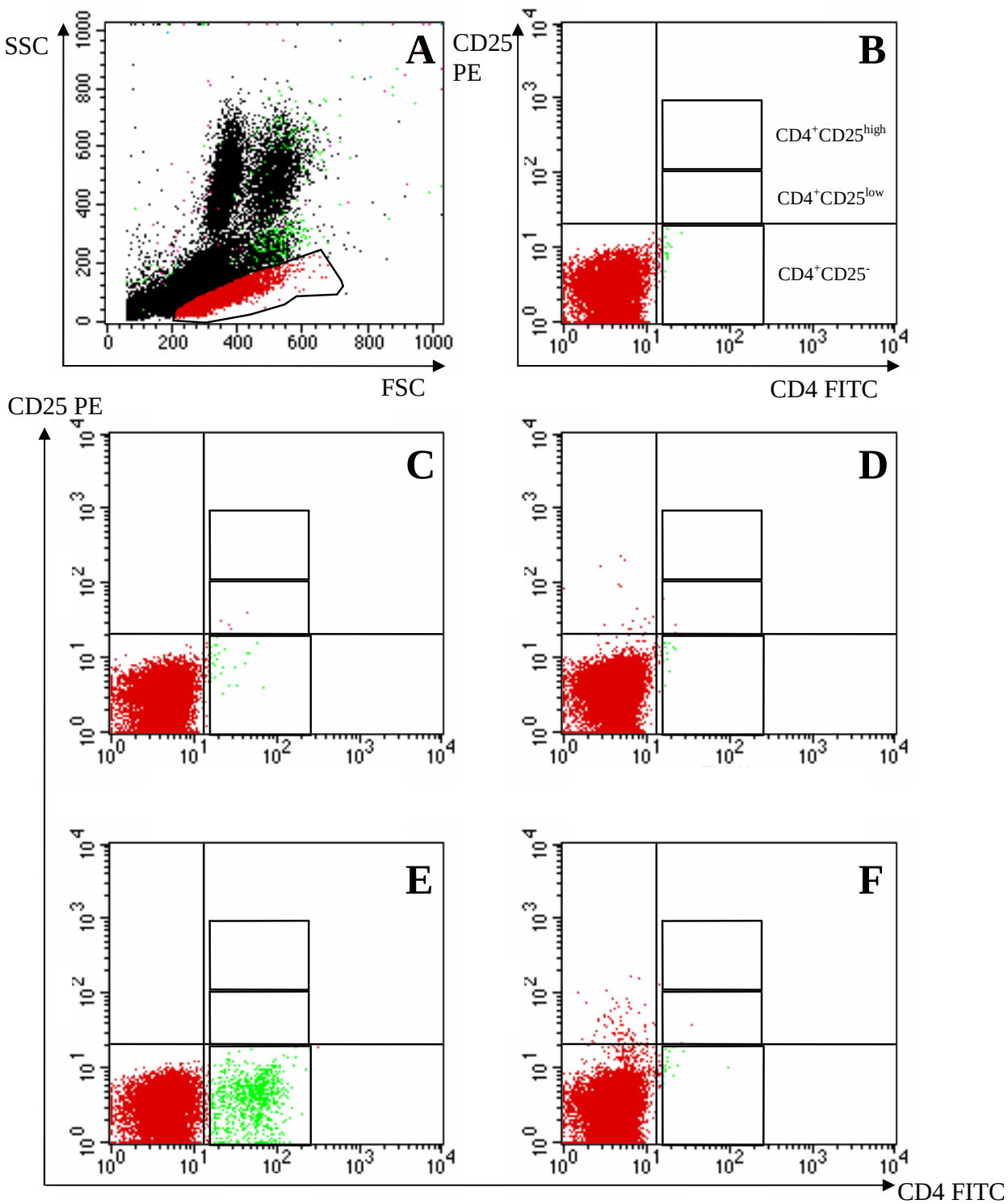

Figure 3.4: FACS analysis of PBMC from a representative IM patient. Lymphocyte gate (**A).** Unstained (**B**), IgG_{2a} FITC isotype (**C**), IgG_{2a} PE isotype (**D**), CD4 FITC (**E**), CD25 PE (**F**).

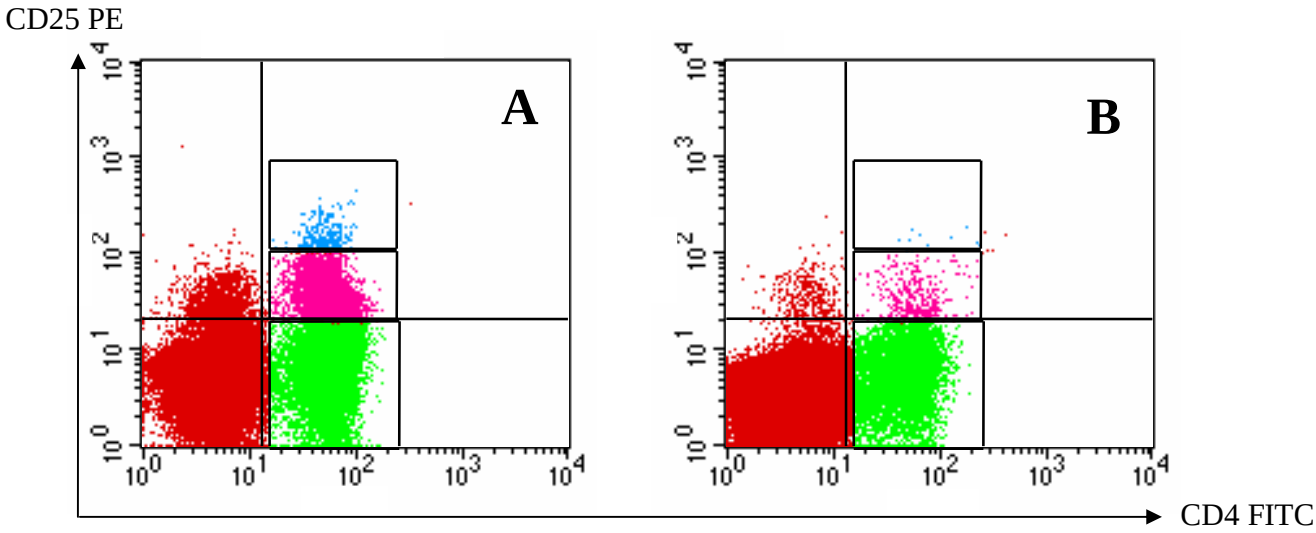

Figure 3.5: FACS analysis of representative PBMC from a healthy seropositive control (**A**) and an IM patient (**B**), stained with CD25 PE and CD4 FITC.

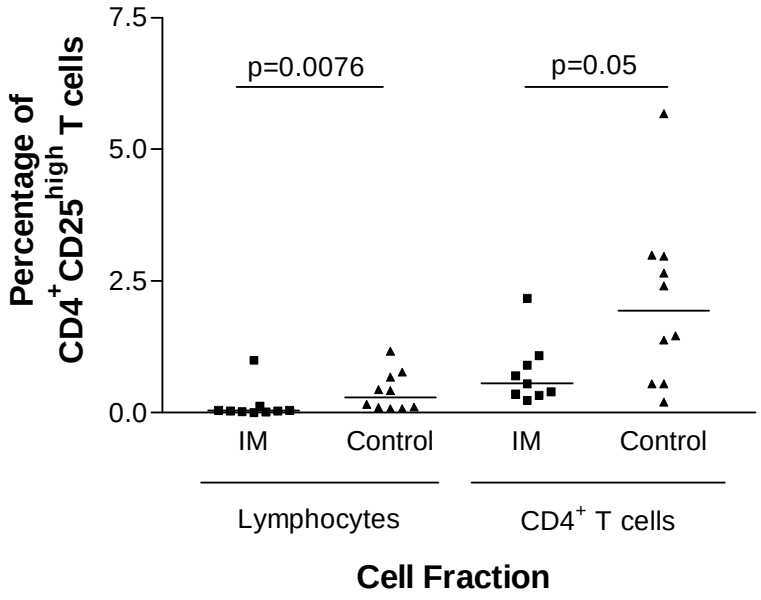

Figure 3.6: Frequency of $CD4^{+}CD25^{high}$ T cells in acute IM patients (closed squares) and healthy seropositive controls (closed triangles) within both the lymphocyte and $CD4^{+}$ T cell population. Median is indicated by horizontal bars.

The frequency of $CD4^+CD25^{high}$ T cells in PBMC was investigated in 10 healthy seropositive controls and 9 IM patients (figure 3.6). $CD4^+CD25^{high}$ T cells were found at low frequency in the lymphocyte population of healthy controls (median 0.29%), ranging between 0.08-1.17%. The frequency of these cells was significantly lower (p=0.0076) in PBMC from IM patients (median 0.04%) with a range of 0.01-1%. However, in acute IM there is a massive expansion of cytotoxic CD8-expressing T cells and since FACS measures the percentages of cells and the number of cells collected from each gate remains the same, the expanded $CD8^+$ T cells would result in the apparent reduced frequency of $CD4^+$ T cells. Therefore the percentage of $CD25^{high}$-expressing cells within the $CD4^+$ T cells was compared between IM and controls (figure 3.6) since the $CD4^+$ T cells have been shown not to expand in acute IM (Williams *et al*, 2005).

The frequency of $CD25^{high}$-expressing cells within the $CD4^+$ T cells in healthy seropositive controls (median 1.94%) ranged between 0.06 and 5.68%. In IM patients, the frequency of $CD25^{high}$-expressing cells was reduced compared to healthy controls (median 0.56%, range 0.24 to 2.18%) with borderline significance (p=0.05).

Conclusions

The reduced frequency of $CD4^+CD25^{high}$ T cells in the $CD4^+$ population of IM patients, when compared with healthy seropositive controls, suggests that these T cells might influence the outcome of primary infection with EBV, possibly by allowing the huge expansion of EBV-specific $CD8^+$ T cells found in IM patients. In order to establish

more precisely the role these cells play in IM, the function of $CD4^+CD25^{high}$ T cells was addressed.

3.2.3 Regulatory T cell enrichment

Introduction

$CD4^+CD25^{high}$ T cells are found at low frequency in human peripheral blood, limiting the number of experiments that can be carried out. To address this issue, a T_{reg} enrichment kit was used to enrich for these particular cells.

Summary of $CD4^+CD25^{high}$ T cell enrichment from 5 healthy controls

$1x10^8$ PBMC from 5 healthy seropositive donors were used to optimise the T_{reg} enrichment procedure (see *Materials and Methods*, section 2.9). The Miltenyi Biotec T_{reg} Isolation kit uses a negative selection step, resulting in unmanipulated $CD4^+$ T cell enrichment, followed by a positive selection based on $CD25^{high}$ expression. At each step a portion of cells were removed for FACS analysis and stained for CD4 and CD25 expression, as previously described (see *Results*, section 3.2.2). This provided a set of data for each of the following; unseparated PBMC, $CD4^-$ cells, $CD4^+$ T cells, $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells. A representative FACS plot for each cell subset is shown in figure 3.7 (colours indicate gating strategies).

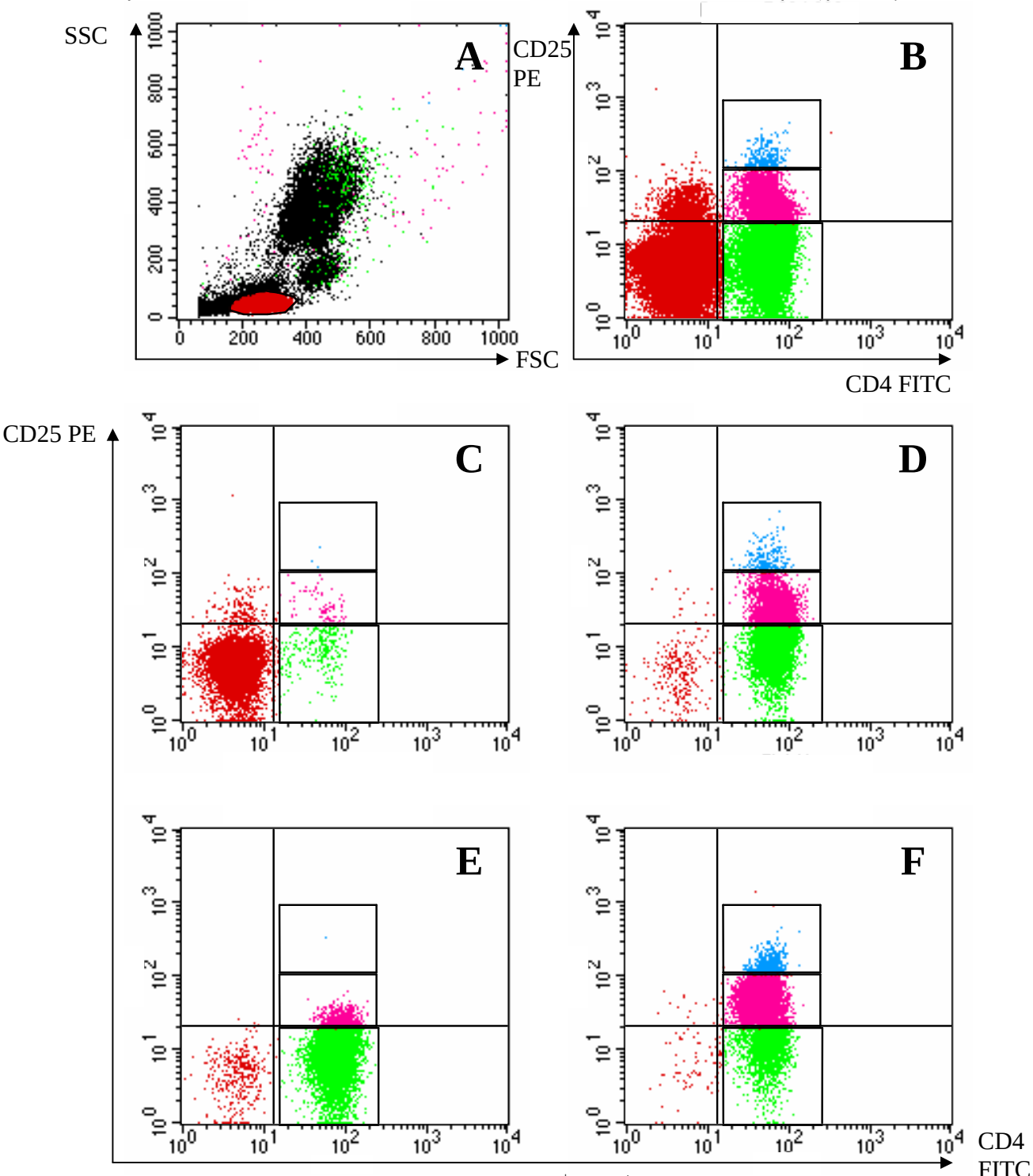

Figure 3.7: Representative FACS analysis for $CD4^+CD25^+$ T cell frequency at each step of the T_{reg} enrichment procedure from a healthy seropositive donor. Unseparated PBMC (**B**), CD4-depleted cells (**C**), CD4-enriched cells (**D**), $CD4^+CD25^-$ T cells (**E**) and $CD4^+CD25^+$ T cells (**F**) were stained with a combination of CD25 PE and CD4 FITC and gated on lymphocytes (**A**).

In addition, the $CD4^+CD25^+$-enriched T cell population of each donor was analysed for purity and percentage recovery (i.e. number of enriched T_{reg}). The mean percentage purity of $CD4^+CD25^{high}$ T cells was 79.18% and the mean number of enriched cells was $5.36x10^5$ cells. Individual data are shown in table 3.4 for each of the 5 donors. Due to restrictions in cell numbers this experiment could not be carried out on cells from IM patients.

Table 3.4: *Percent purity and number of $CD4^+CD25^{high}$ T cells enriched for each of the 5 donors.*

donor	percent $CD4^+CD25^{high}$ T cell purity %	number of $CD4^+CD25^{high}$ T cells enriched	% recovery
D14	69.61	$4x10^5$	0.40
D15	94.15	$4.2x10^5$	0.42
D16	68.13	$2.8x10^5$	0.28
D17	87.25	$7x10^5$	0.70
D18	76.77	$8.8x10^5$	0.88

3.2.4 Proliferation Assays

Introduction

Proliferation assays were employed to measure the effect of enriched $CD4^+CD25^+$ T cells on cell populations activated by a variety of stimulants. Thymidine incorporation is the most widely used assay to measure cell proliferation. However, the CMV laboratory does not support the equipment required for this procedure hence an alternative measure of proliferation was sought.

MTT proliferation

In order to establish the best density of cells to use for this assay, doubling dilutions from $5x10^6$ to $5x10^3$/200µl well of 2 established lymphoblastoid cell lines (LCL) were used. Figure 3.8 shows the mean absorbance for two LCL lines at different cell densities incubated for 24 hours. Cell proliferation increased as the cell density increased, as evidenced by the rise in mean absorbance. The peak absorbance is 0.75 for both cell lines as stated.

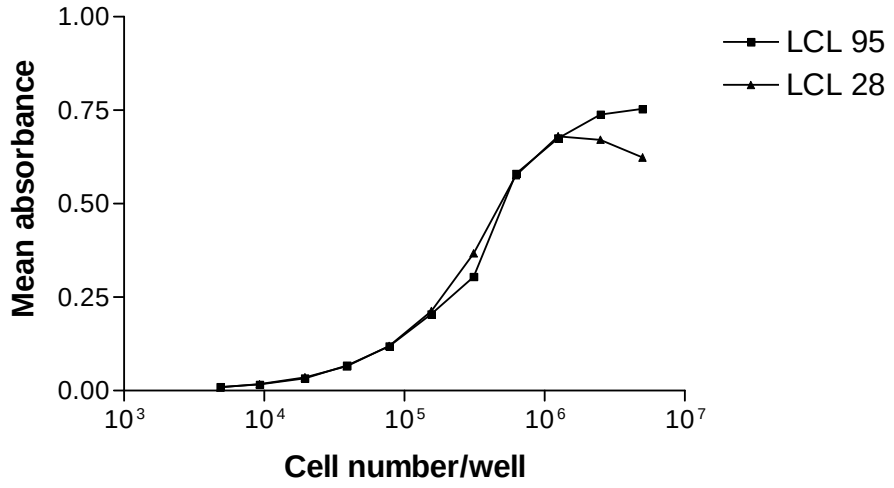

Figure 3.8: Mean absorbance of established LCL 95 (closed squares) and LCL 28 (closed triangles) at different cell densities using MTT assay.

Serial dilutions of PBMC, ranging from $5x10^6$ to $5x10^3$/200µl, from 2 donors were either cultured alone or stimulated with PHA at 1µg/ml or 10µg/ml for 24 hours in order to establish how well the assay worked when using this cell type. Results are shown in figure 3.9. Mean absorbance increased over a range from 0 to 0.55, as cell density increased. PBMC from donor 23 had a maximum absorbance of 0.55 when PBMC were

stimulated with 1µg/ml PHA and seeded at $5x10^6$ cells/well, whereas PBMC from donor 24 gave a maximum absorbance reading of 0.019 under the same experimental conditions. Untreated PBMC from both donors (mean absorbance 0.53 and 0.16 for D23 and D24, respectively) proliferated as well as PBMC stimulated with 1µg/ml and 10µg/ml PHA.

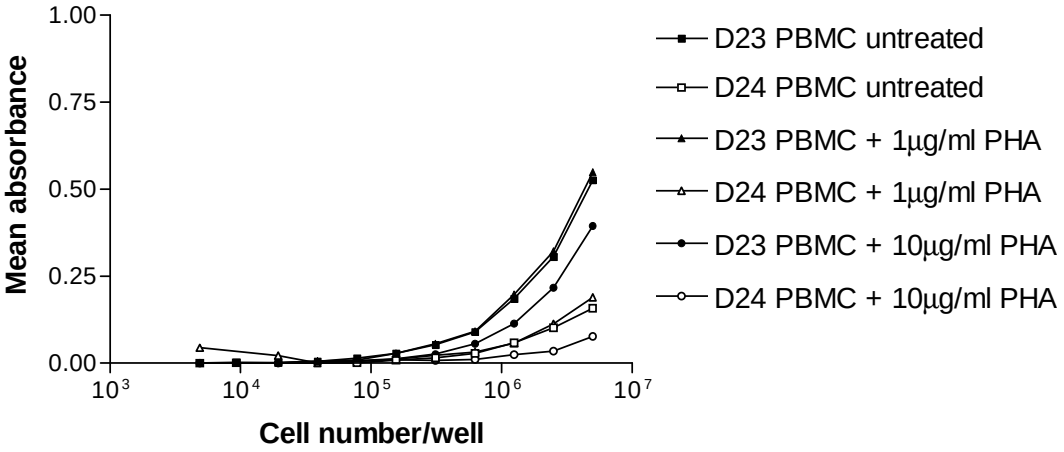

Figure 3.9: Mean absorbance of healthy control peripheral blood mononuclear cells from donor 23 (closed symbols) and 24 (open symbols). Untreated (squares), stimulated with 1µg/ml PHA (triangles) or 10µg/ml PHA (circles) using MTT assay.

The low level of proliferation observed for both donors, even when PBMC were stimulated with high concentrations of PHA, indicated that the assay was not sensitive enough. The assay was to be used for the investigation of human $CD4^+CD25^{high}$ T cell function and since these cells are found at low frequency in the peripheral blood, the insensitivity would be a problem and so an alternative proliferation assay was sought.

BrdU proliferation

The BrdU proliferation assay works on the principle that BrdU (a synthetic thymidine analogue) is incorporated directly into the cell's DNA, which is then recognised by an anti-BrdU antibody. Similar to the MTT assay, a substrate is added and coloured reaction is observed which can be read on a microplate reader. Absorbance is proportional to cell proliferation (see *Materials and Methods*, section 2.12.2).

The BrdU proliferation assay was tested on 2 established LCL. 10-fold dilutions from $1x10^5$ to $1x10^2$ cells/100µl well were set up and incubated for 24 hours prior to the addition of BrdU, whereupon the cells were incubated for a further 24 hours. Results are shown in figure 3.10.

Both LCL proliferated at $1x10^3$ (mean absorbance 0.15 and 0.19 for LCL 48 and 117, respectively) and $1x10^4$ cells/well (mean absorbance 1.86 and 0.97 for LCL 48 and 117, respectively) with maximum absorbance readings occurring when LCL were plated at $1x10^5$ cells/well (mean absorbance 2.02 and 1.54 for LCL 48 and 117, respectively). The maximum mean absorbance of 2.02 in the BrdU assay compared with 0.75 in the MTT assay, suggesting that the BrdU assay is the more sensitive of the two assays. In addition, cells had to be plated at $5x10^6$ cells/well in the MTT assay to give maximum proliferation whereas in the BrdU assay, the cells could be plated at the lower density of $1x10^5$ cells/well to give maximum proliferation.

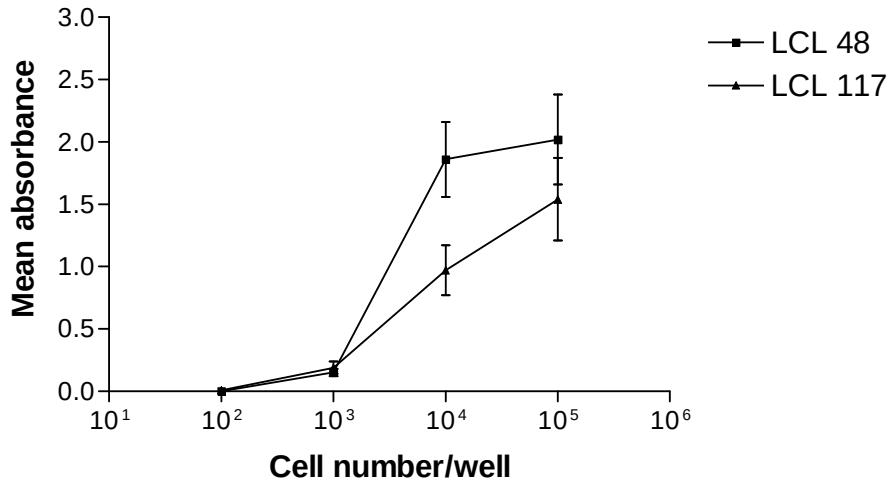

Figure 3.10: Mean absorbance of LCL 48 (squares) and LCL 117 (triangles) at different densities using BrdU assay. Bars indicate standard deviation.

Next, PBMC were set up in doubling dilutions ranging from $2x10^5$ to $1x10^4$ cells/well. Each density of cells was stimulated with 1, 5 or 10µg/ml PHA or left untreated and incubated for 24, 48, 72, 96 or 120 hours. Untreated PBMC provided a baseline mean absorbance and this was subtracted from the absorbance observed when PBMC were stimulated with PHA. Figure 3.11 shows the results for this optimisation.

No significant proliferation was observed when PBMC were treated with 1µg/ml PHA (figure 3.11A). An increase of PHA concentration to 5µg/ml resulted in a slight increase in proliferation, with a peak mean absorbance of 0.33 when PBMC were plated at $5x10^4$ cells/well and incubated for 96 hours (figure 3.11B).

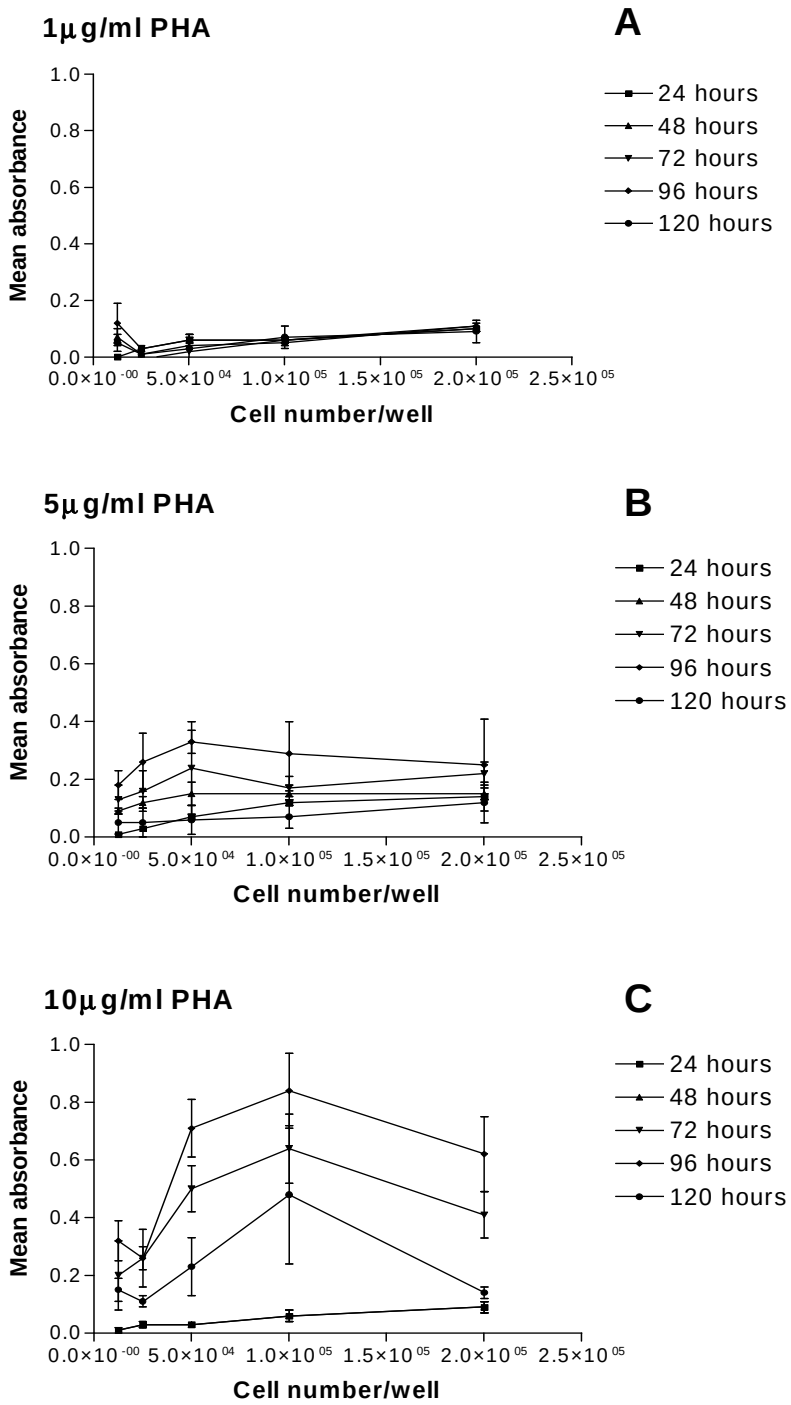

Figure 3.11: Mean absorbance of PBMC stimulated for 24 (squares), 48 (up triangles), 72 (down triangles), 96 (diamonds) or 120 (circles) hours of incubation with PHA at 1µg/ml (**A**), 5µg/ml (**B**) or 10µg/ml (**C**). Bars indicate standard deviation.

A further increase in PHA concentration to 10μg/ml resulted in a high level of proliferation for incubation periods of 72, 96 and 120 hours, with peak mean absorbance values of 0.64, 0.84 and 0.48, respectively. Similar proliferation was observed when the incubation time was 24 or 48 hours with a peak mean absorbance of 0.09 when cells were plated at $2x10^5$ cells/well. The optimal conditions under which the highest proliferation rate of 0.84 occurred when PBMC were plated at $1x10^5$ cells/well and incubated for 96 hours (figure 3.11C). This assay gave better results when using lower cell numbers than the MTT assay and hence the BrdU assay was used for the remainder of the proliferation experiments.

In order to examine the possibility that the FCS in culture medium could stimulate PBMC, another optimising experiment was set up. PBMC were either stimulated with PHA at 10μg/ml in TCM 10% or resuspended in TCM containing 5 or 10% FCS and proliferation was measured in triplicate wells at 48, 96 and 144 hours of incubation. Figure 3.12 shows results for this experiment.

PBMC incubated for 48 hours proliferated only when stimulated with 10μg/ml PHA (figure 3.12A) with a maximum mean absorbance of 1.51 when cells were seeded at $1x10^5$ cells/well. PBMC treated with 5% FCS and 10% FCS for 48 hours displayed low proliferative responses, with peak mean absorbance values of 0.105 and 0.103, respectively, occurring when cells were plated at $2x10^5$ cells/well.

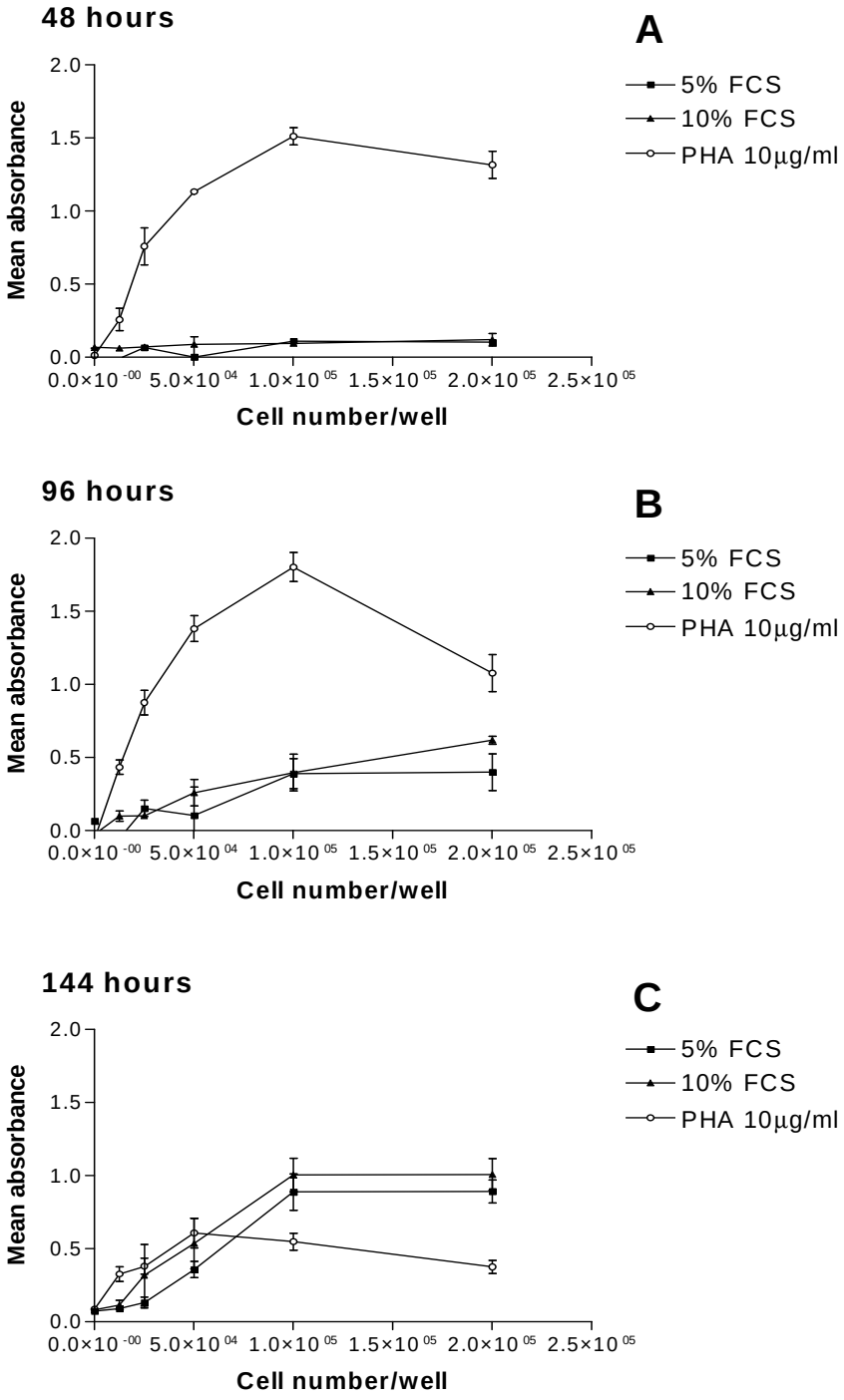

Figure 3.12: Mean absorbance of PBMC treated with 5% FCS (closed squares), 10% FCS (closed triangles) or 10µg/ml PHA (open circles) for 48 (**A**), 96 (**B**) or 144 (**C**) hours of incubation. Bars indicate standard error.

At 96 hours post-treatment, PBMC treated with 5% FCS demonstrated increased proliferation with a maximum mean absorbance of 0.401 when cells were seeded at $2x10^5$ cells/well. Similarly, PBMC treated with 10% FCS increased proliferation with a peak absorbance of 0.619 under the same experimental conditions. PBMC treated with 10µg/ml PHA demonstrated optimal proliferation at all cell densities, with a peak mean absorbance of 1.803 when cells were seeded at $1x10^5$ cells/well. This is an approximate 4.5-fold increase compared to proliferation observed with either of the FCS treatments (figure 3.12B). By 144 hours of stimulation, increases in proliferation were observed when PBMC were treated with 5% FCS (mean absorbance 0.889) and 10% FCS (mean absorbance 1.004) when cells were plated at $1x10^5$ cells/well. Treatment of PBMC with PHA under the same experimental conditions resulted in a reduced level of proliferation in comparison to 48 and 96 hour incubation, with a peak mean absorbance of 0.548 (figure 3.12C).

Having established the criteria for proliferation of PBMC stimulated with PHA and determined that FCS contributes to proliferation not before but only when incubated for 120 hours, the next step was to optimise the conditions for measuring proliferation when PBMC were stimulated with plate-bound anti-CD3 (see *Materials and Methods*, section 2.12.3).

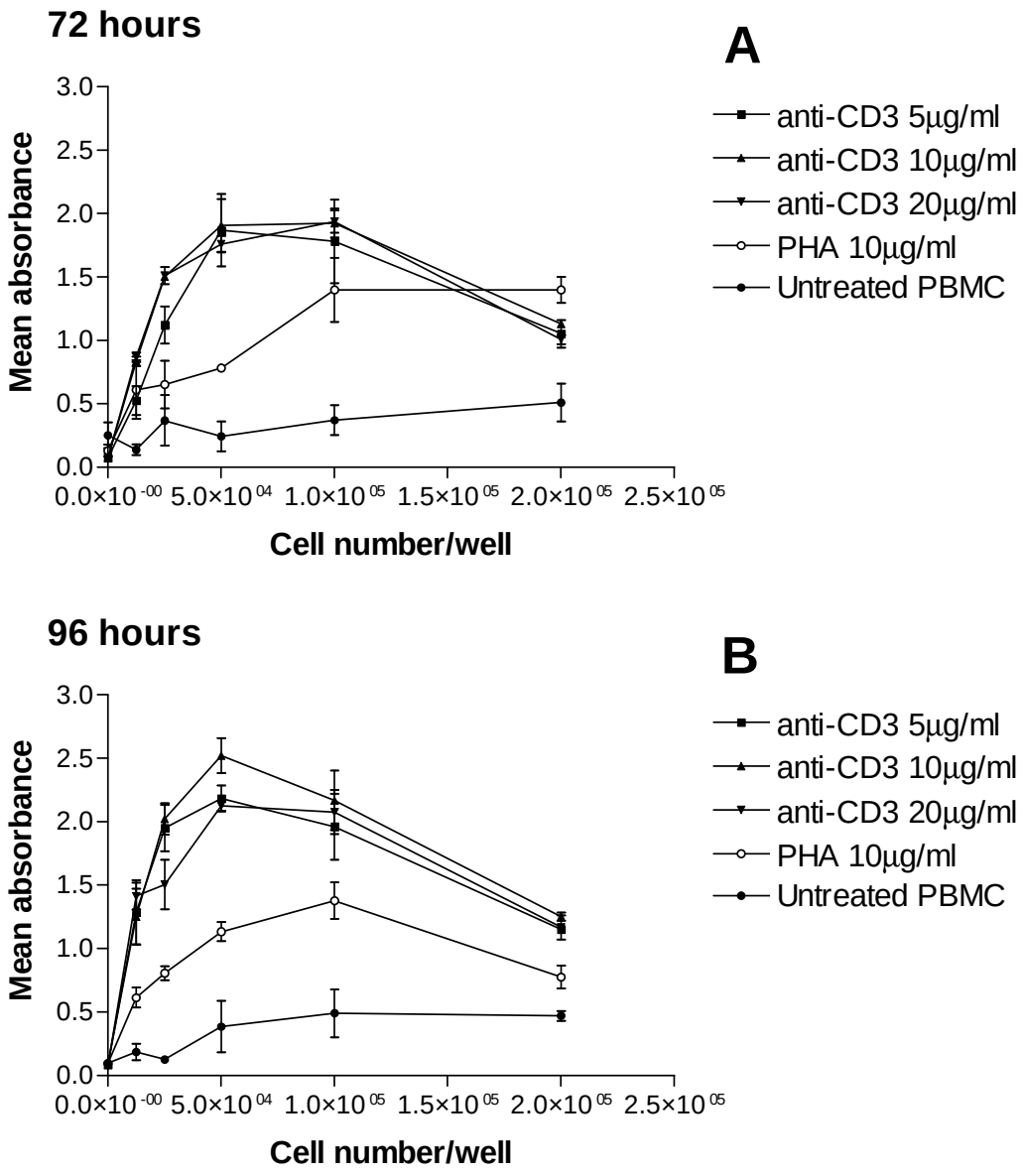

Figure 3.13: Mean absorbance of PBMC in response to stimulation with 5µg/ml (closed squares), 10µg/ml (up closed triangles) or 20µg/ml (down close triangles) plate-bound anti-CD3, 10µg/ml PHA (open circles) or left untreated (close circles) for 72 (**A**) or 96 (**B**) hours of incubation. Bars indicate standard error.

PBMC were set up in doubling dilutions ranging from $2x10^5$ to $1x10^4$ cells/well and stimulated with plate-bound anti-CD3 at 5, 10 or 20μg/ml for 72 and 96 hours of incubation and proliferation measured in triplicate. Stimulation of PBMC with 10μg/ml PHA was used as the positive control whereas untreated PBMC acted as the negative control. Figure 3.13 shows the results of this experiment.

At 72 hours of incubation, untreated PBMC proliferated to a very low level at every cell density, with a maximum mean absorbance of 0.511 when plated at $2x10^5$ cells/well. When stimulated with PHA at 10μg/ml for 72 hours, proliferation increased, in comparison to untreated PBMC, at every cell density with a peak mean absorbance of 1.399 when cells were plated at $1x10^5$ cells/well. PBMC stimulated with 5μg/ml anti-CD3 for 72 hours proliferated at high levels, with the peak mean absorbance values of 1.781 and 1.870 observed when cells were plated at $1x10^5$ and $5x10^4$ cells/well, respectively. This level of proliferation was also observed when the concentration of anti-CD3 was increased to 10μg/ml (mean absorbance 1.925 and 1.906, respectively) and 20μg/ml (mean absorbance 1.936 and 1.759, respectively) and the incubation period kept constant (figure 3.13A).

Similarly, untreated PBMC proliferated to a very low level at every cell density when incubated for 96 hours with a peak mean absorbance of 0.49 when cells were plated at $1x10^5$ cells/well. High levels of proliferation were observed when PBMC were stimulated with PHA at 10μg/ml for 96 hours, peaking at 1.378 when cells were plated

at $1x10^5$ cells/well. PBMC proliferated equally well when stimulated with 5, 10 or 20 µg/ml anti-CD3, with a maximum mean absorbencies of 2.183, 2.521 and 2.125, respectively, when plated at $5x10^4$ cells/well (figure 3.13B).

Since equal levels of proliferation were observed when anti-CD3 was used at 5, 10 and 20µg/ml, a similar experiment was set up using lower concentrations of anti-CD3. Figure 3.14 shows results for PBMC proliferating in response to 0.5, 1 and 2µg/ml anti-CD3, 10µg/ml PHA or left untreated, for incubation periods of 72, 96 and 144 hours.

Unstimulated PBMC proliferated poorly when incubated for 48 or 96 hours, with mean absorbance values of 0.278 and 0.464, respectively, when plated at $2x10^5$ cells/well (figure 3.14A and 3.14B). Peak mean absorbance increased to 0.823 when untreated PBMC at $2x10^5$ cells/well were incubated for 144 hours (figure 3.14C). PBMC stimulated with 10µg/ml PHA proliferated well at 48, 96 and 144 hours of incubation with maximum mean absorbance values of 0.571, 1.689 and 1.154, respectively, when plated at $2x10^5$ cells/well (figure 3.14B and 3.14C).

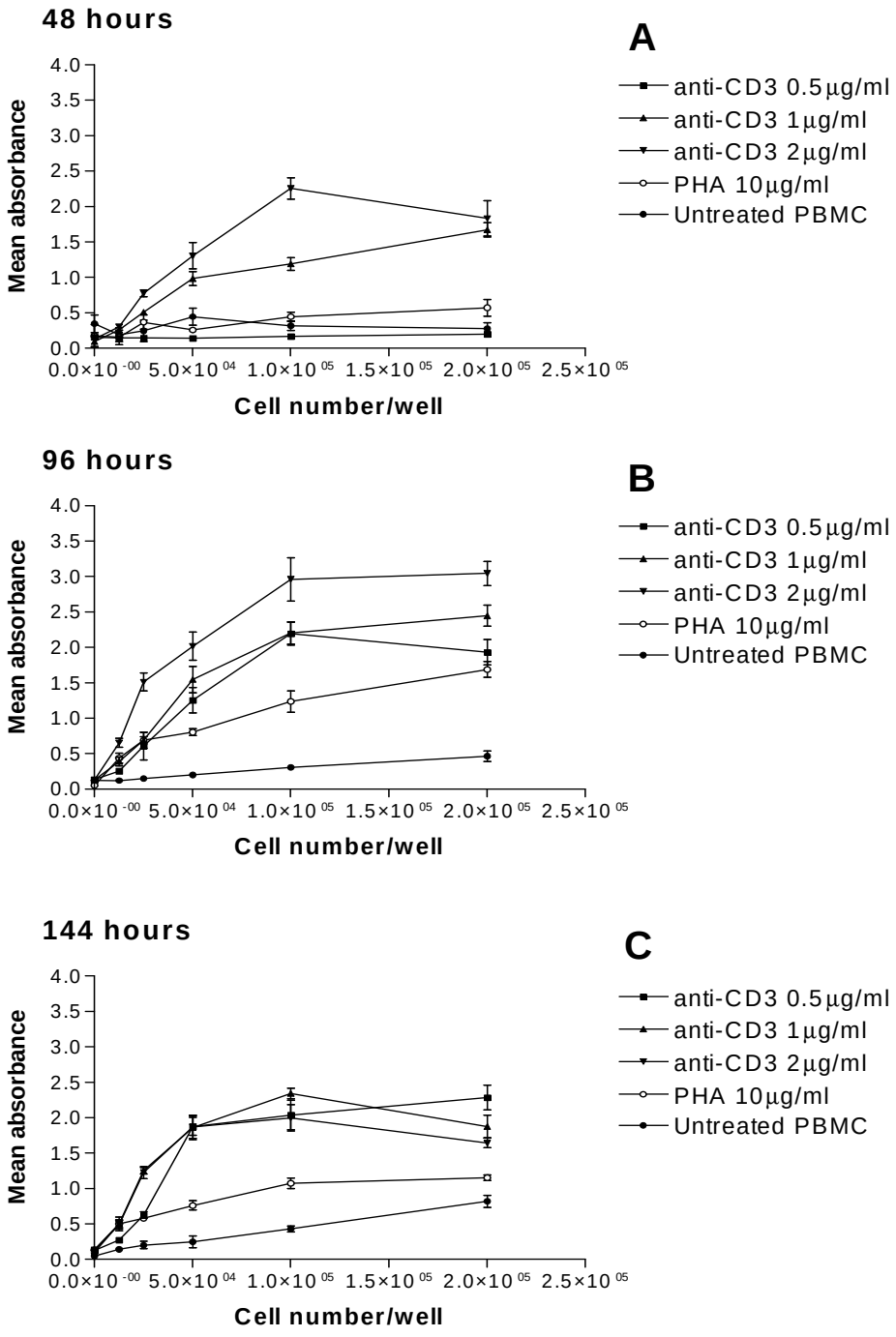

Figure 3.14: Mean absorbance of PBMC in response to stimulation with anti-CD3 at 2µg/ml (closed squares), 1µg/ml (up closed triangles) or 0.5µg/ml (down closed triangles) or 10µg/ml PHA (open circles) or untreated (closed circles) for 48 (**A**), 96 (**B**) or 144 (**C**) hours of incubation. Bars indicate standard error.

PBMC stimulated with 0.5µg/ml anti-CD3 had a low maximum mean absorbance of 0.202 at 48 hours post-stimulation when plated at $2x10^5$ cells/well (figure 3.14A). However, this concentration of anti-CD3 did result in better proliferation with peak mean absorbance values of 2.195 and 2.038 when cells were plated at $1x10^5$ cells/well and the incubation period was extended to 96 and 144 hours, respectively (figures 3.14B and 3.14C). An increase in anti-CD3 concentration to 1µg/ml resulted in an increased peak mean absorbance values of 1.673 with cells at $2x10^5$ cells/well at the 48 hour point, 2.449 at the 96 hour point and 2.346 when cells were at $1x10^5$ cells/well and incubated for 144 hours. Treatment of PBMC with anti-CD3 at 2µg/ml resulted in peak absorbencies of 2.254 (48 hours), 2.963 (96 hours) and 1.999 (144 hours) when cells were at $1x10^5$ cells/well (figures 3.14A-C).

These optimisation assays were carried out in order to establish the ideal experimental conditions to measure the proliferative response of PBMC when stimulated with anti-CD3. The incubation period was chosen to be 96 hours with cells plated at $1x10^5$ cells/well. The concentration of anti-CD3 was chosen to be 5µg/ml and, as a positive control, PHA would be used at 10µg/ml.

Suppression Assay in 3 healthy controls

In order to investigate whether cells isolated using the T_{reg} enrichment procedure (see *Materials and Methods*, section 2.9) had regulatory function, a suppression assay was set up on 3 healthy seropositive controls. Cells from these donors were separated into fractions; unseparated PBMC, $CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells and recombined

cocultures of $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells. These fractions were cultured at $1x10^5$ cells per well in 200µl of TCM 10%, stimulated with either 5µg/ml plate-bound anti-CD3 or left untreated, for 5 days. If cell numbers permitted, the same cell fractions were also stimulated with 10µg/ml PHA. After 5 days of incubation, cell proliferation was measured using the BrdU assay and mean absorbance, proportional to cell proliferation, was established using triplicate values.

Figure 3.15 shows the response of cell fractions from 3 seropositive donors to 5µg/ml anti-CD3 stimulation for the incubation period of 96 hours. Unseparated PBMC proliferated to a high level, with a mean absorbance of 2.84. The $CD4^+CD25^-$ T cells demonstrated relatively high levels of proliferation with a mean absorbance of 1.24, albeit to a lesser extent than the PBMC fraction. Proliferation of $CD4^+CD25^+$ T cells was reduced (mean absorbance 0.24) in comparison with PBMC and $CD4^+CD25^-$ T cells. Furthermore, the addition of $CD4^+CD25^+$ T cells to the $CD4^+CD25^-$ T cells reduced their proliferative response, resulting in a mean absorbance of 0.43. Statistical analysis was not possible since the numbers in each group were too small.

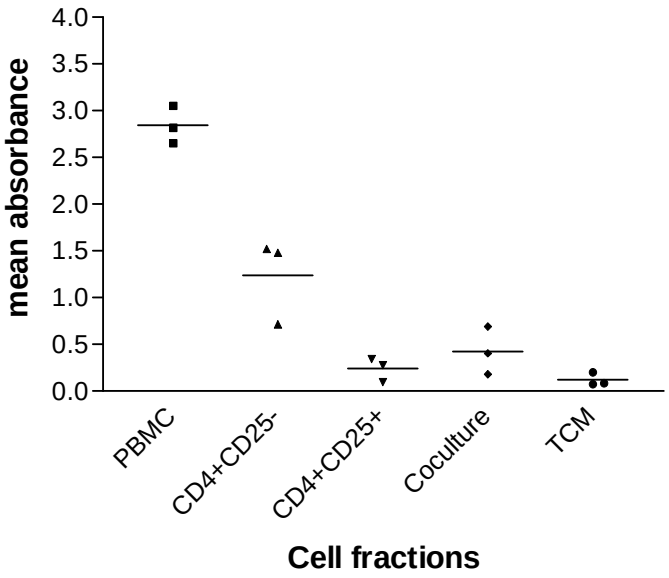

Figure 3.15: Proliferation of cell populations, from 3 seropositive donors (D27, D28 and D29) run in triplicate, in response to stimulation with 5μg/ml plate-bound anti-CD3 for an incubation period of 96 hours. Horizontal bars indicate mean.

Conclusions

The $CD4^+CD25^+$ T cells are poor at proliferation, even in the presence of anti-CD3 antibodies. Furthermore, coculture of $CD4^+CD25^+$ T cells with $CD4^+CD25^-$ T cells results in reduced proliferation by the latter cell population. Hence $CD4^+CD25^+$ T cells, enriched from healthy seropositive donors, are functionally capable of suppressing proliferation of other cell populations. Due to restrictions in cell number this experiment could not be carried out on cell fractions from IM patients.

3.2.5 ELISPOT

Introduction

After establishing that $CD4^+CD25^+$ T cells, enriched from healthy seropositive controls, were capable of suppressing the proliferation of other cell fractions in culture, the next step was to see whether these cells were capable of affecting the response of PBMC to stimulation with EBV-specific antigens. The IFN-γ ELISPOT measures IFN-γ released by cells, which is then bound by anti-IFN-γ antibody, visualised, and subsequently read using an ELISPOT plate reader. This was the assay selected to evaluate whether PBMC responses to EBV antigens are altered by the addition or removal of $CD4^+CD25^+$ T_{reg} (see *Materials and Methods*, section 2.9)

Optimising the IFN-γ ELISPOT assay with LCL

Initially LCL were chosen to stimulate PBMC since they express all latent EBV antigens (Kieff & Rickinson, 2001). In order to establish the experimental conditions required for a successful assay, PBMC and LCL were optimised for cell density and stimulation ratio.

LCL and PBMC were cocultured for 48 hours in U-bottomed 96-well plates in volumes of 100µl in duplicate (at ratios and cell densities of PBMC shown in table 3.5) and the ELISPOT assay was carried out as previously described (*Materials and Methods*, section 2.10). The experiment was carried out using healthy seropositive control PBMC

(D30) and PBMC from an IM patient (IM9). LCL were established whilst PBMC were stored in liquid nitrogen.

Table 3.5: *Combinations of lymphoblastoid cells and peripheral blood mononuclear cells used to optimise the IFN-γ ELISPOT assay*

lymphoblastoid cell lines density/50µl	Peripheral blood mononuclear cell density/50µl				
	1:1	**1:2**	**1:5**	**1:10**	**1:20**
$1x10^5$	$1x10^5$	$2x10^5$	$5x10^5$	$1x10^6$	$2x10^6$
$1x10^4$	$1x10^4$	$2x10^4$	$5x10^4$	$1x10^5$	$2x10^5$
$1x10^3$	$1x10^3$	$2x10^3$	$5x10^3$	$1x10^4$	$2x10^4$

Figure 3.16A shows results for the healthy seropositive control. When LCL were used at a density of $1x10^5$, mean SFC/million PBMC was 1845, 1468, 561, 184 and 52 as the ratio of PBMC:LCL increased from 1:1 to 20:1, respectively. Overall range was between 0 and 1980 SFC/million PBMC. Reducing the density of LCL to $1x10^4$/well resulted in an increased mean SFC/million PBMC at each ratio, from 1:1 to 20:1 of 2700, 1575, 920, 605 and 558, respectively and an increase in variation to a range of between 430 to 3640 SFC/million PBMC. A further reduction in LCL density to $1x10^3$/well had different effects at each ratio, resulting in an increase in mean SFC/million PBMC at some ratios and a decrease in others: 3250, 3750, 720, 150 and 325 (from 1:1 to 20:1, respectively). The greatest variation in IFN-γ production occurred when LCL were used at $1x10^3$/well, where the results ranged from 0 to 6660 SFC/million PBMC.

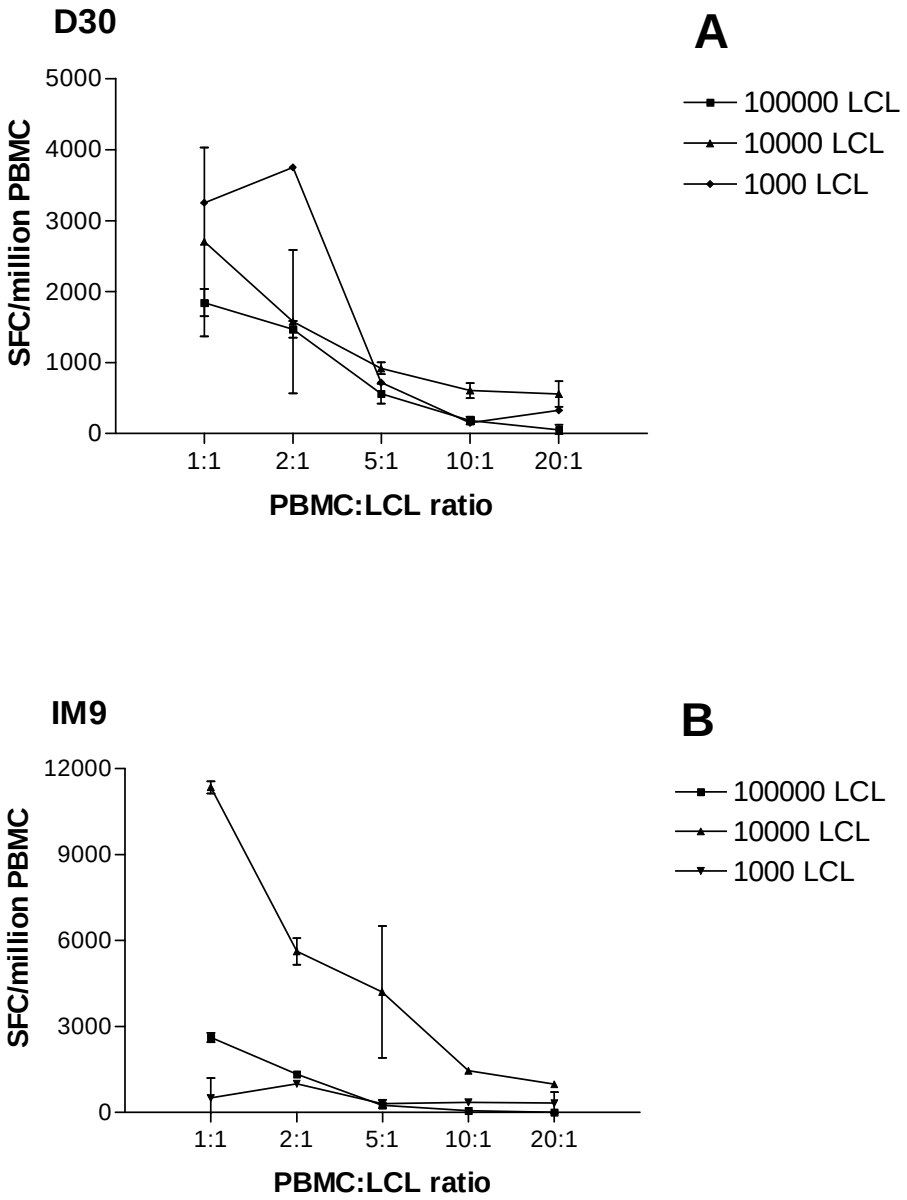

Figure 3.16: IFN-γ ELISPOT results for healthy seropositive control D30 (**A**) and IM patient, IM9 (**B**). PBMC stimulated with LCL at $1x10^5$ (squares), $1x10^4$ (upward triangles) or $1x10^3$ (downward triangles) per well. Bars indicate standard error.

The IFN-γ response seen in IM patients is shown in figure 3.16B. When LCL were used at a density of $1x10^5$, mean SFC/million PBMC was 2615, 1338, 251, 69 and 0 as the

ratio increased from 1:1 to 20:1, respectively, showing a similar trend to the healthy seropositive control. The range at this LCL density was between 0 and 2730 SFC/million PBMC.

When LCL were used at a density of $1x10^4$ LCL, the mean SFC/million PBMC of 11350, 5625, 4210, 1450 and 325 at each ratio was increased in comparison to the healthy control, as was the variation, with an increased range of between 935 and11500 SFC/million PBMC. At a density of $1x10^3$ LCL, mean SFC/million PBMC at each ratio was 500, 1000, 300, 350 and 325, respectively. These results are similar to the healthy control since there appears to be no particular trend. Range was reduced in comparison to the control at this density of LCL, ranging from 0 to 1000 SFC/million PBMC.

As a result of this experiment the conditions chosen for all future experiments were that LCL, at a density of $1x10^4$/well, would be used to stimulate doubling dilutions of PBMC ranging from $1x10^5$ to $1.25x10^4$/well.

Initially it was intended that PBMC from IM patients and healthy controls would be in liquid nitrogen whilst LCL were developed. When the LCL had been grown up, the PBMC would be revived and separated into two portions; PBMC with $CD4^+CD25^+$ T cells and PBMC without $CD4^+CD25^+$ T cells. Both cultures would then be stimulated with LCL and their response measured using the IFN-γ ELISPOT. To this end, LCL were established for 10 IM patients and 10 healthy seropositive controls. However, the

process of freezing samples from IM patients considerably reduced the number of viable PBMC revived. It was therefore decided to use an alternative approach to assess the effect of $CD4^{+}CD25^{+}$ T cells on PBMC stimulated with EBV antigens that could be carried out on fresh IM samples.

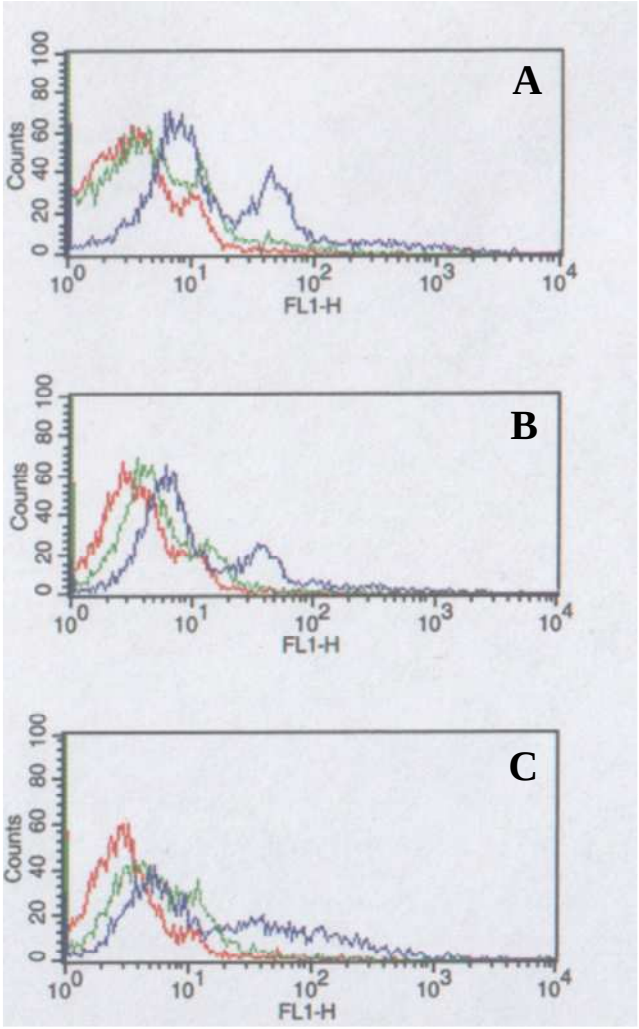

Figure 3.17: Flow cytometry staining for HLA A2 status on PBMC homozygous for HLA A2 (**A**), PBMC heterozygous for HLA A2 (**B**) and non-HLA A2 PBMC (**C**), showing unstained PBMC (red), isotype control stained PBMC (green) and HLA A2 stained PBMC (blue). FL1-H indicates HLA-A2 FITC.

The next option was to use EBV peptides to stimulate PBMC. This required HLA typing of PBMC since the peptides are HLA restricted. It was decided to use HLA A2 positive donors since it occurs in 40-50% of the population worldwide (Mahdavi & Monk, 2005a;Botten *et al*, 2007b). Figure 3.17 shows results for HLA A2 staining on an A2 homozygous PBMC, an A2 heterozygous PBMC and a non-A2 PBMC.

Once this technique was established on PBMC with known HLA A2 expression, HLA staining was carried out on all IM samples and healthy controls. The HLA A2 positive donors were used for further experiments.

Optimising the IFN-γ ELISPOT assay with peptides

PBMC from an EBV-positive HLA A2 heterozygous donor (D4) were set up at $2x10^5$ cells/well, $1x10^5$ cells/well and $5x10^4$ cells/well and treated with one of the following peptides; GLC, SVR or QAK at 5μg/ml and 10μg/ml, PHA at 1μg/ml or left untreated, and incubated for 48 hours (See *Materials and Methods*, section 2.10.3 for more details). The ELISPOT assay was then run and IFN-γ spots counted. Figure 3.18 shows the results of this optimisation.

The mean IFN-γ response by untreated PBMC peaked at 20 SFC/million PBMC when cells were plated at $1x10^5$ cells/well. The total range of SFC/million for all cell densities ranged from 0 to 50 SFC/million PBMC (figure 3.18A).

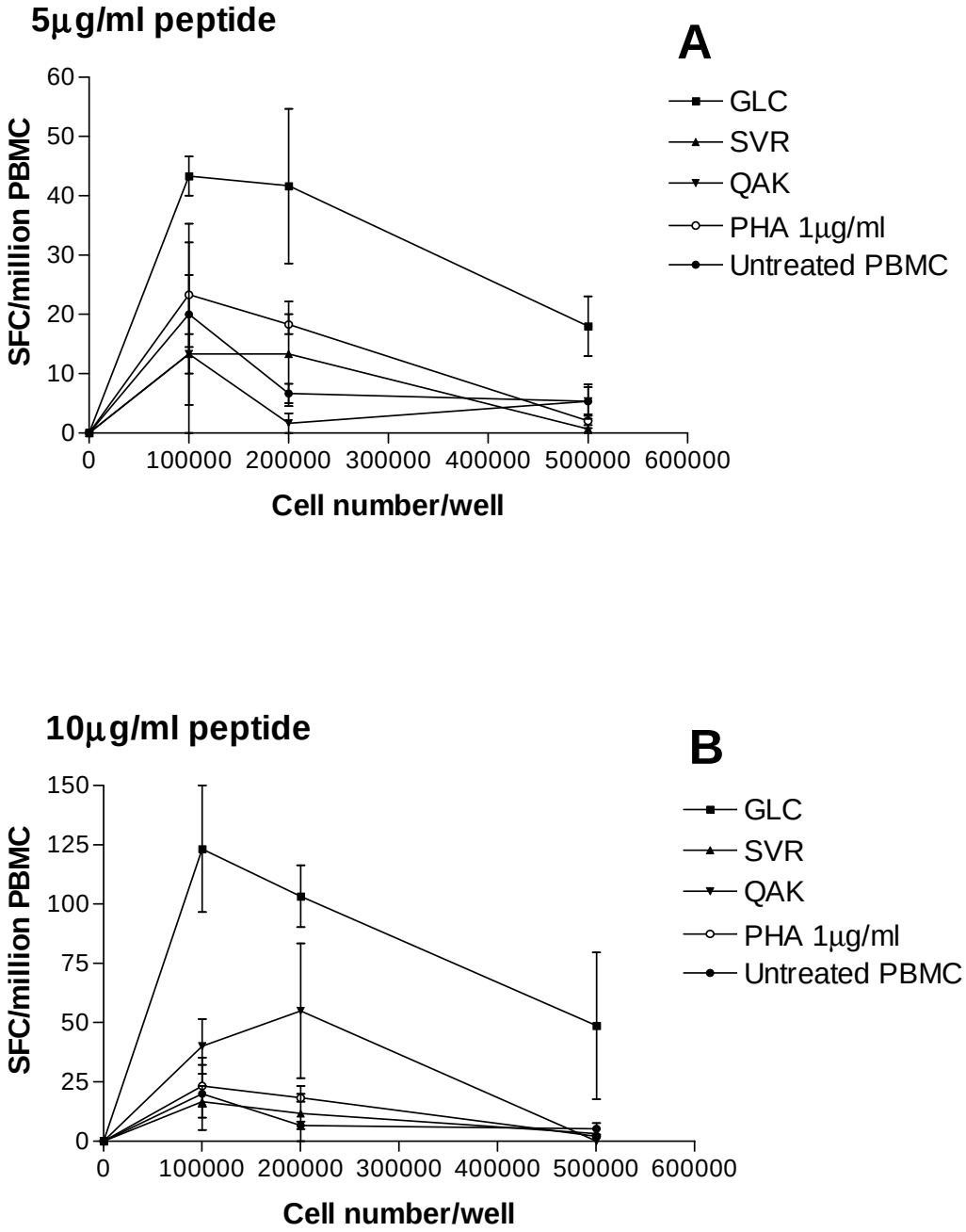

Figure 3.18: IFN-γ ELISPOT results for PBMC from a HLA A2 donor stimulated with peptides GLC (closed squares), SVR (closed up triangles) or QAK (closed down triangles), 1µg/ml PHA (open circles) or left untreated (closed circles). Peptides were used at 5µg/ml (**A**) or 10µg/ml (**B**). Bars indicate standard error.

A mean SFC/million PBMC of 13.3 was observed for PBMC treated with 5µg/ml of the non-A2 peptide, QAK, with a density of $5x10^5$ cells/well (figure 3.18A). The overall range for treatment with QAK was between 0 and 40 SFC/million PBMC. When QAK was used at 10µg/ml an increased mean SFC/million PBMC of 55 was observed at a cell density of $2x10^5$ cells/well (figure 3.18B). Total range for all cell densities ranged between 0 to 95 SFC/million PBMC.

Treatment with 1µg/ml PHA did not increase the IFN-γ release (peak mean SFC/million PBMC of 23.3 when cells were at a density of $1x10^5$ cells/well) and since this was to act as the positive control, the higher concentration of 5µg/ml was used in future ELISPOT assays. Overall, the results from PHA-treated PBMC ranged from 0 to 40 SFC/million PBMC, with higher responses observed at densities of $1x10^5$ cells/well (mean SFC/million PBMC 23.3) in comparison with cell densities of $2x10^5$ cells/well and $5x10^5$ cells/well, mean SFC/million PBMC 18 and 2, respectively (figure 3.18A).

A low level of IFN-γ was released in response to 5µg/ml SVR (mean SFC/million PBMC 13.3) at densities of $1x10^5$ and $2x10^5$ cells/well. Very little response was observed when cell density was increased to $5x10^5$ cells/well (mean SFC/million PBMC 0.67). Results ranged from 0 to 30 SFC/million PBMC for all cell densities (figure 3.18A). On increasing the SVR to 10µg/ml, the IFN-γ response was slightly increased, with peak mean SFC/million PBMC of 16.7 was observed when cells were at $1x10^5$

cells/well. However, the overall range of data remained similar to treatment with SVR at 5µg/ml, from 0 to 35 SFC/million PBMC (figure 3.18B).

High levels of IFN-γ were released in response to 5µg/ml GLC at densities of $1x10^5$ and $2x10^5$ cells/well (mean SFC/million PBMC 43.3 and 41.7, respectively). Some response was observed when cell density was increased to $5x10^5$ cells/well (mean SFC/million PBMC 18) but not to the same extent as lower cell densities. Results ranged from 0 to 65 SFC/million PBMC for all cell densities (figure 3.18A). Increasing the GLC concentration to 10µg/ml increased the response, with maximum mean SFC/million PBMC of 123.3, 103.3 and 48.7 being recorded when cells were at $1x10^5$, $2x10^5$ and $5x10^5$ cells/well, respectively. The overall range also increased, ranging from 0 to 150 SFC/million PBMC (figure 3.18B).

Since GLC induced a high IFN-γ response, experimental conditions were established with regards to this peptide. The higher concentration of 10µg/ml of peptide was opted for since it provoked a higher IFN-γ response to GLC (mean SFC/million PBMC 103.3). 1µg/ml PHA did not induce an IFN-γ response so the concentration was increased to 5µg/ml in all future experiments. IFN-γ release reduced when PBMC density was increased from $2x10^5$ to $5x10^5$ (SFC/million PBMC 103.3 and 48.7, respectively) so the lower of the two cell densities was used.

ELISPOT results with & without T_{reg} for 8 healthy controls

HLA A2 positive healthy seropositive donors were used as controls for the IFN-γ ELISPOT assay and were provided by a frozen bank of PBMC already established in the research group. Once revived, all donor PBMC were subjected to the T_{reg} enrichment procedure. The T_{reg}-depleted PBMC were then separated into two samples and half of the enriched $CD4^+CD25^+$ T cells were added back to one sample. The IFN-γ ELISPOT was then set up using the following fractions: T_{reg}-depleted PBMC with $CD4^+CD25^+$ T cells and PBMC without $CD4^+CD25^+$ T cells. HLA information on each of the 8 donors is shown in table 3.6.

Table 3.6: *Information on healthy seropositive donors used as controls in the IFN-γ ELISPOT assays*

donor number	HLA A type
D5	A1-A2
D6	A2-A31
D7	A1-A2
D8	A2- A11
D9	A2- A3
D10	A2-A28
D11	A2- A11
D12	A2- A2

Figure 3.19 shows the IFN-γ response by all donor PBMC if left untreated (panel A) or in response to stimulation with QAK (panel B), PHA (panel C), SVR (panel D), LLD (panel E) or GLC (panel F). PHA was used at 5μg/ml and all peptides were used at 10μg/ml.

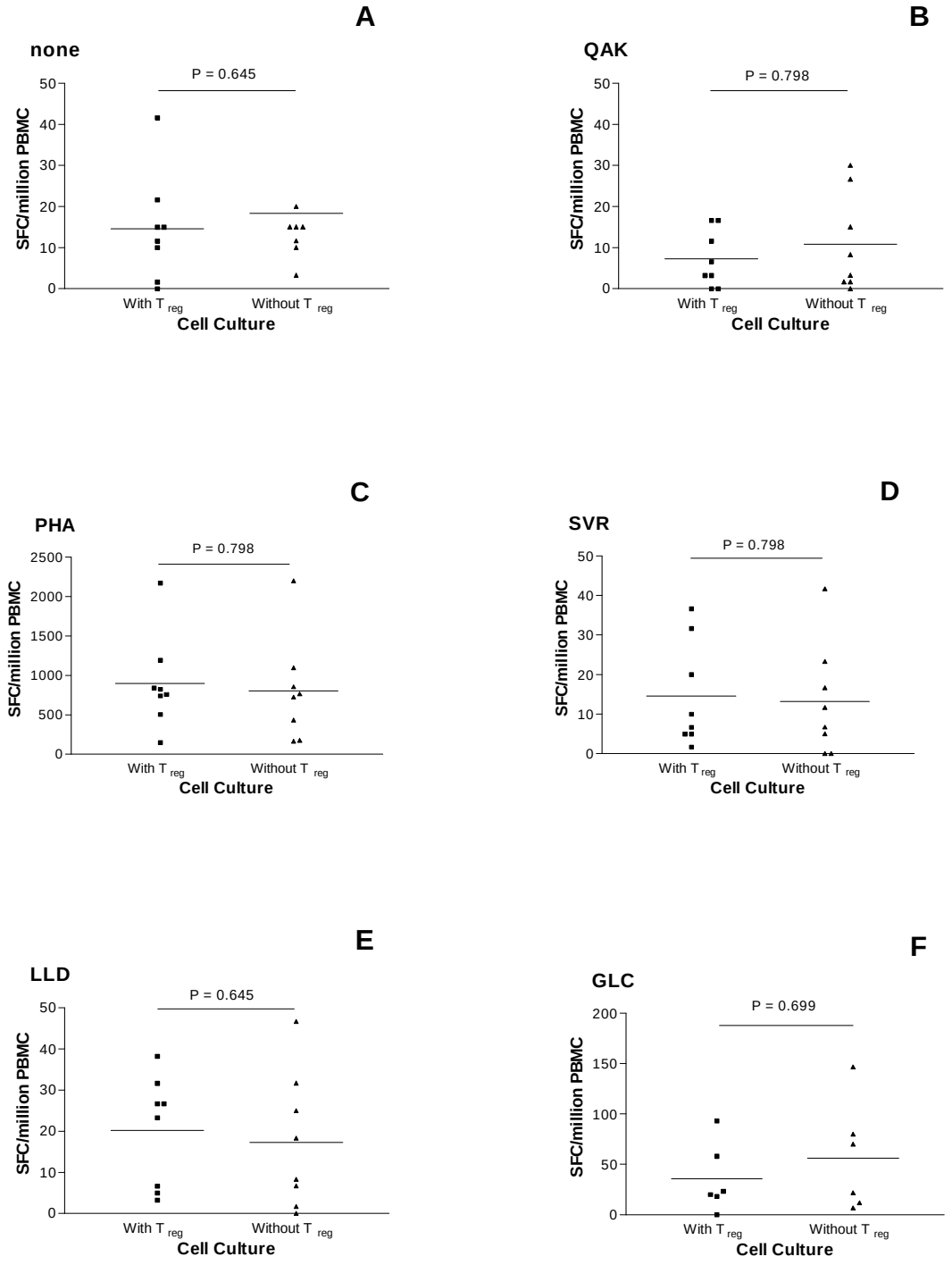

Figure 3.19: IFN-γ ELISPOT results for 8 healthy seropositive PBMC cultures in the presence (closed squares) or absence (closed triangles) of T_{reg}, left untreated (**A**), QAK (**B**), 5µg/ml PHA (**C**), SVR (**D**), LLD (**E**) or GLC (**F**). Horizontal bars indicate the median SFC/million PBMC.

Untreated PBMC released a low level of background IFN-γ, with a range of results between 0 and 56.7 SFC/million PBMC. Comparing the presence of T_{reg} (median SFC/million PBMC 13.4) to T_{reg} absence (median SFC/million PBMC 15), no significant difference was observed (p=0.645). PBMC stimulated with QAK, the non-A2 peptide, had a range of 0 to 30 SFC/million PBMC. The presence of T_{reg} (median SFC/million PBMC 5) or absence of T_{reg} (median SFC/million PBMC 5.8) did not significantly affect IFN-γ release (p=0.798). PBMC treated with PHA resulted in the largest IFN-γ response with a result range between 148.3 to 2201.7 SFC/million PBMC. No significant difference was observed (p=0.798) between PBMC with T_{reg} (median SFC/million PBMC 793.4) and PBMC without T_{reg} (median SFC/million PBMC 745.9).

No significant difference was observed when PBMC were stimulated with SVR (p=0.798), with low levels of IFN-γ produced by both cultures containing T_{reg} (median SFC/million PBMC 8.35) and lacking T_{reg} (median SFC/million PBMC 9.2). Results ranged from 0 to 41.7 SFC/million PBMC. Stimulation with LLD resulted in a slight difference in IFN-γ response, albeit not significant (p=0.654), between cultures containing T_{reg} (median SFC/million PBMC 25) and those lacking T_{reg} (median SFC/million PBMC 13.3). The results ranged between 0 and 46.7 SFC/million PBMC.

In the absence of T_{reg}, PBMC released an increased level of IFN-γ in response to GLC when compared to cultures containing T_{reg} (median SFC/million PBMC 45.85 and 21.65, respectively) although this was not significant (p=0.699). Results ranged between

0 and 146.7 SFC/million PBMC (figure 3.19). A summary of the data displayed in figure 3.19 is shown in figure 3.21.

ELISPOT results with & without T_{reg} for 7 IM patients

Figure 3.20 shows the IFN-γ response by PBMC from IM patients if left untreated (panel A) or in response to stimulation with QAK (panel B), PHA (panel C), SVR (panel D), LLD (panel E) or GLC (panel F). PHA was used at 5µg/ml and all peptides were used at 10µg/ml.

A low background level of IFN-γ was released by untreated PBMC, with a range of data between 1.7 and 48.3 SFC/million PBMC. No significant difference was observed ($p=0.710$) when T_{reg} presence (median SFC/million PBMC 13.3) was compared to T_{reg} absence (median SFC/million PBMC 8.3). Stimulation of PBMC with the non-A2 peptide, QAK, also resulted in a low level of response with a result range of 0 to 42.5 SFC/million PBMC. IFN-γ production was not significantly affected ($p=0.318$) by the presence (median SFC/million PBMC 5) or absence of T_{reg} (median SFC/million PBMC 6.7). PBMC treated with PHA resulted in the same high IFN-γ response from both culture with and without T_{reg} (median SFC/million PBMC 66.7) with no significant difference ($p=1$). The result range for this treatment was 16.7 to 881.7 SFC/million PBMC.

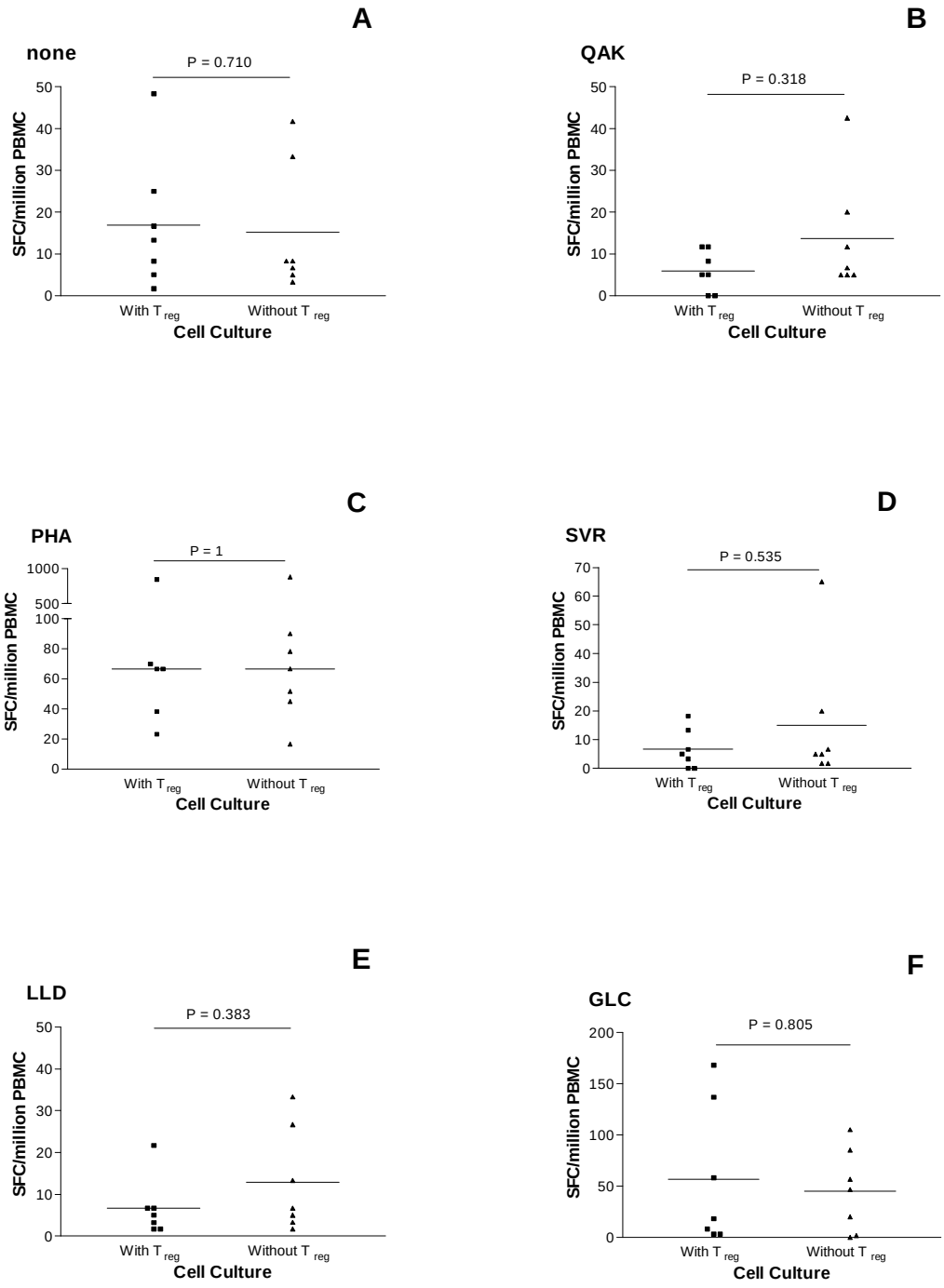

Figure 3.20: IFN-γ ELISPOT results for 7 IM patient PBMC cultures in the presence (closed squares) or absence (closed triangles) of T_{reg}, left untreated (**A**), QAK (**B**), 5µg/ml PHA (**C**), SVR (**D**), LLD (**E**) or GLC (**F**). Horizontal bars indicate the median SFC/million PBMC.

Equally low levels of IFN-γ were produced by PBMC with and without T_{reg} (median SFC/million PBMC 5) when stimulated with SVR, with no significant difference observed (p=0.535). The range of results was 0 to 65 SFC/million PBMC. LLD stimulation of PBMC resulted in a low, non-significant response (p=0.383) by both cultures containing T_{reg} (median SFC/million PBMC 5) and those lacking T_{reg} (median SFC/million PBMC 6.7), with a result range of 0 to 33.3 SFC/million PBMC. In response to GLC stimulation, an increased IFN-γ response was observed in PBMC lacking T_{reg} when compared to cultures containing T_{reg} (median SFC/million PBMC 46.7 and 18.3, respectively) although this was not significant (p=0.805). Results ranged between 0 and 168.3 SFC/million PBMC. A summary of the data displayed in figure 3.20 is shown in figure 3.22.

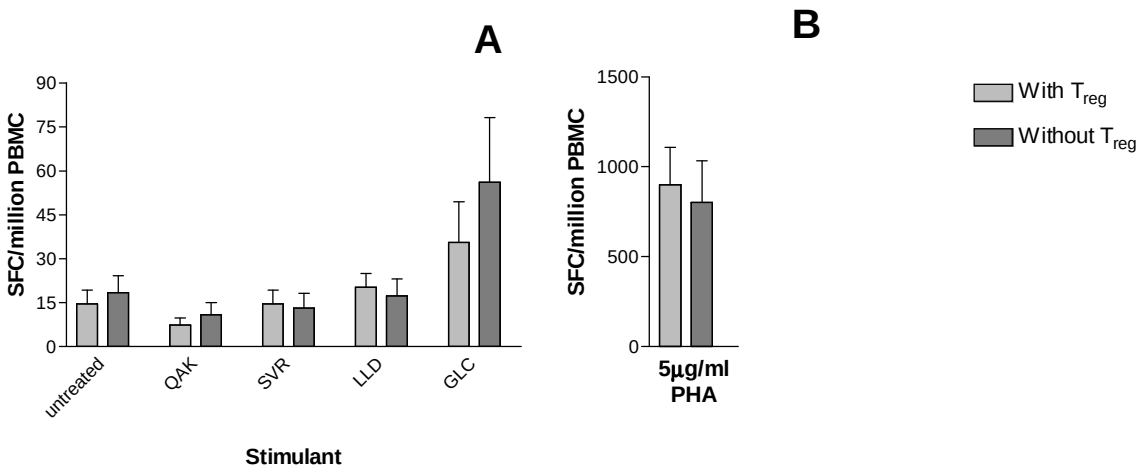

Figure 3.21: Combined IFN-γ ELISPOT results for 8 healthy seropositive PBMC cultures in the presence (light grey) or absence (dark grey) of T_{reg}, left untreated or stimulated with 10µg/ml of peptide; QAK, SVR, LLD or GLC (**A**) or 5µg/ml PHA (**B**). Bars represent standard error.

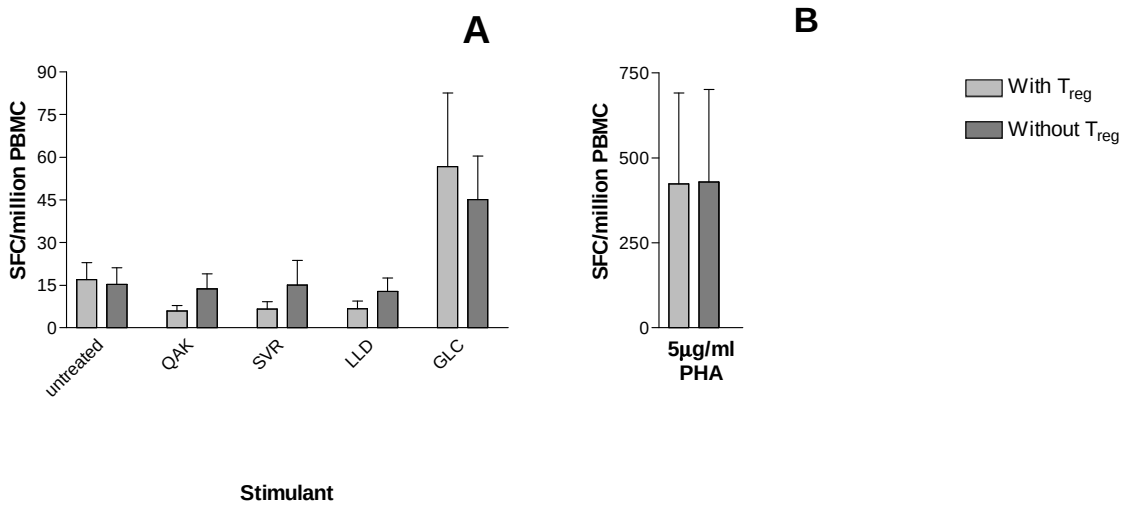

Figure 3.22: Combined IFN-γ ELISPOT results for 7 IM patient PBMC cultures in the presence (light grey) or absence (dark grey) of T_{reg}, left untreated or stimulated with 10µg/ml of peptide; QAK, SVR, LLD or GLC (**A**) or 5µg/ml PHA (**B**). Bars represent standard error.

Comparing the response of untreated PBMC in the presence of T_{reg} from healthy seropositive controls and IM patients reveals a similar level of IFN-γ production (median SFC/million PBMC 13.4 and 13.3, respectively, p=0.78). This was also observed in cultures lacking T_{reg} (median SFC/million PBMC 15 and 8.3, respectively, p=0.28).

IFN-γ production by PBMC with T_{reg} stimulated with QAK did not vary between healthy controls and IM patients (median SFC/million PBMC 5 and 5, respectively, p=0.87) or in cultures lacking T_{reg} (median SFC/million PBMC 5.8 and 6.7, respectively, p=0.46).

Stimulation of PBMC with PHA in the presence of T_{reg} resulted in a significantly higher IFN-γ response in healthy seropositive controls (median SFC/million PBMC 793.4) than the same culture in IM patients (median SFC/million PBMC 66.7, p=0.009). Similarly, PBMC stimulated with PHA in the absence of T_{reg} resulted in a significantly higher production of IFN-γ in healthy seropositive controls (median SFC/million 745.9) than PBMC without T_{reg} from IM patients (median SFC/million PBMC 66.7, p=0.009).

Equally low levels of IFN-γ production were observed by PBMC from healthy seropositive controls and IM patients, with (median SFC/million PBMC 8.35 and 9.2, respectively) and without T_{reg} (median SFC/million PBMC 5 and 5, respectively) in response to stimulation with SVR (with T_{reg} p=0.19 and without T_{reg} p= 0.87).

Stimulation of PBMC with LLD in cultures containing T_{reg} resulted in a significantly increased response (p=0.04) in healthy seropositive controls (median SFC/million PBMC 25) when compared with IM patients (median SFC/million PBMC 5). No significant difference in IFN-γ production was observed in cultures lacking T_{reg} from either sample group (median SFC/million PBMC in healthy seropositive controls 13.3 and IM patients 6.7, p=0.78).

Comparing IFN-γ production in cultures containing T_{reg} between healthy seropositive controls and IM patients stimulated with GLC revealed no significant differences (median SFC/million PBMC 21.65 and 18.3, respectively, p=1). Similar results were

observed in cultures lacking T_{reg} (SFC/million PBMC 45.85 and 46.7, respectively, p=0.73).

Conclusions

The removal of $CD4^+CD25^+$ T cells from PBMC did not significantly alter the production of IFN-γ in response to stimulation with PHA or HLA A2-restricted peptides derived from EBV antigens. PHA and LLD responses were significantly lower in IM patients than healthy controls but were unaffected by the presence or absence of T_{reg}.

3.2.6 FOXP3 Staining

Introduction

To date one of the most specific markers for T_{reg} is the expression of FOXP3, a transcriptional factor involved in the generation and function of regulatory T cells (reviewed in Campbell & Ziegler, 2007a;Kim & Rudensky, 2006). The expression of this marker was assessed to provide further evidence that the population of cells used in the experiments were conventional regulatory T cells.

Optimisation of FOXP3 staining in Tonsil sections

Published work on FOXP3 staining used tonsil sections as control material since T_{reg} expressing this marker are almost always found in this lymphoid tissue (Roncador *et al*, 2005). Negative controls consisted of replacing the primary or a combination of primary and secondary antibody with PBS. An antibody binding the B-cell membrane antigen (L26) was employed as the positive control.

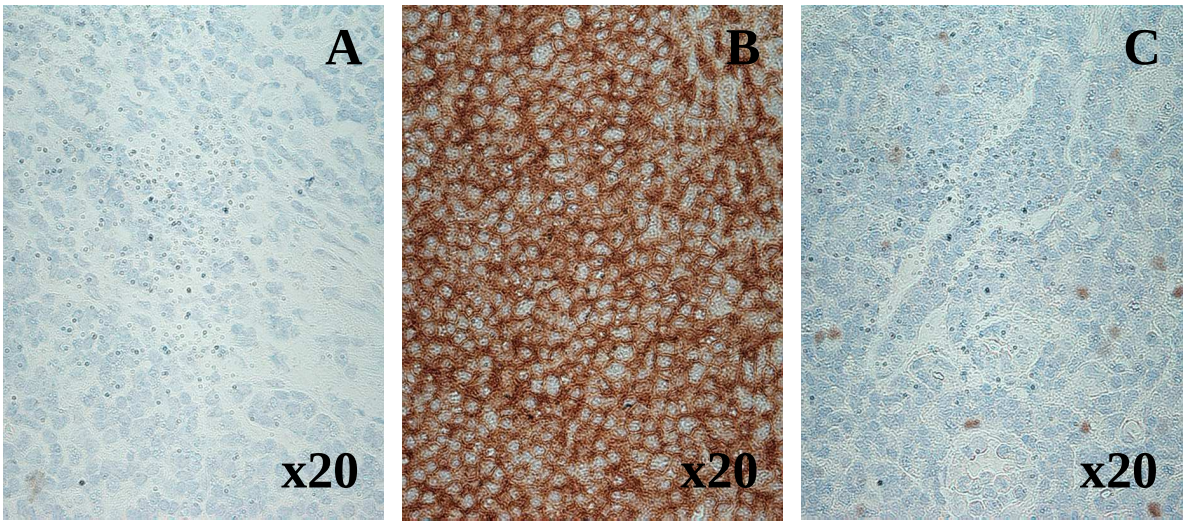

Figure 3.23: FOXP3 staining in paraffin-embedded tonsil sections (**C**). Controls consisted of replacing primary and secondary antibodies with PBS (**A**) and L26 which binds B-cells (**B**).

Figure 3.23 shows results for the staining procedure on tonsil sections. No staining was seen in the negative control, whereas staining was widespread in the positive control. Nuclear-stained FOXP3 positive cells were apparent throughout the section.

FOXP3 staining in PBMC from healthy controls

Having established that the FOXP3 antibody could be used successfully in control tonsil sections, the next step was to look for FOXP3 in PBMC from healthy seropositive controls. PBMC were separated into fractions; unseparated PBMC, $CD4^+$-depleted cells, $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells and cytospins were made from each cell population and stained with FOXP3 antibody. The negative controls consisted of replacing primary only or primary and secondary antibody with PBS. Staining with L26 was used as the positive control.

Figure 3.24 shows the results for staining on PBMC. The negative control shows no staining. When PBS is used to replace the primary antibody alone, slight non-specific staining is observed although not in the cells themselves. L26 provides a good positive control in this case since there will be B cells in the PBMC, the staining indicates that the kit is functioning. When the FOXP3 antibody is used, no staining above background was observed.

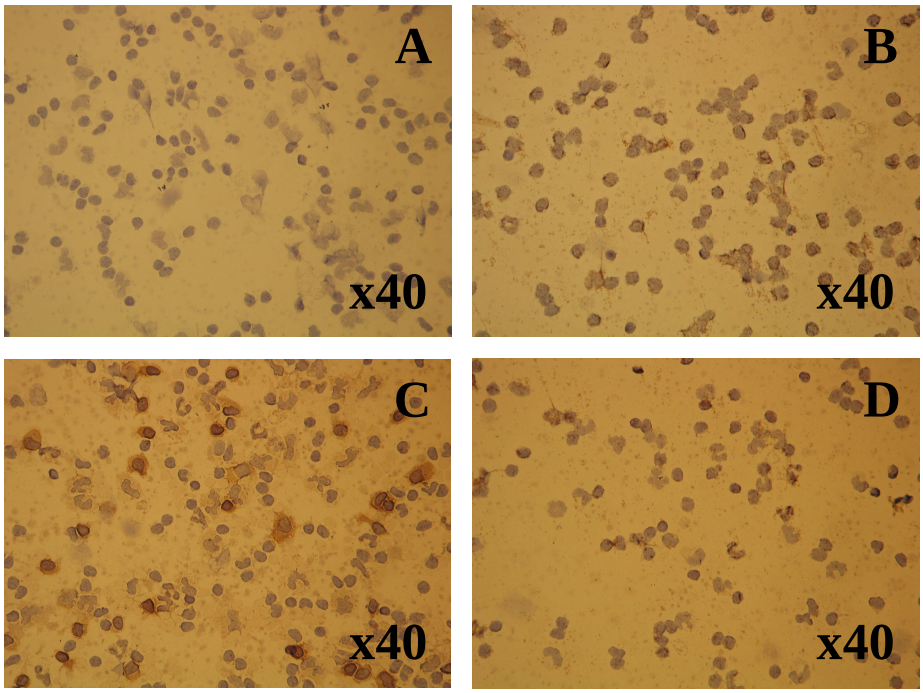

Figure 3.24: FOXP3 staining in PBMC cytospins (**D**). Controls consisted of PBS replacing the primary and secondary antibodies (**A**), PBS replacing the primary antibody only (**B**) and L26 (**C**). Magnification x40.

Figure 3.25 shows results for staining on CD4-depleted cells. Similar to PBMC, no staining is seen when PBS is used to replace both the primary and secondary antibody. Non-specific staining is increased somewhat when PBS replaces only the primary antibody. Some staining is apparent with L26, and non-specific staining with FOXP3

makes it difficult to see whether any cells are positive. In addition, this fraction of cells looks far less healthy than the PBMC.

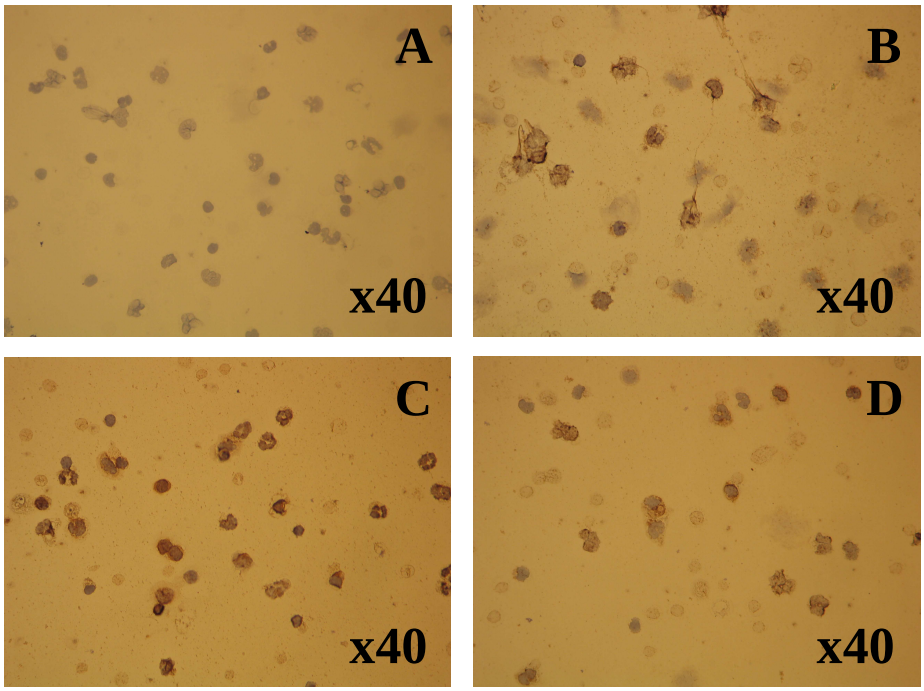

Figure 3.25: FOXP3 staining in CD4-depleted cytospins (**D**). Controls consisted of PBS replacing the primary and secondary antibodies (**A**), PBS replacing the primary antibody only (**B**) and L26 (**C**).

Figure 3.26 shows results for the $CD4^{+}CD25^{-}$ T cell fraction. No staining is observed in the negative or positive controls, or indeed with the FOXP3 antibody. Of note is the observation that the cells in this fraction look healthier than the previous fraction.

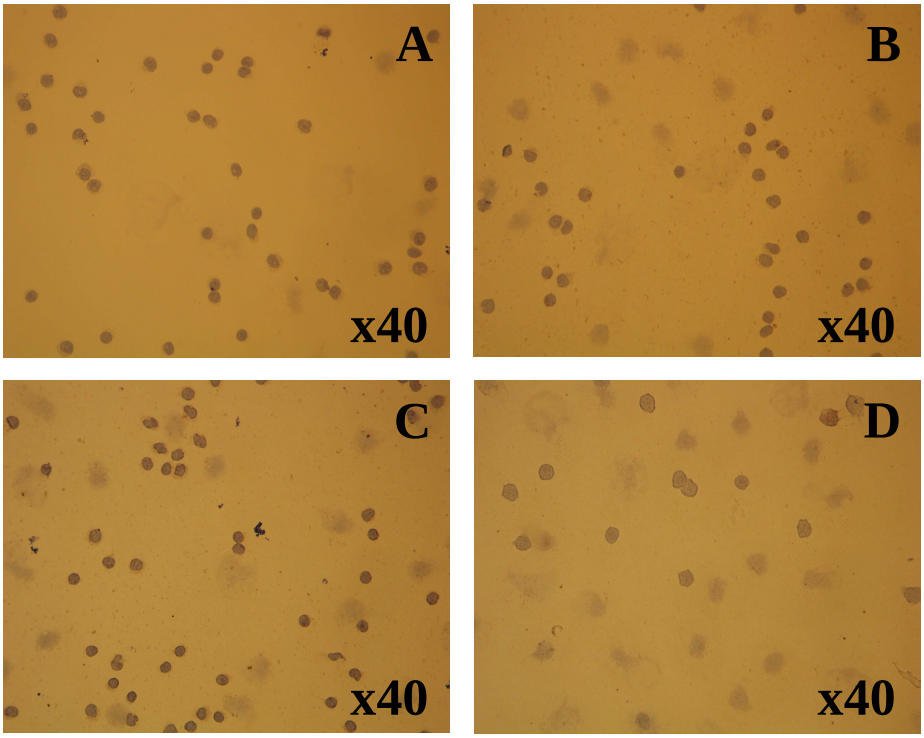

Figure 3.26: FOXP3 staining in CD4+CD25- cytospins (**D**). Controls consisted of PBS replacing the primary and secondary antibodies (**A**), PBS replacing the primary antibody only (**B**) and L26 (**C**).

Figure 3.27 shows results for the $CD4^{+}CD25^{+}$ T cell fraction. Again, no staining is observed in any of the control slides, however, there are clearly positive cells when FOXP3 antibody is used. Similarly to the previous fraction, the cells look healthy.

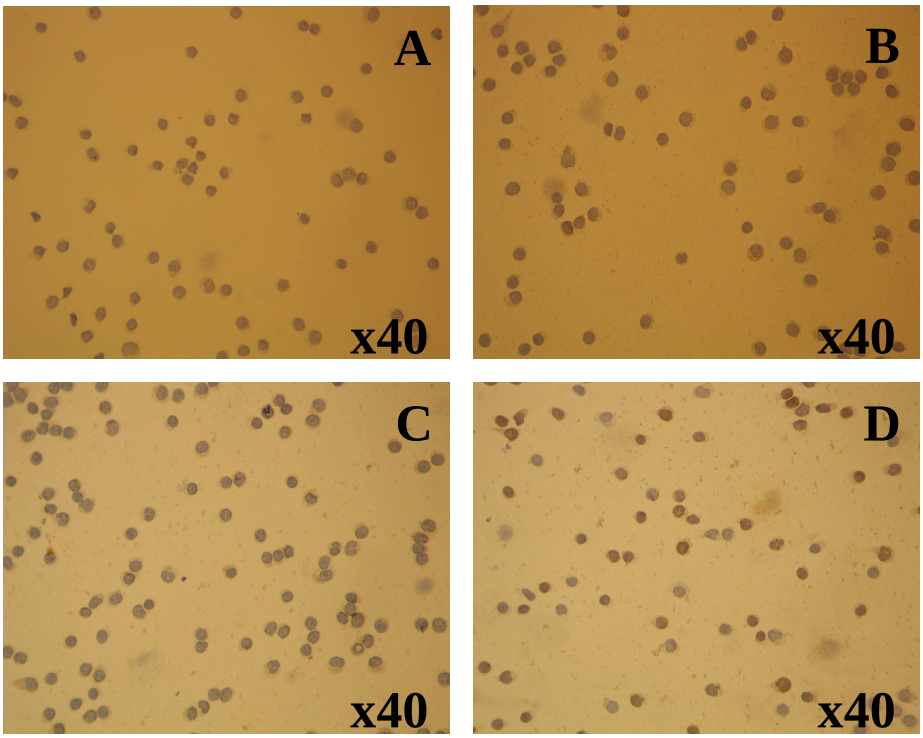

Figure 3.27: FOXP3 staining in CD4+CD25+ cytospins (**D**). Controls consisted of PBS replacing the primary and secondary antibodies (**A**), PBS replacing the primary antibody only (**B**) and L26 (**C**).

This staining was carried out in order to investigate whether the $CD4^{+}CD25^{+}$ T cells enriched by the isolation procedure expressed FOXP3, one of the most specific markers for regulatory T cells. Figures 3.24-3.27 show representative results for healthy control donor. All three donors used for this staining procedure showed similar results. Limitations in cell number meant this procedure could not be carried out on IM patients. However, due to non-specific staining the positive cells on these slides could not be accurately quantified.

FOXP3 staining of activated cells

Although FOXP3-positive cells appeared to be at a higher frequency in the $CD4^+CD25^+$ T cell fraction, high levels of non-specific staining on unstimulated cell fractions meant interpretation of the results was difficult. On advice from Dr Bridget Fox in Dr Alison Banham's Oxford Laboratory, an adaptation was made to the protocol involving the stimulation of PBMC, CD4-depleted cells, $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells with T cell expander beads and 500IU/ml rIL-2 for 3 days prior to making cytospins. FOXP3 staining was then carried out in order to determine which population of cells gave rise to the highest frequency of FOXP3-positive cells.

Figure 3.28 shows results for staining on PBMC. When PBS is used to replace both the primary and secondary antibodies no staining is observed. Similarly, when PBS was used to replace the primary antibody no staining was observed. The same level of staining was observed in each cell fraction using these negative controls so the figures below do not show all of these results. L26 staining was strong and observed throughout the cell population, indicating the kit was working. FOXP3 staining resulted in a few positive cells throughout the slide, accounting for 9.0% of the cells in any given view.

Figure 3.29 shows results for staining on CD4-depleted cells. Similar to PBMC alone, no staining was observed when PBS was used to replace the primary alone, or primary and secondary antibodies (figures not shown). L26 showed strong staining throughout the cells. FOXP3-positive cells accounted for 7.9% of the cells, a slight decrease in comparison to PBMC.

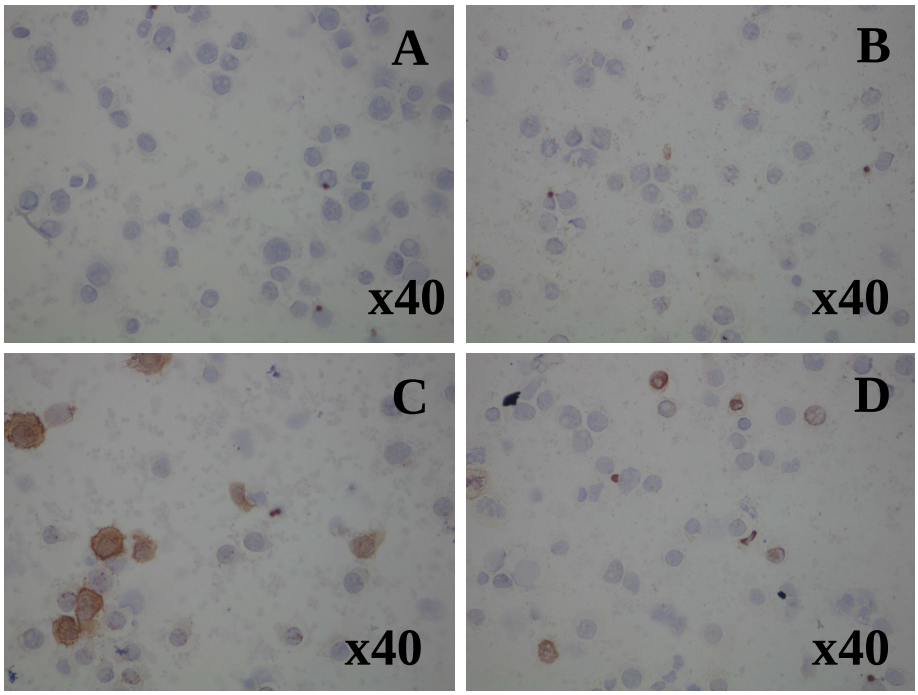

Figure 3.28: Immunohistochemistry for PBMC cytospins with primary and secondary antibodies replaced with PBS (**A**), PBS replacing the primary alone (**B**), the B-cell antibody, L26 (**C**) and FOXP3 (**D**).

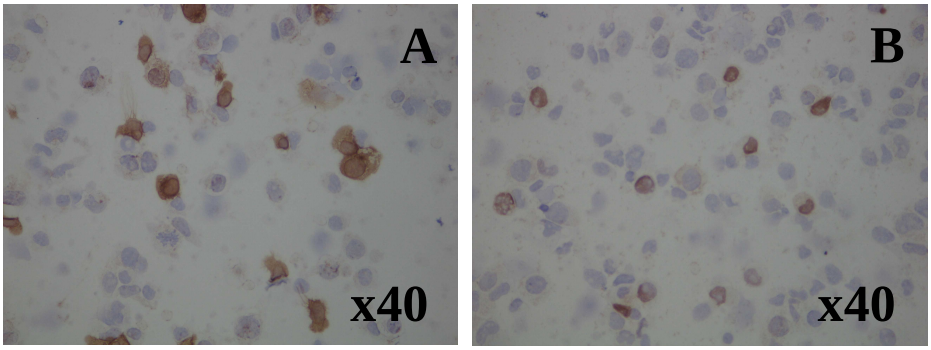

Figure 3.29: Immunohistochemistry for CD4-depleted cytospins with the B-cell antibody, L26 (**A**) and FOXP3 (**B**)

Figure 3.30 shows results for staining on $CD4^{+}CD25^{-}$ T cells. No staining was observed with PBS replacing the primary and secondary (not shown) or with PBS replacing the primary antibody alone (figure 3.30A). L26 could not be used as a positive control since

few B cells would be expected in this cell fraction. FOXP3-positive cells occurred more frequently than in PBMC or CD4-depleted cell fractions, accounting for 17.7% of the cells.

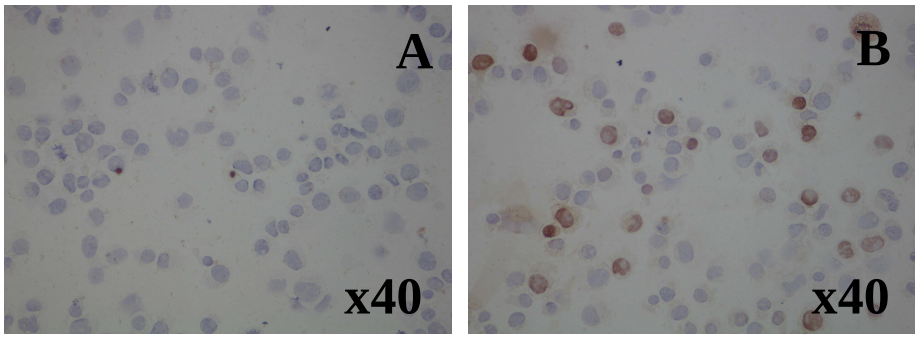

Figure 3.30: Immunohistochemistry for CD4+CD25- T cell cytospins with PBS replacing the primary antibody alone (**A**) and FOXP3 (**B**).

Figure 3.31 shows results for staining on $CD4^{+}CD25^{+}$ T cells. No staining was observed with PBS replacing the primary and secondary (not shown) or with PBS replacing the primary antibody alone (figure 3.31A). Again, L26 could not be used as a positive control since B cells would not be in this cell fraction. Out of all the cell fractions, this cell population contained the highest frequency of FOXP3-positive cells, 63.8% of the cells stained for this protein. Figure 3.32 summarises the percentage of FOXP3-positive cells in each fraction.

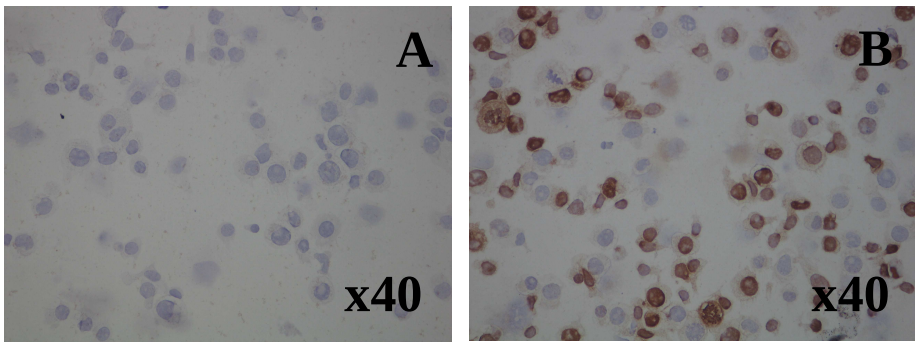

Figure 3.31: Immunohistochemistry for CD4+CD25+ T cell cytospins with PBS replacing the primary antibody alone (**A**) and FOXP3 (**B**).

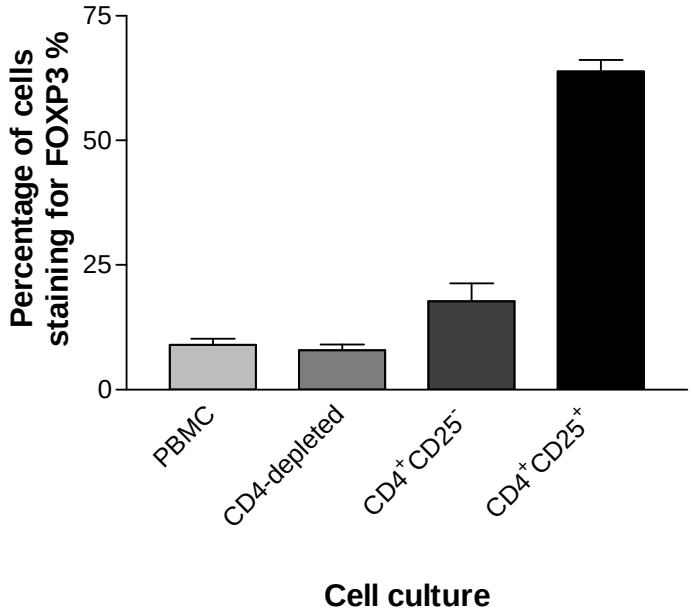

Figure 3.32: Percentage of FOXP3-positive cells in PBMC, CD4-depleted, CD4+CD25- T cells and CD4+CD25+ T cells stimulated with T cell expansion anti-CD28/anti-CD3 beads and 500IU/ml human recombinant interleukin-2 for 3 days. Bars indicate standard error.

Although when used in culture, the cell fractions were not stimulated in the same way, this experiment established that of all the cell fractions, $CD4^+CD25^+$ T cells contained the highest frequency of cells expressing FOXP3, providing further proof that the Treg isolation procedure did enrich $CD4^+CD25^+FOXP3^+$ T cells with regulatory function as shown in suppression assays.

FOXP3 staining in frozen lymph node and IM tonsil sections

Having established that the enriched cells expressed FOXP3, and the staining procedure was successful, expression of this transcriptional regulator by cells in IM tonsils (kindly provided by A. Akbar) was investigated. The IM tonsil sections available for staining

were frozen and as such required an alternative control to the previous staining of tonsil, which had been carried out on paraffin-embedded sections. Frozen lymph node sections were chosen since they were also available.

Negative controls consisted of PBS replacing either both primary and secondary antibodies or the primary alone. L26 was again used as a positive control. Figure 3.33 shows that no staining was observed in the negative control (PBS used in place of the primary antibody, figure 3.33, panel A & B) or when FOXP3 antibody was used (figure 3.33, panel C & D). The tissue morphology of the lymph nodes suggests that these frozen blocks have deteriorated during storage.

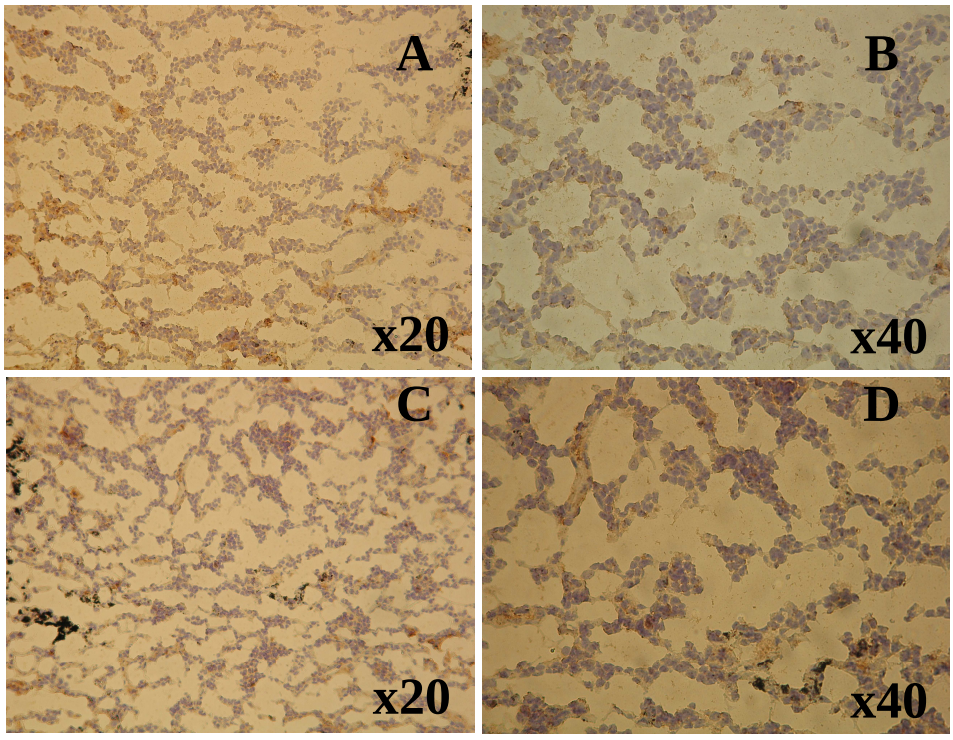

Figure 3.33: FOXP3 staining in healthy lymph node sections (**C** & **D**) with the primary antibody replaced with PBS as a control (**A** & **B**).

Figure 3.34 shows the staining results for IM59. No staining was apparent in either of the negative controls (panels A and B). The intact structure of the tonsil was clearly visible with a dense mass of cells in the follicular regions. Upon staining with L26 these follicular regions are strongly stained (panel C). FOXP3 positive cells were widespread and restricted to the extrafollicular areas of the tissue.

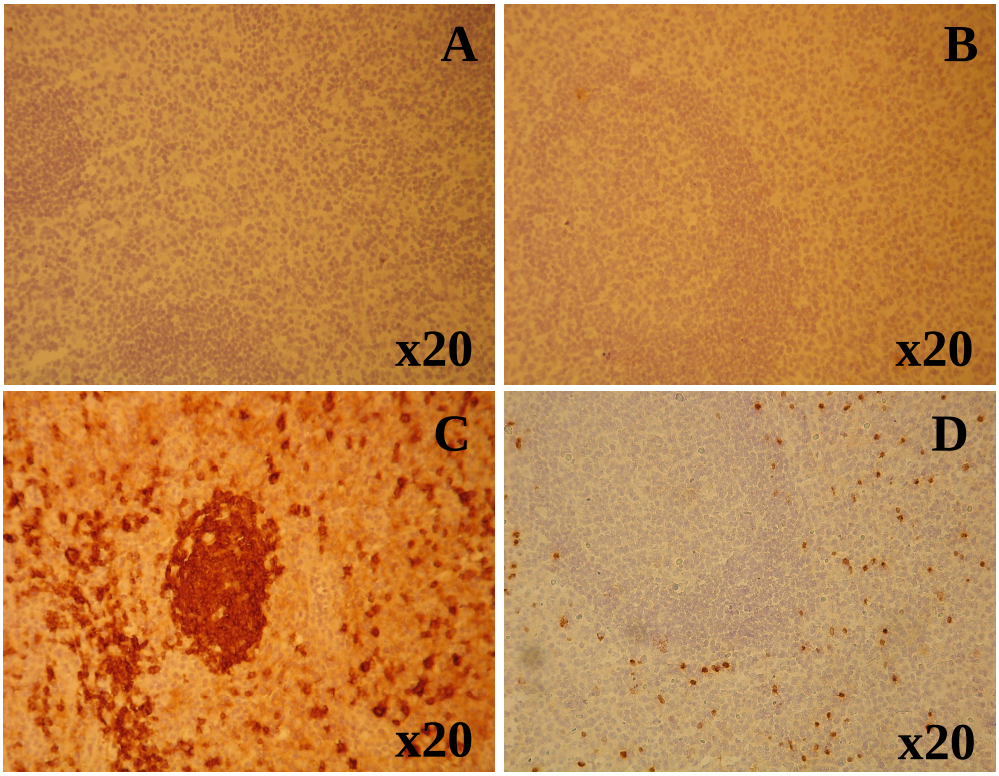

Figure 3.34: FOXP3 staining in IM tonsil sections from IM59 (**D**). Controls consisted of the primary and secondary antibodies replaced with PBS (**A**), PBS replacing the primary antibody only (**B**) and L26 (**C**).

Figure 3.35 shows the staining results for IM60. Again, the structure of the tonsil was intact and cells expressing FOXP3 were restricted to the extrafollicular areas of the tissue.

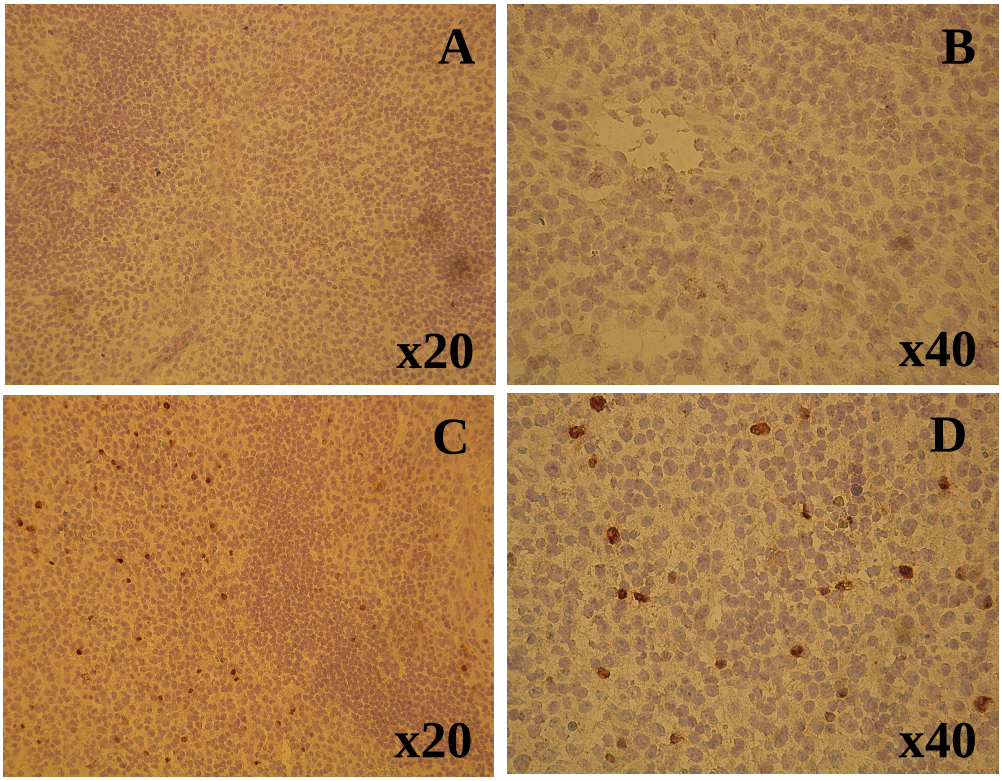

Figure 3.35: FOXP3 staining in IM tonsil sections from IM60 (C & D) with controls consisting of replacing the primary antibody with PBS (A & B).

Conclusions

FOXP3-positive cells are abundant scattered throughout the extrafollicular tissue of the tonsils from two IM patients. As a control, frozen lymph nodes were used although the tissue structure had degraded during storage. FOXP3-positive cells appear to be less abundant in paraffin-embedded tonsil sections from healthy controls, implying that cells expressing FOXP3 may be recruited to the primary site of infection during acute IM.

3.3 Cytotoxic T lymphocyte (CTL) growth

3.3.1 Introduction

Members of the CMV laboratory have previously been involved in the generation of a bank of CTL, which were subsequently used to treat patients suffering Post-Transplant Lymphoproliferative disease (PTLD) (Haque *et al*, 2007a). The growth of each of these cell lines was well documented and although the same procedure was used to develop each line, their ability to lyse target cells varied. This prompted the suggestion that regulatory T cells may persist in some lines and affect their level of cytotoxicity. To address this question, CTL lines were grown from 2 healthy seropositive donors (D35 and D36). T_{reg} were initially depleted from all PBMC on day 0, half the cultures had half the T_{reg} added back prior to stimulation, allowing the development of two cultures from each donor; CTL with T_{reg} and CTL without T_{reg}.

The dynamics of growth were assessed for each of the CTL lines. Figure 3.36 shows the absolute number of CTL at day 0 and henceforth for each stimulation (panel A) and fold-increase in CTL (panel B). All CTL increased in number until stimulation 7 or 8. Removal of T_{reg} at the start of culture had no affect on CTL number for either cell line (figure 3.36, panel A). Different numbers of PBMC were used to initiate cultures so fold-increase in cell numbers CTL was considered by dividing cell number at each stimulation by the starting number of PBMC (figure 3.36, panel B). However, this revealed no difference in growth dynamics either.

3.3.2 Growth curves

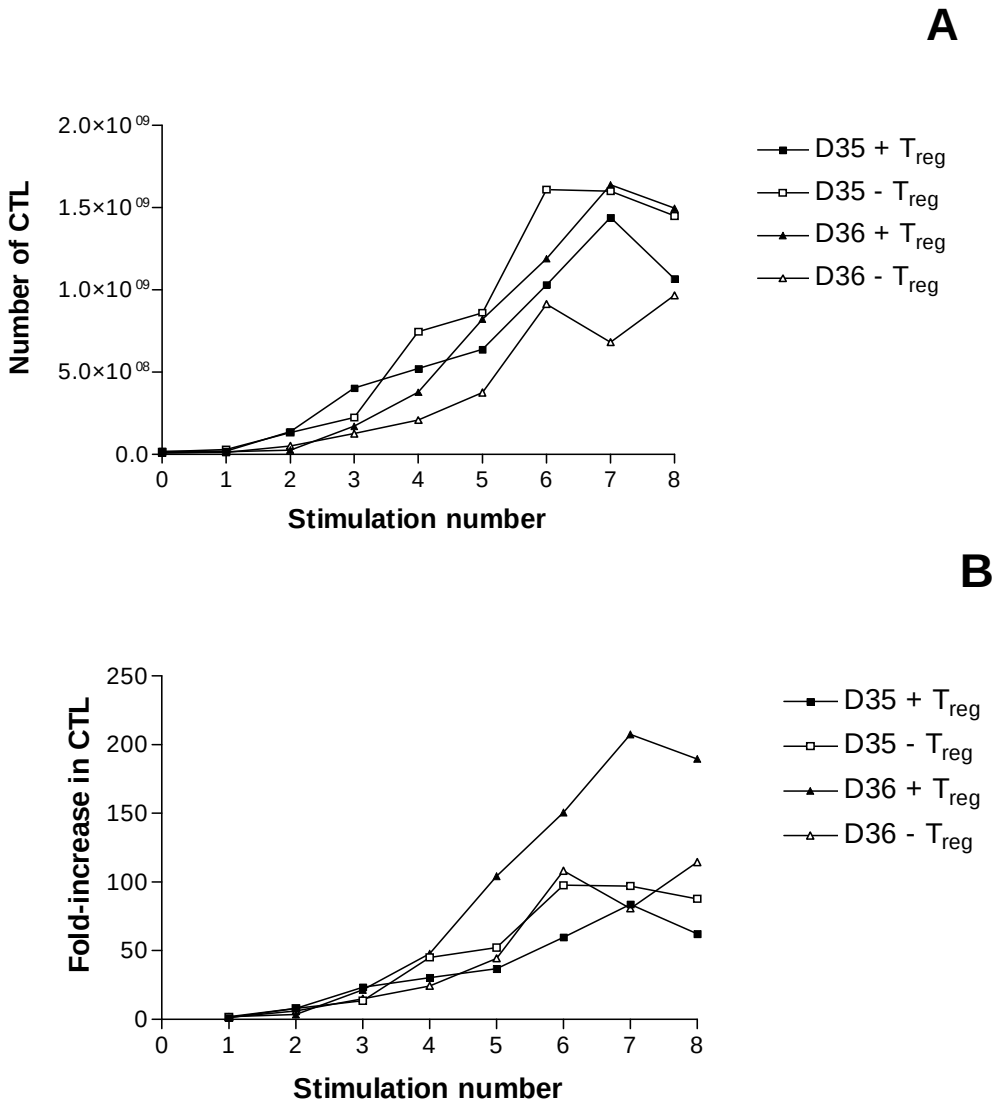

Figure 3.36: Growth of CTL developed from two healthy donors D35 (squares) and D36 (triangles) in the presence (closed symbols) or absence (open symbols) of T_{reg}. Figure shows absolute number of CTL (**A)** and fold-increase in CTL (**B**).

3.3.3 Regulatory T cell frequency

The frequency of T_{reg} was determined by flow cytometry at stimulation 4 and 8 for both CTL lines, in order to establish whether these regulatory cells had expanded in culture.

Figure 3.37 shows results for T_{reg} frequency in both CTL lines, with and without T_{reg}, at stimulation 4 and 8, within the lymphocyte population (panel A) and within the $CD4^+$ T cell population (panel B). At stimulation 4, the frequency of $CD4^+CD25^+$ T cells within the lymphocyte population is low within both T_{reg}-depleted and T_{reg}-repleted CTL lines for both donors (D35, 1.26% and 1.75%, respectively; D36, 0.14% and 0.17%, respectively). At stimulation 8, T_{reg} frequency is further reduced in both T_{reg}-depleted and T_{reg}-repleted CTL lines (D35, 0.15% and 0.01%, respectively; D36, 0.03% and 0.18%, respectively). In the D35 CTL cultured with T_{reg}, a moderately higher frequency of these cells is recorded at stimulation 4 (1.75%) although by stimulation 8, no $CD4^+CD25^+$ T cells can be found (0.01%). The $CD4^+CD25^+$ T cells remain at a low level in D36 CTL, irrespective of culture type and stimulation number (panel A).

Figure 3.37, panel B shows the equivalent T_{reg} frequencies within the $CD4^+$ T cell population. At stimulation 4, there is a high T_{reg} frequency within the D35 CTL lines, irrespective of whether $CD4^+CD25^+$ T cells were removed from or reintroduced to the original culture (28.8% and 28.8%, respectively). A reduced T_{reg} frequency was found in D36 CTL (without T_{reg} 5.3%; with T_{reg} 6.4%) in comparison with D35 at stimulation 4, again regardless of original culture status. At stimulation 8, a reduction in T_{reg} frequency is observed in all cultures with no particular pattern emerging as to which cultures contain the most (D35, with T_{reg} 0.3%, without T_{reg} 4.3%; D36, with T_{reg} 3.0%, without T_{reg} 0.7%).

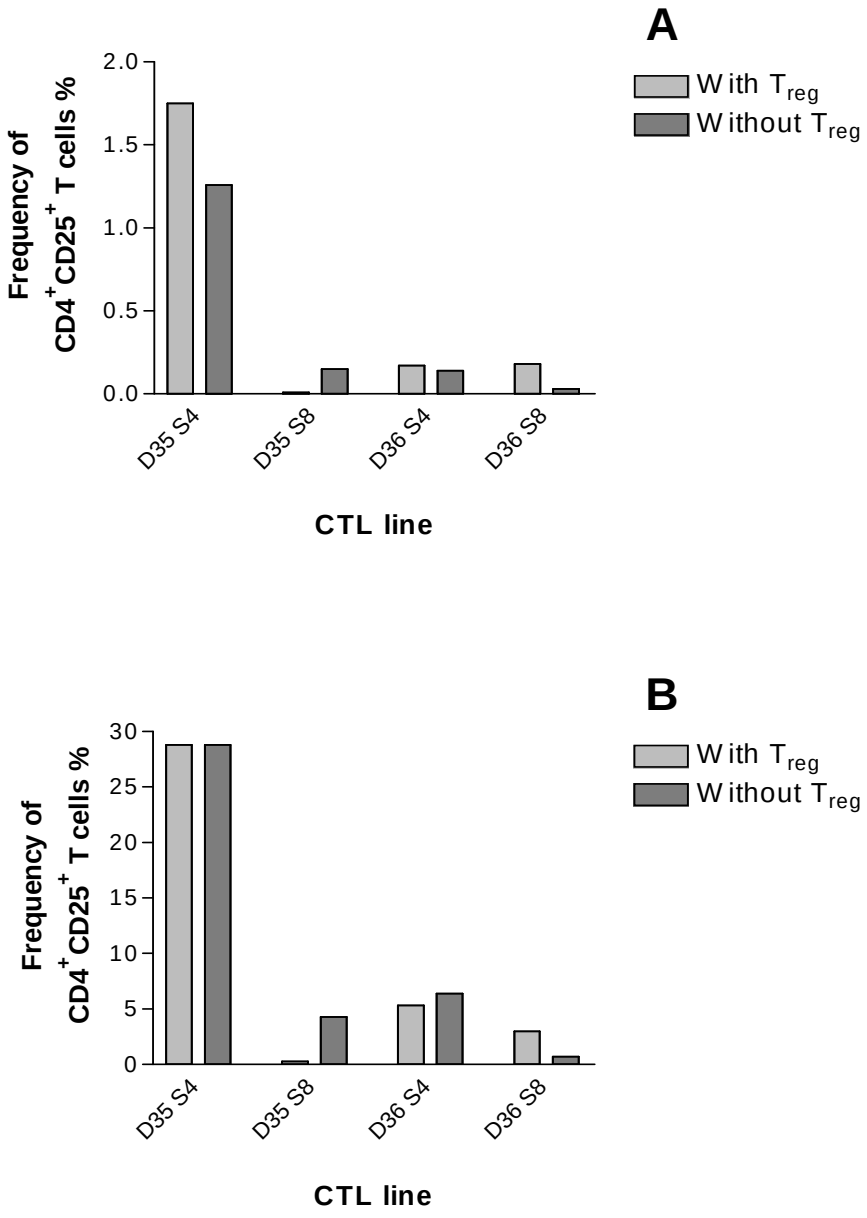

Figure 3.37: Frequency of $CD4^+CD25^+$ T cells shown in CTL developed from two healthy donors D35 and D36, in the presence (light grey) or absence (dark grey) of Treg, at week 4 (S4) and 8 (S8). Figure shows frequency of T_{reg} within the lymphocyte population (**A)** and within the $CD4^+$ T cell population (**B**).

Cytotoxicity

T_{reg} depletion did not affect growth of the CTL or $CD4^+CD25^+$ T cells frequency. To address their functional capacity, samples of CTL were taken at stimulations 4 and 8 and used in cytotoxicity assays against a panel of target cells: autologous LCL, mismatched LCL and K562, at ratios of 20:1, 10:1 and 5:1.

Figure 3.38 shows results for all CTL lines at stimulation 4. D35 CTL with T_{reg} exhibit a low level of specificity demonstrated by the equally high specific lysis of both autologous (41.2% at a 20:1 ratio) and mismatched LCL (51.9% at a 20:1 ratio). This is further evidenced by a particularly high level of killing when the targets were K562 cells (76.7% at a 20:1 ratio). D35 CTL lacking T_{reg} exhibit a similar level of killing for autologous LCL (48.5% at a 20:1 ratio), mismatched LCL (35.7% at a 20:1 ratio) and K562 (86.8% at a 20:1 ratio). D36 CTL with T_{reg} display high K562 killing (60.2% at a 20:1 ratio) as do CTL without T_{reg} (71.9% at a 20:1 ratio). In the presence of T_{reg}, CTL have high autologous LCL killing (47.7% at a 20:1 ratio) whereas absence of T_{reg} results in no visible lysis of the same target. Mismatched LCL killing is relatively low in both D36 CTL with T_{reg} (6.1% at a 20:1 ratio) and CTL without T_{reg} (8.5% at a 20:1 ratio).

Figure 3.39 shows results for all CTL lines at stimulation 8. A marginal increase in killing of autologous LCL (48.7% at a 20:1 ratio) was observed by D35 T_{reg}-repleted CTL at stimulation 8 in comparison with stimulation 4 (41.2% at a 20:1 ratio) whereas a reduction in lysis of mismatched LCL and K562 (7.8% and 26.6%, respectively, at a

20:1 ratio) was observed compared to stimulation 4 (51.9% and 76.7%, respectively, at a 20:1 ratio), highlighting the improvement in specificity by this line.

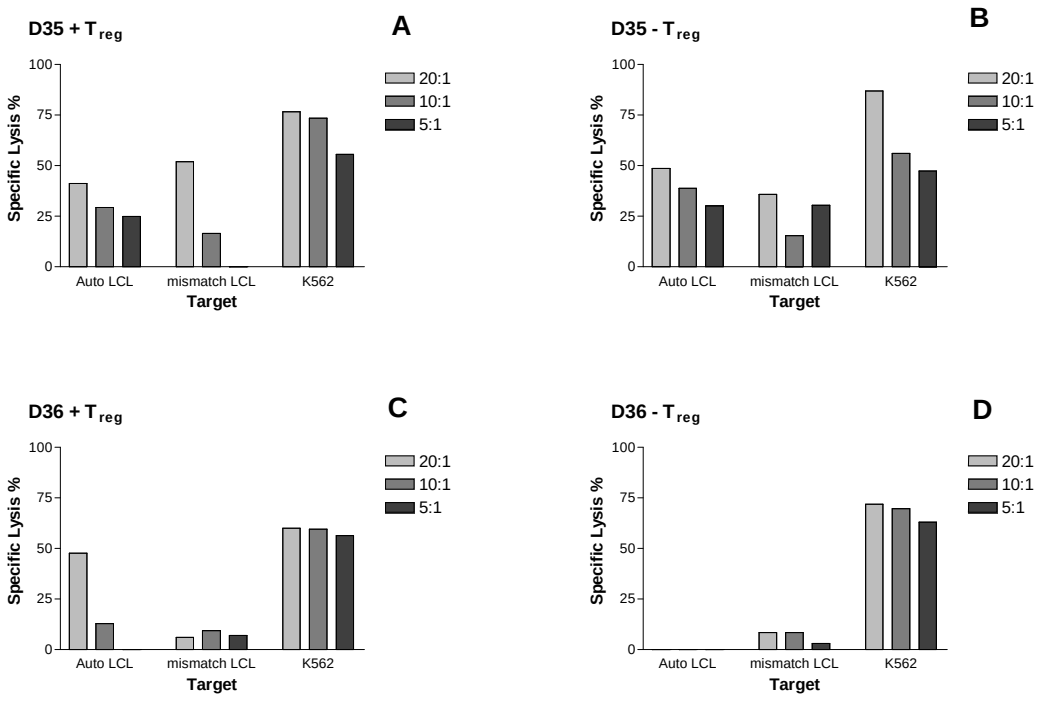

Figure 3.38: Cytotoxicity results for D35 (panels A & B) and D36 (panels C & D), in the presence (panels A & C) and absence (panels B & D) of T_{reg} at stimulation 4.

At stimulation 8, D35 T_{reg}-depleted CTL no improvement in lysis of autologous LCL was observed (48.1% at a 20:1 ratio) in comparison with the same culture at stimulation 4 (48.5% at a 20:1 ratio). However, there were reductions in specific lysis of both mismatched LCL (13.4% from 35.7%, at a 20:1 ratio) and K562 (25.4% from 86.8%, at a 20:1 ratio).

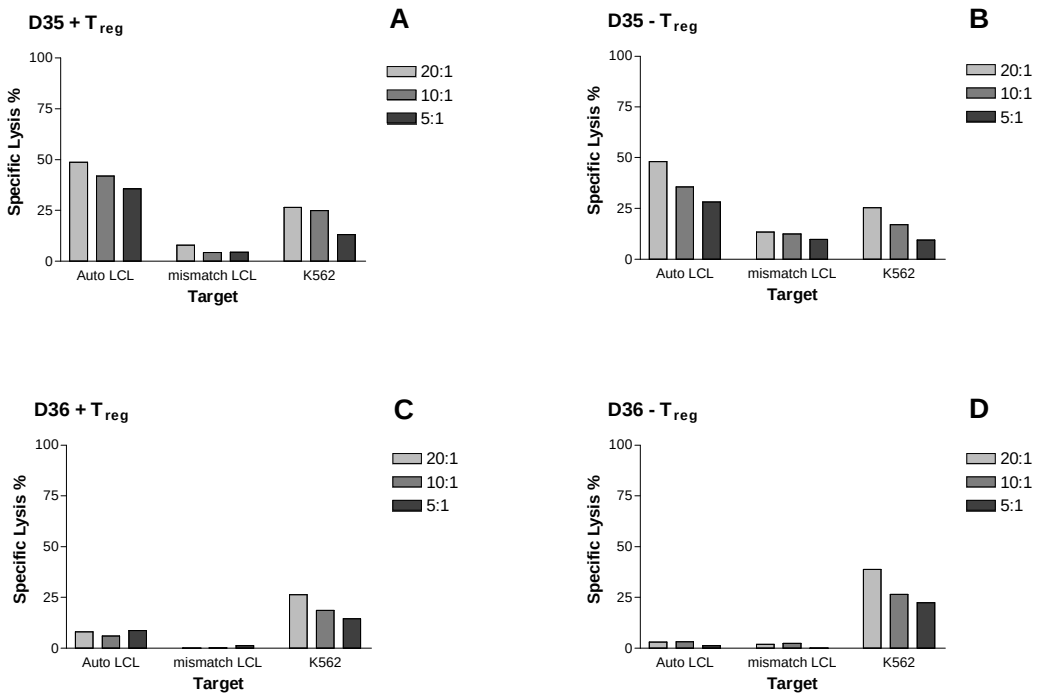

Figure 3.39: Cytotoxicity results for D35 (panels A & B) and D36 (panels C & D), in the presence (panels A & C) and absence (panels B & D) of T_{reg} at stimulation 8.

D36 T_{reg}-repleted CTL exhibited reduced specificity, demonstrated by a decrease in killing of autologous LCL at stimulation 8 (8.2% at a 20:1 ratio) in comparison with stimulation 4 (47.7% at a 20:1 ratio). Specific lysis of mismatched LCL was reduced at stimulation 8 (0% from 6.1%, at a 20:1 ratio) as was the killing of K562 (26.4% from 60.2%, at a 20:1 ratio). D36 T_{reg}-depleted CTL demonstrated slightly improved specific lysis of autologous LCL at stimulation 8 (3.0% at a 20:1 ratio) when compared to stimulation 4 (0%). Reductions in specific lysis were observed at stimulation 8 against mismatched LCL (2.0% from 8.5%, at a 20:1 ratio) and K562 (38.8% from 71.9%, at a 20:1 ratio).

3.3.4 Conclusions

Four CTL were developed from 2 healthy seropositive controls, one culture from each containing T_{reg}, the other depleted of these cells. These results show that in preliminary experiments, T_{reg} depletion has no effect on the development or function of CTL.

3.4 *In vivo* Experiments

3.4.1 Introduction

In order to assess the ability of T_{reg} to suppress EBV-driven proliferation *in vivo* we used the SCID mouse model set up in the CMV laboratory where injected PBMC from healthy seropositive donors results in EBV-driven tumours developing in approximately 50% of cases (Picchio *et al*, 1992b). In this study, PBMC, T_{reg}-depleted PBMC and T_{reg}-repleted PBMC were injected IP into SCID mice and tumour development and time to tumour were measured. Results are shown in figure 3.40.

3.4.2 Results

At day 50 post-injection, all groups of mice had high survival rates (90%), regardless of whether they were injected with unmanipulated PBMC, T_{reg}-depleted PBMC or T_{reg}-repleted PBMC. By day 75, mice injected with unmanipulated PBMC had a much poorer survival rate (35%) in comparison to T_{reg}-repleted mice (73%) and T_{reg}-depleted mice (73%). On day 100, the survival rate of mice injected with unmanipulated PBMC was further reduced at 27%, whereas the mice injected with T_{reg}-repleted PBMC remained at 73% survival and the third group, mice treated with T_{reg}-repleted PBMC

was slightly reduced with 62% survival, however, the differences observed did not significantly differ (p=0.213).

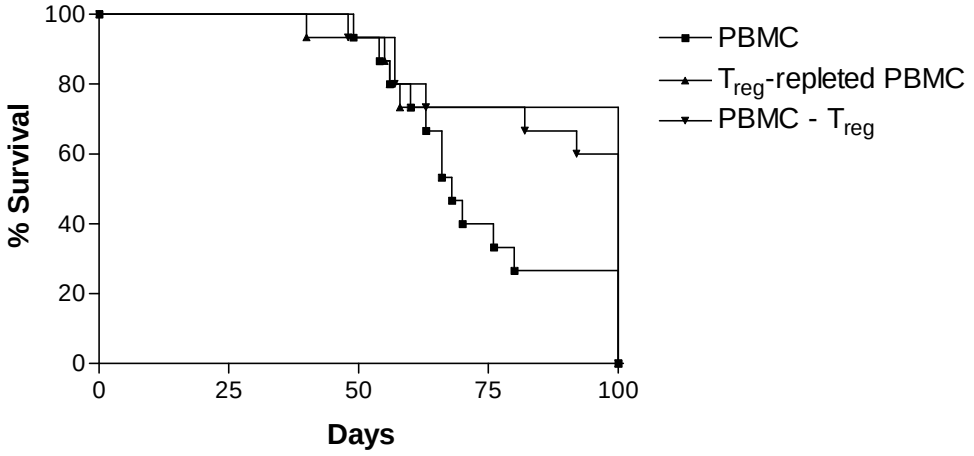

Figure 3.40: Survival curve for SCID mouse model where mice were injected with unmanipulated peripheral blood mononuclear cells (squares), peripheral blood mononuclear cells repleted with T_{reg} (upward triangles) or peripheral blood mononuclear cells depleted of T_{reg}. Mice were monitored for 100 days for tumour development.

3.4.3 Conclusions

The removal of T_{reg} from PBMC alters the time to tumour formation although not significantly, and to the equal extent of T_{reg}-repleted PBMC. This preliminary result probably indicates that the procedure by which the T_{reg} are removed has an effect on tumour formation, rather than the lack of a particular cell population.

3.5 LMP experiments

3.5.1 Introduction

Published data have demonstrated that stimulation of PBMC from healthy seropositive donors with LMP peptides resulted in the release of IL-10 by $CD4^+$ T cells. In addition, PBMC stimulated in this way suppressed IFN-γ production by and proliferation of cells to non-EBV antigens (Marshall *et al*, 2003e). Initially we set out to see if the experimental procedure resulted in similar results in our hands. We also wanted to assess whether PBMC from IM patients would react similarly.

3.5.2 Cytokine levels in healthy controls and IM patients

PBMC from 8 healthy seropositive donors and 7 IM patients were plated out and stimulated with PRG peptide, PHA, PPD, or a combination of PRG and PPD, for 5 days. The *Mycobacterium tuberculosis* antigen PPD was used because it should induce recall T cell responses in PBMC *in vitro* since the majority of UK citizens have been immunized with bacillus Calmette-Guérin. Samples were taken from triplicate wells and levels of IL-10 and IFN-γ were measured using an ELISA. Levels of cytokines found in unstimulated PBMC were subtracted from other readings.

<u>IL-10 production</u>

Treatment of PBMC from healthy seropositive donors (figure 3.41) with PRG resulted in a low level of IL-10 production (median 0pg/ml) with a range of 0 to 600pg/ml. Stimulation of PBMC with PHA (median 2073.5pg/ml, range 1178.4 to 3082.4pg/ml)

resulted in a very significant increase in IL-10 production in comparison with PRG (p=0.0002). PPD alone resulted in very low IL-10 production with a median of 5.88pg/ml and a range of 0 to 273.3pg/ml. Although marginally more IL-10 was produced by PBMC treated with PPD and PRG (median 41.8pg/ml, data range 0 to 275.3pg/ml), this was not significant (p=0.442).

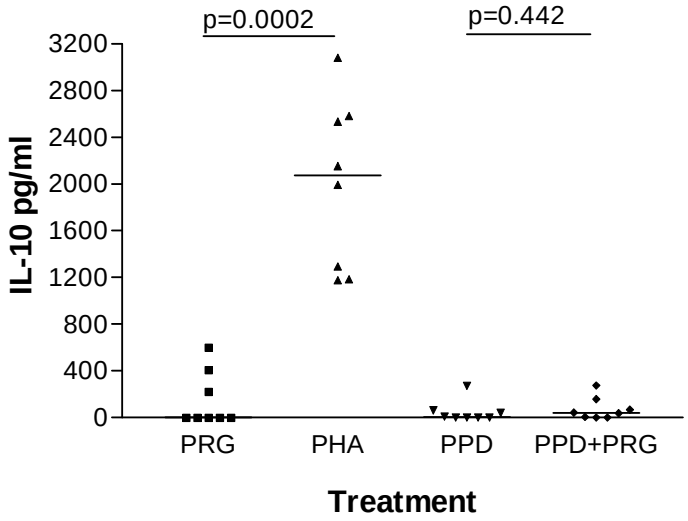

Figure 3.41: IL-10 concentration (pg/ml) produced by peripheral blood mononuclear cells from healthy seropositive donors in response to stimulation with PRG (squares), phytohaemagglutin (upward triangles), recall antigen PPD (downward triangles) and PRG + PPD (diamonds) for 5 days. Horizontal bars indicate median.

PBMC from IM patients (figure 3.42) treated with PRG resulted in a low level of IL-10 production in (median 0pg/ml) with a range of 0 to 415.7pg/ml. PHA stimulation of IM PBMC resulted in an increased level of IL-10 production in comparison to stimulation with PRG (median 89.0pg/ml, range 0 to 452.9pg/ml) although this was not significant (p=0.208). Treatment of IM PBMC with PPD alone resulted in low levels of IL-10 (median 20.4pg/ml, range 0 to 147.1pg/ml). The level of IL-10 was further reduced

when IM PBMC were treated with PPD and PRG (median 0pg/ml, data range 0 to 37.3pg/ml), although this was not found to be significant (p=0.259).

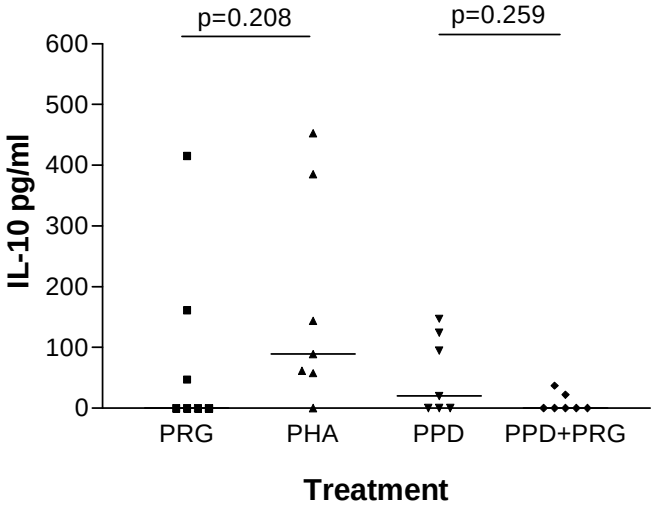

Figure 3.42: IL-10 concentration (pg/ml) produced by peripheral blood mononuclear cells from IM patients in response to stimulation with PRG (squares), phytohaemagglutin (upward triangles), recall antigen PPD (downward triangles) and PRG + PPD (diamonds) for 5 days. Horizontal bars indicate median.

On comparing the IL-10 response between healthy seropositive controls and IM patients, stimulation of PBMC with PRG resulted in a non-significant (p=0.955), equally low production of IL-10 (median 0pg/ml for both donor groups). Stimulation of PBMC with PHA resulted in a highly significant difference (p=0.0003) in IL-10 production, with healthy seropositive donors producing more IL-10 (median 2073.5pg/ml) in comparison IM patients (median 89.02pg/ml). PBMC from healthy seropositive donors produced lower levels of IL-10 (median 5.88pg/ml) in response to PPD stimulation than PBMC from IM patients (median 20.4pg/ml), although the difference was not significant (p=0.779). Conversely, IM patients PBMC produced less IL-10 (median 0pg/ml) in

response to treatment with PRG and PPD than healthy seropositive controls (median 41.2pg/ml) with borderline significance (p=0.054).

IFN-γ production

Results for the IFN-γ ELISA are shown in figure 3.43 and figure 3.44. PBMC from healthy seropositive donors stimulated with PRG resulted in a very low level of IFN-γ production (median 0pg/ml) with a range of 0 to 11.2pg/ml. Equally low levels of IFN-γ were produced by PBMC in response to PHA stimulation (median 0pg/ml, range 0 to 12pg/ml) with no significant difference observed when compared to stimulation with PRG (p=0.959). A high level of IFN-γ was produced by PBMC when stimulation with PPD alone with a median of 569.8pg/ml and a range of 0 to 1378.8pg/ml. This was somewhat reduced when PBMC were stimulated with a combination of PRG and PPD (median 470.4pg/ml, range 34 to 1407.2pg/ml), although the difference was not significant (p=0.959).

PRG stimulation of IM patient PBMC resulted in undetectable levels of IFN-γ (median 0pg/ml). Stimulation of PBMC with PHA, on the other hand, resulted in high levels of IFN-γ production (median 949.6pg/ml, range 0 to 1316.8pg/ml). A high level of IFN-γ was also produced by PBMC in response to PPD stimulation (median 479.2pg/ml, data range 0 to 1309.6pg/ml). This was significantly reduced (p=0.0262) when PBMC were stimulated with a combination of PRG and PPD (median 0pg/ml, range 0 to 465.6pg/ml).

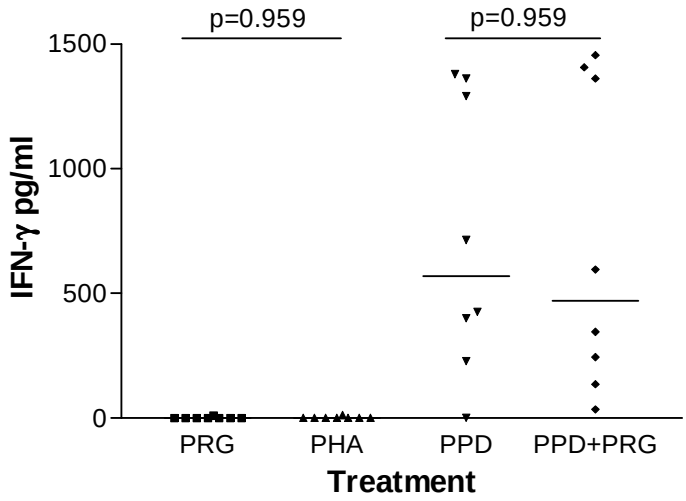

Figure 3.43: IFN-γ concentration (pg/ml) produced by peripheral blood mononuclear cells from healthy seropositive donors in response to stimulation with PRG (squares), phytohaemagglutin (upward triangles), recall antigen PPD (downward triangles) and PRG + PPD (diamonds) for 5 days. Horizontal bar indicates median.

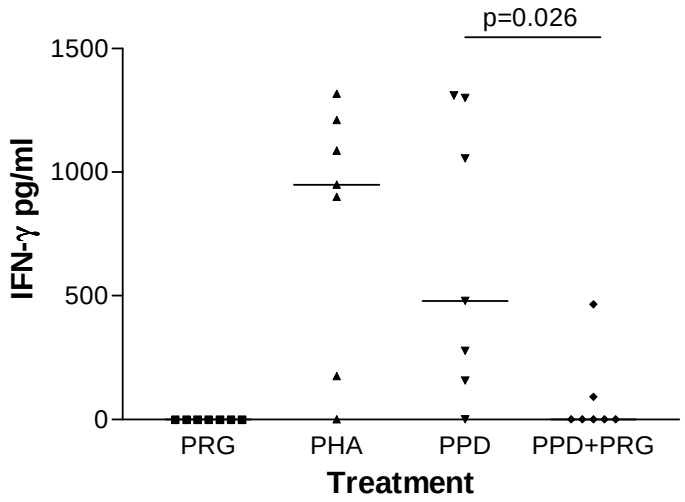

Figure 3.44: IFN-γ concentration (pg/ml) produced by peripheral blood mononuclear cells from patients with infectious mononucleosis in response to stimulation with PRG (squares), phytohaemagglutin (upward triangles), recall antigen PPD (downward triangles) and PRG + PPD (diamonds) for 5 days. Horizontal bar indicates median.

Comparison of the PBMC response from healthy seropositive donors and IM patients to stimulation with PRG reveals similarly low levels of IFN-γ production, the median being 0pg/ml for both groups. In response to PHA stimulation, PBMC from IM patients produced significantly higher (p=0.0037) levels of IFN-γ (median 949.6pg/ml) in comparison to PBMC from healthy seropositive donors (median 0pg/ml). Equally high levels (p=0.694) of IFN-γ were produced in response to PPD alone by PBMC from both healthy seropositive donors (median 569.8pg/ml) and IM patients (479.2pg/ml). When PPD and PRG were used in combination to stimulate PBMC, the IFN-γ response by healthy seropositive controls (median 470.4pg/ml) was significantly higher (p=0.006) than the response by IM patients (median 0pg/ml).

3.5.3 Cell proliferation in healthy controls and IM patients

PBMC from the same 8 healthy seropositive donors and 7 IM patients were plated out at a density of $1.25x10^6$ cells/ml in 1ml aliquots in a 24 well plate. PBMC were stimulated with 15µg/ml PRG, a LMP peptide, 10µg/ml PHA, 10µg/ml PPD, the recall antigen, or a combination of PRG and PPD, for 5 days. Proliferation of the PBMC in response to stimulation was established using the BrdU proliferation assay. Results for healthy seropositive controls and IM patients are shown in figure 3.45 and figure 3.46, respectively.

The proliferative response of healthy seropositive control PBMC to stimulation with PRG was low with a median absorbance of 0.146 and a range between 0 and 0.38. In

comparison, stimulation with PHA resulted in a significantly higher level (p=0.0499) of proliferation (median absorbance 1.34, range 0 to 2.6).

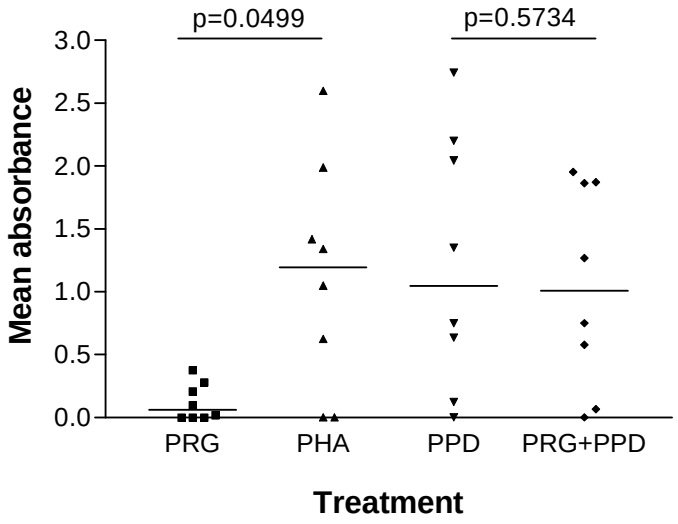

Figure 3.45: Proliferative response of PBMC from healthy seropositive controls to stimulation with 15µg/ml PRG, 10µg/ml PHA, 10µg/ml PPD or a combination of PRG and PPD. Cells were incubated for 5 days. Horizontal bars indicate median.

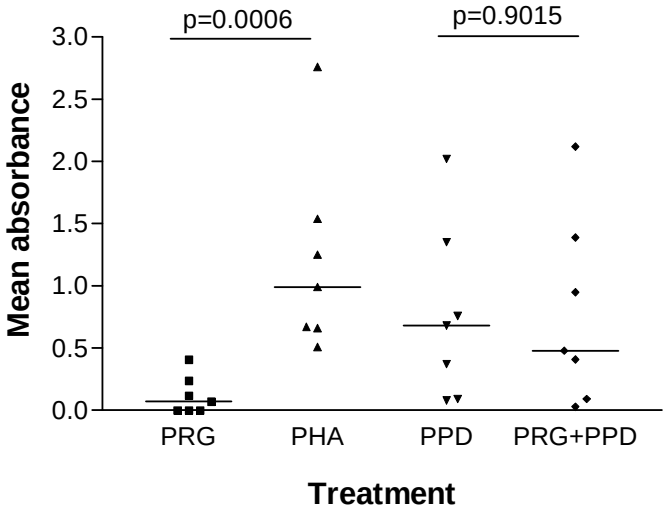

Figure 3.46: Proliferative response of PBMC from infectious mononucleosis patients to stimulation with 15µg/ml PRG, 10µg/ml PHA, 10µg/mlPPD or a combination of PRG and PPD. Cells were incubated for 5 days. Horizontal bars indicate median.

A similarly high level of proliferation was observed when PBMC were treated with PPD (median absorbance 1.05, range 0 to 2.74). Stimulation of PBMC with a combination of PPD and PRG slightly reduced the level of proliferation (median absorbance 1.01, range 0 to 1.95), although this was not significant (p=0.5734).

PBMC from IM patients proliferated poorly in response to stimulation with PRG (median absorbance 0.07, range 0 to 0.41). Stimulation with PHA resulted in a very significant increase (p=0.0006) in proliferation (median absorbance 0.99, range 0.51 to 2.76). PPD treatment resulted in a reduced level of proliferation in comparison to PHA with a median absorbance of 0.68 and range of between 0.08 and 2.02, however, this was not statistically significantly (p=0.383). A further reduction in proliferation was observed when PBMC were treated with a combination of PPD and PRG (median absorbance 0.48, range 0.03 to 2.12), although this was not significant (p=0.9015).

PBMC from both healthy seropositive controls and IM patients responded with low levels of proliferation to stimulation with PRG (median absorbance 0.1 and 0.07, respectively), and this was not found to be significantly different (p=0.9551). Stimulation with PHA resulted in a higher proliferative response by PBMC from healthy seropositive controls (median absorbance 1.34) in comparison to PBMC from IM patients (median absorbance 0.99), although the difference was not significant (p=0.9551). Treatment with PPD also induced higher levels of proliferation by PBMC from healthy seropositive controls (median absorbance 1.35) than IM patient PBMC

(median absorbance 0.68) but again, this observed difference was not significant (p=0.3969). Similarly, a higher level of proliferation, although not significant (p=0.6943), was observed by PBMC from healthy seropositive controls (median absorbance 1.27) than PBMC from IM patients (median absorbance 0.48) in response to combined treatment with PPD and PRG.

3.5.4 Conclusions

Stimulation of PBMCs from healthy seropositive controls and IM patients with the recall antigen PPD and LMP peptide PRG did not significantly alter cell proliferation or IL-10 production when compared to PPD stimulation alone. However, in the same cultures IFN-γ production was significantly decreased in IM cases, and reduced, but not significantly so, in cultures from controls. With the exception of IFN-γ production from healthy controls, PHA significantly induced responses (IL-10 production and proliferation) which were greater in controls than IM cases.

4 Discussion

4.1 Role of Regulatory T cells

Regulatory T cell activity has been determined to affect the outcome of infection with a number of persistent viruses. The role these suppressive cells play can be beneficial to the host. Suvas *et al* were investigating the role of T_{reg} in a mouse model of herpes simplex-induced stromal keratitis, an immunopathological disease mediated by $CD4^+$ T cells. Their results showed that the immunopathology was markedly increased in T_{reg}-depleted animals, with increased lesion severity and a lower dose of virus was required to cause disease (Suvas *et al*, 2004a). However, T_{reg} can also suppress T cell responses to viral antigens, as demonstrated by *in vitro* experiments with HCV antigens. The coculture of $CD4^+CD25^+$ regulatory T cells with $CD8^+$ T cells isolated from chronic HCV patients resulted in a reduced proliferative and IFN-γ response to HCV peptides as measured by flow cytometry. Furthermore, depletion of T_{reg} cells from PBMC increased the frequency of HCV-peptide tetramer binding, a result that was reversed by the addition of T_{reg} at 1:2 and 1:10 ratios (Rushbrook *et al*, 2005f). These two publications indicate that T_{reg} activity influences viral immunopathology and persistence.

The severity of primary EBV infection varies from subclinical seroconversion, with no apparent symptoms, to mild, severe or occasionally, fatal infectious mononucleosis (Wick *et al*, 2002b). The symptoms of IM, including fever, lymphadenopathy, sore throat and fatigue, are thought to be caused by cytokines including TNF-α, TNF-β, IL-6

and IL-1β, produced by the over-whelming T cell response to EBV antigens (Callan *et al*, 1996;Foss *et al*, 1994c). It is therefore possible that T_{reg} are activated and play a role in the eventual subsidence of the $CD8^+$ T cell response to EBV. In this regard, a number of recent publications have investigated T_{reg} responses to EBV antigens. Stimulation of PBMC from healthy seropositive individuals *in vitro* with LMP-1 results in the suppression of IFN-γ production, and cell proliferation by a population of cells expressing CD4, via an IL-10 mediated mechanism (Marshall *et al*, 2003d). In addition, Voo *et al* demonstrated the *in vitro* outgrowth of T cell clones with suppressive activity when PBMC were stimulated with an EBNA-1 peptide that carried out their regulatory activity via a cell-cell contact mediated route (Voo *et al*, 2005e). To date one study has demonstrated T_{reg} activity after IM in 4 subjects (Marshall *et al*, 2007). We wanted to expand on this work in an attempt to elucidate what role T_{reg} play in primary infection with EBV in this disease setting.

The mechanisms by which T_{reg} suppress cell responses is still the subject of intense scrutiny. Currently two major regulatory T cell subgroups have been demonstrated that primarily mediate their activity via cytokine release; IL-10-producing Tr1 cells and Th3 cells that secrete TGF-β_1. Hence the level of these cytokines was investigated in IM patient plasma to provide indirect proof for the presence of either of these regulatory T cell populations.

IL-10 was found to be significantly elevated in patients who had experienced IM symptoms for 1 week or less (median 71.8pg/ml) in comparison to healthy seropositive controls (median 11.3pg/ml). When symptoms had persisted over a week, although marginally reduced (median 48.4pg/ml), IL-10 levels were still significantly higher than healthy seropositive controls. TGF-β_1 levels in patients who had suffered symptoms for a week or less (median 18ng/ml) were also significantly higher than healthy seropositive controls (median 9.3ng/ml) and this statistical significance increased further when symptoms had been endured for over a week (median 26.9ng/ml).

The high levels of both IL-10 and TGF-β during the first week of symptoms imply, but do not prove, the presence of T_{reg}. Over a week of symptoms resulted in a reduction of IL-10, possibly indicating a reduced presence of IL-10-producing T_{reg}, whereas the increased levels of TGF-β_1 suggests cells secreting this cytokine, perhaps of the Th3 regulatory lineage, become more abundant as disease progresses. However, a number of different cell types are known to produce IL-10 and TGF-β_1, including macrophages, monocytes and $CD8^+$ T cells when activated. Thus to provide firm proof of the cytokine origin, the identity of the cells producing these soluble mediators in IM must be established via intracellular staining and flow cytometry.

Nevertheless, evidence supporting a link between the presence of IL-10 and TGF-β_1 and T_{reg} presence in persistent virus infections does exist. Cabrera *et al* demonstrated *in vitro* IL-10 release by $CD4^+CD25^+$ T_{reg} from in response to HCV antigens in chronically

infected patients (Cabrera *et al*, 2004d), Weiss *et al* demonstrated both IL-10 and TGF-β_1 release by $CD4^+CD25^+$ T_{reg} in response to the HIV antigen, p24, in HIV-infected patients undergoing HAART therapy (Weiss *et al*, 2004d) and Marshall *et al* identified IL-10-producing cells in response to stimulation with the EBV antigen, LMP-1, as CD4-expressing T cells (Marshall *et al*, 2004c). In addition, since it is known that EBV encodes a viral homologue of IL-10 (Hsu *et al*, 1990a), it is possible that this could induce T_{reg} at the site of primary infection.

Natural human T_{reg} are identified as $CD4^+CD25^{high}$ T cells, hence investigating the role of T_{reg} in IM was complicated by the high expression of CD25 on non-regulatory, activated $CD4^+$ T cells as well as $CD8^+$ T cells circulating during early infection (Tomkinson *et al*, 1987). In an attempt to overcome this issue when investigating the frequency of T_{reg} in the peripheral blood of IM patients, cells were gated using expression of $CD4^+$ and very high levels of CD25, in the hope that this would represent the constitutively high CD25 expressers and thus T_{reg}.

$CD4^+CD25^{high}$ T cell frequency in IM patients was investigated to establish if there was a difference in cell numbers when compared to healthy virus carriers and to provide further proof that these regulatory cells might influence the outcome of primary infection with EBV. By flow cytometry, $CD4^+CD25^{high}$ T cells were found at low frequency (median 0.29%) in the lymphocyte population of healthy controls, similar to the previous studies (1-3%, Boettler *et al*, 2005c;Cabrera *et al*, 2004c;Baecher-Allan *et*

al, 2001b). Slight differences in this frequency are likely to reflect differences in the gating strategy during flow cytometry. However, since the same gating strategy was applied to both IM and healthy seropositive controls in this study, this was deemed a valid comparison. The frequency of $CD4^{+}CD25^{high}$ T cells within the lymphocyte population was significantly lower in IM patients (median 0.04%) when compared to healthy seropositive controls. This is likely to have arisen because the total number of $CD8^{+}$ T cells increases dramatically in acute IM (lymphocyte counts of up to $15x10^{9}$/litre) hence apparently reducing the $CD4^{+}$ T cell count. Thus the percentage of $CD25^{high}$-expressing cells within the $CD4^{+}$ T cell population was compared since this population of cells, although activated, do not expand in acute IM (Williams *et al*, 2005). The frequency of $CD25^{high}$-expressing cells within the $CD4^{+}$ T cells in IM patients (median 0.56%) was reduced in comparison to healthy seropositive controls (median 1.94%) with borderline significance ($p=0.05$); hence T_{reg} are present but may be depleted in the blood of acute IM patients.

Previous studies looking at roles for human T_{reg} in persistent viral infections have found differences in the frequency of these regulatory cells in the peripheral blood of infected individuals when compared with healthy, uninfected controls. The frequency of $CD4^{+}CD25^{+}$ T_{reg} was found to be elevated in chronic HCV infection in comparison to recovered and normal controls (Cabrera *et al*, 2004b;Boettler *et al*, 2005b). Similarly, Levy *et al* determined that $CD4^{+}CD25^{+}$ T cells were expanded in HIV patients undergoing HAART (Levy *et al*, 2003). Weiss *et al* demonstrated these cells were

regulatory and suggested that persistence of HIV antigens might trigger their expansion (Weiss *et al*, 2004e). Furthermore, Peng *et al* demonstrated higher frequencies of T_{reg} in chronic HBV patients in comparison with healthy controls and acute HBV patients (Peng *et al*, 2000). Our data is consistent with this, since we found a significantly higher frequency of $CD4^{+}CD25^{+}$ T cells in healthy EBV seropositive individuals (i.e. chronically infected subjects), in comparison acute IM patients. However, it is important to note that these previous studies only investigated the presence of T_{reg} in peripheral blood of patients, and the majority did not detect alterations in T_{reg} frequency when healthy controls were compared with patients with acute infections.

Since the majority of studies evaluating T_{reg} frequency in persistent viral infections indicate that a higher frequency of this population of cells is associated with chronic disease, the investigation of T_{reg} frequency in CAEBV could reveal that higher numbers of T_{reg} are associated with disease duration and poor prognosis. In order to determine this, a larger pool of IM patients would be required, with disease monitoring over a long period of time.

The reduced number of T_{reg} in IM patients may indicate a redistribution of these cells within the body, as shown in studies on patients with ovarian carcinoma (Curiel *et al*, 2004c). Using flow cytometry, Curiel *at al* found T cells expressing CD4 and CD25 at higher frequencies in malignant ascites of patients with untreated ovarian epithelial cancers, particularly at later stages of disease, in comparison to non-malignant ascites,

indicating the accumulation of cells was tumour-related. This finding was confirmed by multicolour confocal microscopy. In addition, they demonstrated high levels of FOXP3 in ovarian tumour tissue but not in normal ovarian tissue. Furthermore, on examining locally draining lymph nodes, fewer $CD4^+CD25^+$ T cells were found in ovarian cancer patients in comparison to normal controls, a fact the authors attributed to preferential recruitment of these cells to the tumour in the cancer patients, rather than the lymph node. The functional activity of $CD4^+CD25^+$ T cells isolated from tumour ascites confirmed their regulatory activity by their ability to suppress cell proliferation of, and IFN-γ production by, $CD3^+CD25^-$ T cells in coculture (Curiel *et al*, 2004b).

Consistent with Curiel's study, studies in our laboratory indicated that FOXP3-positive cells appeared more abundant in IM tonsil (n=2) compared with healthy control sections, although care must be taken with this interpretation since normal control tonsil tissue was paraffin-embedded whereas the IM tonsil tissue were frozen sections. In order to confirm these preliminary results, tissue sections should be subjected to the same procedure and FOXP3-positive cells counted. However, this result does imply that FOXP3-positive cells may be recruited to the site of primary infection in acute IM patients. In future additional markers, including LAG-3, CTLA-4 and GITR, could also be used to aid the identification of T_{reg}.

In human peripheral blood, $CD4^+CD25^{high}$ T cells are found at low frequency (median 0.29%), limiting the type of experiments that can be carried out. These cells were

therefore enriched using a T_{reg} enrichment kit. The mean percentage purity of $CD4^{+}CD25^{high}$ T cells isolated from 5 healthy seropositive donors was 79.18%, consistent with other published results (Rushbrook *et al*, 2005e;Boettler *et al*, 2005a). Thus the procedure used resulted in an enriched population of T_{reg} rather then a totally pure population. The purity of the T_{reg} population could be improved with repeated washing and multiple column runs, however, this resulted in fewer T_{reg} with poor viability, reducing further experimentation. Due to ethical restrictions in the size of blood samples obtained (20ml) T_{reg} enrichment could not be carried out on blood from IM patients.

In order to provide proof that the enriched $CD4^{+}CD25^{high}$ T cells had regulatory activity, by virtue of their ability to suppress anti-CD3-induced proliferation of other T cells in coculture, suppression assays were set up. The first method employed to measure cell proliferation was the MTT assay which was determined to be too insensitive (maximum mean absorbance 0.75 with LCL). Subsequently the BrdU assay was attempted, resulting in an increased maximum mean absorbance of 2.0 with LCL, and this assay was chosen for all future proliferation assays. Initially PHA stimulation was optimised, since this is a polyclonal activator of T cells and was used as a positive control. 10µg/ml resulted in the highest level of proliferation and as such this concentration was used in subsequent experiments.

Having established the conditions under which PHA induced maximum proliferation in PBMC, the next step was to establish the conditions under which PBMC proliferated when treated with anti-CD3. An incubation period of 96 hours and stimulation with 5µg/ml anti-CD3 was chosen and suppression assays were set up using PBMC from 3 healthy seropositive donors in order to determine if the T_{reg} isolated using the enrichment procedure were functional. PBMC from all 3 controls proliferated well in response to anti-CD3 stimulation (median absorbance 2.82) and in coculture, $CD4^+CD25^+$ T cells suppressed the proliferative response of $CD4^+CD25^-$ T cells (median absorbance 0.41), indicating that the enriched T_{reg} were functional. However, it must be noted that the reduced proliferative response of $CD4^+CD25^-$ T cells in coculture could be due to the dilution of the cells themselves. In order to establish if this is the case, $CD4^+CD25^-$ T cells should be set up alone at densities equal to those that are added to the coculture.

The results presented in this current study are consistent with previously published experiments used to provide proof of T_{reg} activity in similar experimental set ups (Hoffmann *et al*, 2004;Dieckmann *et al*, 2002;Curiel *et al*, 2004a). Baecher-Allan *et al* (2002) could not detect suppression in a similar assay although this may have been due to a reduced ratio of responder:regulatory cells (1:1/2) and a lower concentration of anti-CD3 (2.5µg/ml). In future, more samples would be used so that statistical analysis could be carried out, and ideally this experiment should be carried out on PBMC from IM patients in order to establish that T_{reg} are functional in these individuals.

The numbers of markers associated with T_{reg} is still growing although an exclusive marker is yet to be found. To date, the most specific marker remains FOXP3, a transcription factor essential for T_{reg} development (reviewed in Campbell & Ziegler, 2007c;Kim & Rudensky, 2006;Sakaguchi *et al*, 2006). Hence to ensure that cells of this phenotype had been enriched in the $CD4^+CD25^+$ T cell enrichment procedure, staining for FOXP3 was carried out on cells from healthy seropositive donors.

Staining for FOXP3 was optimised using paraffin-embedded tonsil sections in collaboration with Dr Alison Banham and Dr Bridget Fox. FOXP3-positive cells were apparent scattered throughout the tissue sections with no obvious histological pattern (figure 3.23, panel C). PBMC from 3 healthy seropositive controls were then separated into PBMC, CD4-depleted cells, $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells and each population was stained for FOXP3. Initially, high levels of non-specific staining made it difficult to identify FOXP3-positive cells. This is likely to have been due to high levels of cell lysis and cell debris in the samples as a direct result of the long separation procedure using multiple centrifugation steps. Non-specific staining may also have arisen from PBMC engulfing the CD25 microbeads, used for selecting the cells, and then binding by the anti-mouse secondary antibody.

An alternative FOXP3 staining method was employed based on advice from Dr Bridget Fox, involving the use of T cell expander beads and high levels of IL-2, to which each of the populations of cells was subjected. The aim of this procedure was to determine

which population of cells, when activated, contained the highest percentage of FOXP3-expressing cells and this would imply which fraction contained the highest frequency of T_{reg} upon activation.

The results showed a low level of FOXP3-positive cells in unseparated PBMC (9%), which would be expected since T_{reg} are known to be at low frequency in this population of cells. This was enriched to >50% in the $CD4^+CD25^+$ T cell fraction. However, a low level of FOXP3-positive cells was detected in CD4-depleted cells (7.9%) and a higher level was found in the $CD4^+CD25^-$ T cell population (17.7%), indicating that either the sample was contaminated with remaining $CD4^+CD25^+$ T cells or the induction of FOXP3 in other cell subsets.

The exclusivity of FOXP3 expression to the $CD4^+CD25^+$ T cell population has been a matter of debate and the results in this current study, consistent with previous publications (Wang *et al*, 2007;Gavin *et al*, 2006b), suggest that other populations of cells can express FOXP3 under certain conditions. $CD8^+$ T cells have been shown to express FOXP3, possibly accounting for the results seen in the CD4-depleted cell fraction in this study (Gavin *et al*, 2006a). Gavin *et al* demonstrated that FOXP3 expression can be induced in $CD4^+CD25^-$ T cells and $CD8^+$ T cells after 3 days of stimulation with 5, 100 or 1000ng/ml anti-CD3 (Gavin *et al*, 2006c). These results demonstrate that although the enrichment procedure on PBMC from healthy seropositive controls results in $CD4^+CD25^+FOXP3^+$ T cells with regulatory activity, FOXP3

expression is not restricted to this population and can be induced in other cell populations, such as $CD4^+CD25^-$ T cells and $CD8^+$ T cells. Whether this is directly due to the addition of T cell expander beads and IL-2 on PBMC or indirectly via the activity of any remaining $CD4^+CD25^+$ T cells on the surrounding cells leading to the induction of FOXP3 expression is unclear. To establish if the latter event is responsible, the purity $CD4^+CD25^+$ T cell could be improved by repeating the enrichment procedure.

Furthermore, the suppressive potential of the cell populations should be investigated by running suppression assays on cells activated by this procedure, which would also determine a link between FOXP3 expression and functional capacity. Apart from CD25, a variety of markers have been linked with human T_{reg}, including GITR (Shimizu *et al*, 2002b;McHugh *et al*, 2002c), CTLA-4 (Read *et al*, 2000a), and LAG-3 (Huang *et al*, 2004a). Staining for these markers in future experiments would be of interest, especially since a role for T_{reg} expressing LAG-3 but not FOXP3, has been demonstrated in the EBV-associated malignancy, Hodgkin's Lymphoma (Gandhi *et al*, 2006b). In future it would be advisable to use an alternative control to L26, the B cell antibody, to stain $CD4^+CD25^-$ T cell and $CD4^+CD25^+$ T cell populations since these fractions should not contain B cells and hence L26 is not an ideal control antibody.

To carry out functional assays on IM samples, the initial intention was to keep IM PBMC in liquid nitrogen whilst autologous LCL were grown, then PBMC would be revived and could be stimulated using the LCL. However, poor revival of PBMC from

IM patients after freezing meant that too few cells were available to perform the experiment. This cell death is a well established problem in IM, due to the highly activated state of IM $CD8^+$ T cells resulting in rapid apoptosis in culture (Moss *et al*, 1985;Uehara *et al*, 1992). To overcome this problem, an experimental design based on peptide stimulation was chosen since this could be carried out immediately without the need for PBMC storage. In order to determine whether T_{reg} activity affected the T cell response to EBV in IM, PBMC from healthy seropositive controls and IM patients were separated into cultures containing $CD4^+CD25^+$ T cells and cultures lacking $CD4^+CD25^+$ T cells, stimulated with peptides, and the IFN-γ-secreting cells measured by ELISPOT. Peptides restricted to HLA A2 were selected since 40-50% of the population worldwide express this HLA type (Mahdavi & Monk, 2005b;Botten *et al*, 2007a). HLA A2 staining was carried out on cells from each individual IM patient and if found to be positive, the ELISPOT procedure was continued.

Once the experimental conditions were established, PBMC populations, containing or lacking $CD4^+CD25^+$ T cells, were stimulated with 4 different peptides derived from EBV antigens; QAK, an EBNA-3A non-HLA A2 control peptide, SVR, an EBNA-3A peptide, LLD, an EBNA-3C peptide and GLC, a BMFL1 (early lytic antigen) peptide. PHA was used as a positive control whereas untreated PBMC provided a background IFN-γ level.

In healthy seropositive donors, low IFN-γ responses were observed in cultures with and without T_{reg} in response to the non-A2 peptide (median SFC/million PBMC 5 and 5.8, respectively) and no stimulation (median SFC/million PBMC 13.4 and 15, respectively), setting a low background level. A strong IFN-γ response was observed when PBMC with and without T_{reg} were treated with PHA (median SFC/million PBMC 793.4 and 745.9, respectively). A low IFN-γ response was also observed in cultures both with and without T_{reg} in response stimulation to SVR (median SFC/million PBMC 8.35 and 9.2, respectively) and LLD (median SFC/million PBMC 25 and 13.3, respectively) whereas a high level of IFN-γ-secreting cells was recorded in response to GLC (median SFC/million PBMC with T_{reg}; 45.85 and without T_{reg}; 21.65).

EBV in healthy virus-carriers is generally latent and hence, if these individuals did respond to peptide stimulation, they might be expected to react more strongly to the latent peptides, SVR and LLD, than the lytic peptide, GLC. However, Rushbrook *et al* demonstrated a similar level of IFN-γ production in response to GLC in HCV-infected (EBV seropositive) individuals (Rushbrook *et al*, 2005d), as in the present experiments. In another similar study looking at the response of PBMC from healthy seropositive controls to GLC, the authors also demonstrated a response, with an average SFC/million PBMC of 276, although the range of data collected from 11 HLA A2 donors was broad; 13 to 943 SFC/million PBMC (Yang *et al*, 2000).

One explanation for this response to a lytic peptide lies with the fact that EBV reactivates periodically, during which lytic antigens are expressed, hence the high level of IFN-γ produced in response to GLC maybe indicative of lytic viral activity in these individuals. In support of this theory, Tan *et al* found high frequencies of $CD8^+$ T cells in healthy virus carriers binding to lytic epitopes in comparison to latent peptides by tetramer analysis (Tan *et al*, 1999a). Alternatively, the latent peptides used in our analysis were limited to one sequence from each of the EBV antigens and it is plausible that individuals did not possess T cells specific for this particular sequence but may possess T cells specific for a different, as yet unknown epitope from the same antigen.

Overall, no significant differences were observed when PBMC cultures with T_{reg} and PBMC without T_{reg} were compared. There are several possible explanations for these results. It may be that too few T_{reg} were added to the cultures to have a measurable effect. Alternatively, the separation procedure may not have been pure enough, hence some T_{reg} would remain in the depleted cultures. Rushbrook *et al* showed that a ratio of 1 T_{reg} to 10 PBMC resulted in a reduced expansion of $CD8^+$ T cells in HCV-infected patients in response to HCV peptides (Rushbrook *et al*, 2005c), in the current study the ratio was reduced (approximately 1:50-1:80). It is also probable that T_{reg} would have to be activated in order to mediate suppression, and perhaps HLA class I-restricted peptides would not achieve this. In order to investigate this, it would be interesting to carry out the same experiment using HLA class II-restricted peptides since the T_{reg} under investigation in the current study express $CD4^+$. Rushbrook *et al* demonstrated no

difference in $CD8^+$ T cell expansion in response to GLC when comparing cultures containing T_{reg} and those lacking T_{reg} in healthy controls via tetramer analysis. These authors went on to use the same experimental design as the current study and showed that removal of T_{reg} from PBMC from chronic HCV patients did enhance IFN-γ release by $CD8^+$ T cells in response to HCV antigens and GLC, indicating that MHC class II are not required to activate T_{reg} (Rushbrook *et al*, 2005b). Using HCV antigens, Cabrera *et al* showed elevated responses to HLA A2-restricted HCV peptides when T_{reg} were depleted from PBMC and suppressed IFN-γ responses when additional T_{reg} added to PBMC, also inferring that MHC class II restriction is not necessary (Cabrera *et al*, 2004a).

In similar experiments on blood from IM patients, low levels of IFN-γ were released in cultures with and without T_{reg} in response to the non-A2 peptide, QAK (median SFC/million PBMC 5 and 6.7, respectively) and unstimulated cultures (median SFC/million PBMC 13.3 and 8.3, respectively). There was some concern that high background levels of IFN-γ might arise in this group of patients since $CD8^+$ T cell-produced IFN-γ is partly responsible for the symptoms experienced by these patients (Biglino *et al*, 1996;Foss *et al*, 1994d). However, the controls showed this was not the case. PHA stimulation resulted in the same raised level of IFN-γ production in both cultures with T_{reg} and cultures lacking T_{reg} (median SFC/million PBMC 66.7). Stimulation with SVR resulted in a similar level of IFN-γ production by both cultures with and without T_{reg} (median SFC/million PBMC 5). Cultures with and without T_{reg}

also responded similarly to LLD (median SFC/million PBMC 5 and 6.7, respectively). An increased response to GLC was observed in cultures lacking T_{reg} (median SFC/million PBMC 46.7) in comparison to cultures containing T_{reg} (median SFC/million PBMC 18.3) but this was not statistically significant (p=0.805).

Similar levels of IFN-γ were released by PBMC from IM patients and control donors in response to SVR in cultures containing T_{reg} (median SFC/million PBMC 8.35 and 5, respectively). In IM patients, this result can be explained since during primary infection the major $CD8^+$ T cell response is directed against lytic antigens, the response to latent antigen only gaining its majority after recovery from IM (Callan, 2004c). In controls, a similar explanation can be applied as mentioned previously, that the donors in the small data set may not have possessed T cells specific for the particular peptide sequence.

A significantly higher level of IFN-γ was produced by T_{reg}-repleted PBMC from healthy seropositive donors than IM patients in response to LLD. Again, this could be due to the fact that healthy seropositive donors would have more T cells capable of recognising latent peptides than IM patients. However, a similar level of IFN-γ was released in response to GLC, the lytic antigen. As mentioned previously, it was expected that T cell responses in IM patients would be targeted against lytic antigens early during the infection.

This current report demonstrates a significantly reduced (p=0.009) IFN-γ production by PBMC from IM patients (median SFC/million PBMC 66.7) compared to healthy seropositive controls (median SFC/million PBMC 793.4) in response to PHA. Previously published reports also demonstrate a reduced response to PHA stimulation in IM patients when compared to healthy seropositive controls, showing reduced RNA synthesis (Rubin, 1966) and cell proliferation (Sheldon *et al*, 1973), although a further study found no difference in response to PHA after 7 days of stimulation but lymphocytes were hyporesponsive in a mixed lymphocyte reaction (Twomey, 1974). In conjunction with these previous studies, the present results indicate that immunosuppression of the T cell response occurs during early infection with EBV, and, although the present results suggest that T_{reg} are not responsible, the actual mechanism of this remains to be elucidated.

The present results failed to demonstrate significant T_{reg} activity in IM or healthy seropositive donors, however, using a greater pool of peptides as well as HLA class II-restricted peptides would allow for a better understanding of responses to EBV by both healthy virus-carriers as well as IM patients.

The lack of any observed difference between T_{reg}-depleted PBMC and T_{reg}-repleted PBMC does not necessarily indicate that EBV-specific T_{reg} are not active in IM. This study investigated IM during the early stages of the disease. It is plausible that continued antigen presence, in association with the production of viral IL-10, would

create an environment conducive to T_{reg} induction. This may occur at later stages during convalescence, causing the subsidence of the $CD8^+$ T cell response. In order to establish if this is the case, the same experiments could be carried out during later stages of disease. Furthermore, since T_{reg} activity has been demonstrated in chronic infection with HBV and HCV, and during the course of HIV infection, it is possible that T_{reg} play a more active role in the rare cases of CAEBV, and this could also be investigated.

It is also plausible that T_{reg} play a role in silent seroconversion to EBV, rather than IM, where their activity may be responsible for early suppression of $CD8^+$ T cell activity and therefore a reduction in the immunopathological symptoms of IM. To investigate this is more difficult and would necessitate a long term study, requiring patients who are seronegative to be monitored until seroconversion occurs, taking blood samples at regular intervals.

4.2 LMP experiments

Most of the evidence suggesting that T_{reg} activity features in EBV infection points towards a role for LMP-1 in the induction of regulatory T cells (Marshall *et al*, 2003c;Marshall *et al*, 2007a). In particular, Marshall *et al* stimulated PBMC from 20 healthy EBV-positive donors and looked at responses to a range of peptides, spanning the entire length of the LMP-1 protein. They found that a few selected peptides resulted in the production of IL-10, and suppression of the IFN-γ and proliferative responses to non-related antigen stimulation (Marshall *et al*, 2003b). This study was carried out on

healthy seropositive donors and we have extended this work by repeating the experiment on 8 healthy seropositive donors, in order to compare our data with the published results, and on 7 IM patients, in order to determine if the same was detectable in this group of individuals.

The results show that:

- In healthy seropositive controls there was no significant difference in cell proliferation, IFN-γ or IL-10 production between cultures stimulated with PPD and PPD + PRG.
- In IM subjects the addition of PRG to cultures containing PPD significantly reduced IFN-γ production ($p=0.0262$) but not cell proliferation or IL-10 production.
- PHA stimulation of IM PBMCs resulted in significantly less IL-10 production ($p= 0.0003$), significantly higher IFN-γ production ($p=0.0037$) but no difference in proliferation, when compared to healthy controls.

The individual results are shown in figures 3.41-3.46 (see *Results*, pages 142 to 150) and figure 4.1 shows IL-10, IFN-γ and cell proliferation results for PBMC from both healthy seropositive controls and IM patients in response to stimulation with PRG, PPD and PPD in combination with PRG, in a format equivalent to that of Marshall *et al* (2003a) for comparison.

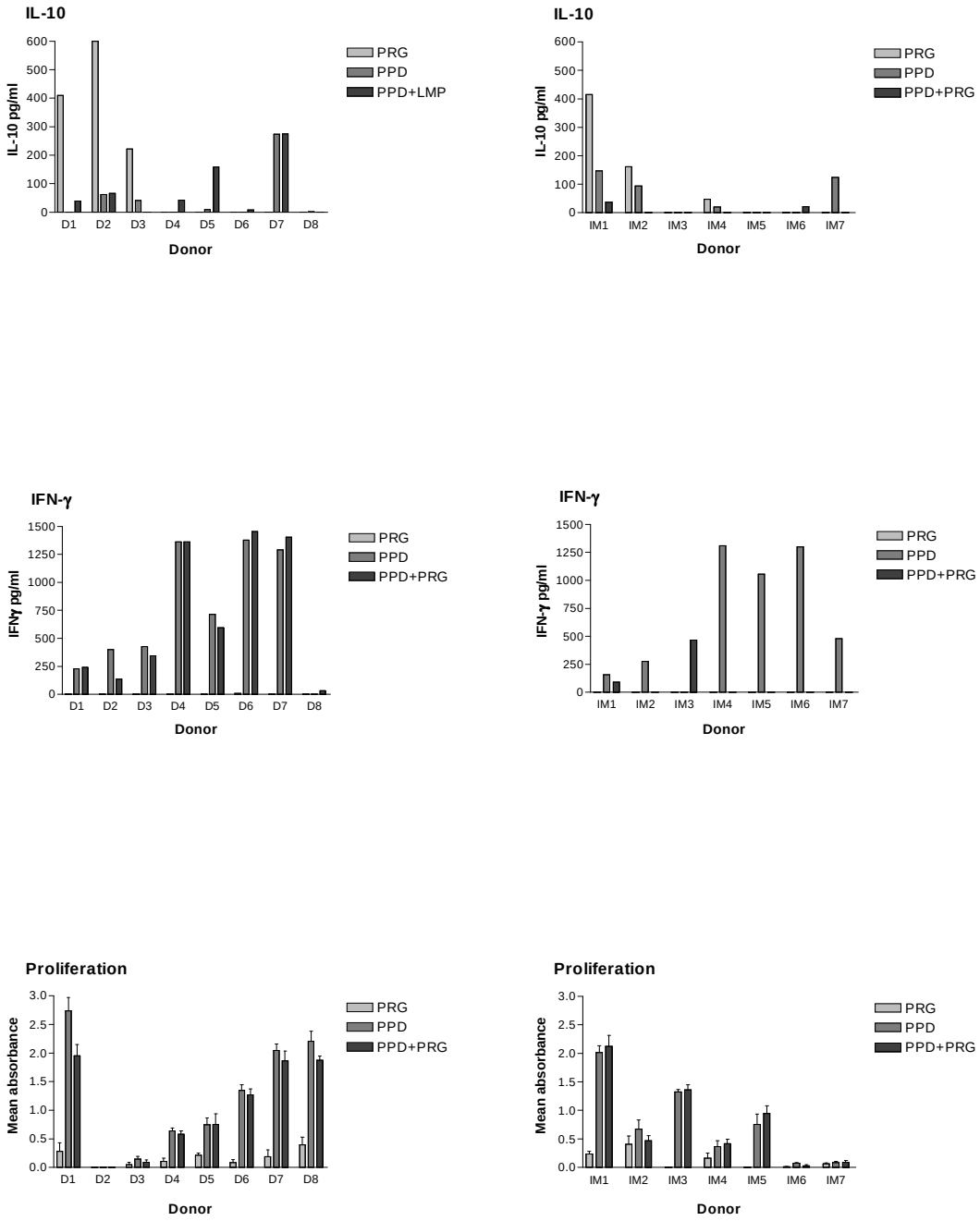

Figure 4.1: IL-10 production (top panels), IFN-γ production (middle panels) and proliferative response (bottom panels) of peripheral blood mononuclear cells from healthy seropositive controls (left panels) and IM patients (right panels) in response to PRG peptide (light grey), recall antigen PPD (medium grey) or PRG in combination with PPD (dark grey). Bars indicate standard error.

We limited the stimulation to the LMP-1 peptide PRG shown by Marshall *et al* to induce IL-10 production, and suppression of both IFN-γ production and cell proliferation in the majority of healthy donors. In our study, PBMC from 5 out of 8 healthy seropositive controls gave no measurable IL-10 production in response to PRG, and 3 out of 8 donors responded with high levels of IL-10 (median 409.8pg/ml). Data published by Marshall *et al* showed that one representative individual responded to PRG with a high level of IL-10 (approximately 150pg/ml). 70% of the 20 seropositive donors responded positively to PRG by producing over double the level of unstimulated controls. In comparison, using these parameters in our study, 38% of donors responded positively.

The IFN-γ response to PRG from all PBMC from the healthy seropositive controls in our study was undetectable. Marshall *et al* detected approximately 15% of donors who responded positively, and since our study was smaller, these results may be regarded as consistent. Cell proliferation in response to PRG the current study cannot be directly compared to findings published by Marshall *et al*, since different methods of measuring cell proliferation were used. However, in the one representative individual shown in their paper, proliferation in response to PRG was not considered positive, and the combined results showed PBMC from fewer than 10% of the 20 seropositive donors responded with cell proliferation. In our study, the proliferation was also low in response to PRG stimulation (median absorbance 0.146, figure 3.45, page 148).

Marshall *et al*'s treatment of seropositive PBMC with PPD alone resulted in very low IL-10 production from 3 individual donors, consistent with our study (median 5.88pg/ml). Treatment with PPD and PRG resulted in increased IL-10 production in all 3 donors from Marshall's study (approximately 800, 1000 and 1000pg/ml, respectively). The results in our study demonstrated a moderate, non-significant increase in IL-10 production with the same stimulus (median 41.8pg/ml) in all donors.

Stimulation with PPD alone resulted in high levels of IFN-γ production from 3 individual donors (20,000, 25,000 and 25,000pg/ml) in Marshall's study, whereas in our study, there was less IFN-γ produced in response to PPD alone (median 569.8pg/ml). Marshall *et al* demonstrated a substantially reduced IFN-γ response from all 3 donor PBMC to treatment with PPD and PRG (almost undetectable) in comparison to PPD alone, whereas the results in this present study found no significant difference in IFN-γ response in 8 donors (median 470.4pg/ml).

PBMC from all 3 donors proliferated to a high level (each approximately 20 mean counts per minute $x10^{-3}$) in response to PPD alone, whereas the addition of PRG reduced this substantially (approximately 2.5, 7 and 7.5 mean counts per minute $x10^{-3}$, respectively) in Marshall's study. In our study, a high level of proliferation was observed in response to PPD alone (median absorbance 1.05) and this was also reduced with the addition of PRG, although not significantly (median absorbance 1.01).

Overall our results agree with those of Marshall *et al*, and the differences between the studies may reflect differences in methodology and/or sample size. In addition, we were only able to run single ELISA samples whereas Marshall *et al* ran samples in duplicate and cell proliferation samples in triplicate, providing a more accurate result. In future, more donors would be recruited and samples would be run in duplicate or triplicate.

Our experiments were also carried out on PBMC from IM patients (figure 4.1). PBMC from 4 out of 7 IM patients did not responded to PRG whereas 3 out of 7 responded with high levels of IL-10 (median 162pg/ml). Using the same method as Marshall *et al* to determine positive results, 43% of IM patients responded positively, similar to the healthy seropositive controls (38%). The IFN-γ response to PRG from all IM patients' PBMC was undetectable, again similar to results from healthy seropositive donors. Cell proliferation by PBMC from IM patients was low (median absorbance 0.07) this was also found to be low in healthy seropositive controls (median absorbance 0.146).

Treatment of IM patients' PBMC with PPD alone resulted in very low IL-10 production (median 20.4pg/ml), increased in comparison to the level produced by PBMC from healthy seropositive controls (median 5.88pg/ml), although not significantly so. Treatment with PPD and PRG reduced IL-10 production to an undetectable level in IM patients whereas these stimuli caused an increased IL-10 production from healthy seropositive donor PBMC (median 41.8pg/ml).

Stimulation with PPD alone resulted in high levels of IFN-γ production from IM patients PBMC (median 479.2pg/ml), and the addition of PRG reduced IFN-γ in 6 out of 7 IM patients PBMC, in 5 cases to an undetectable level. This is the only significant reduction in response to PPD induced by the addition of PRG in our study (p=0.026). This suggests T_{reg} activity induced by PRG, again indicating a role for LMP in controlling the T cell immune response to EBV.

4.3 CTL Growth

The CMV laboratory has developed a bank of cytotoxic T lymphocyte (CTL) lines for use in the treatment of post-transplant lymphoproliferative disease (Wilkie *et al*, 2004). The growth of these CTL lines differs greatly in absolute number of CTL at the final stimulation and the frequency of $CD4^+$ T cell and NK cell within the final population of cells, as does their ability to lyse specific EBV-positive target cells. In order to establish if cell growth characteristics and the observed inability to lyse target cells was due to the presence of T_{reg}, CTL from two donors were developed, one line from each being depleted of T_{reg} and another line of T_{reg}-repleted CTL.

No difference in growth dynamics was observed from either of the lines, indicating that T_{reg} presence or absence in the initial population did not affect the growth of CTL. T_{reg} frequency varied between the CTL although no trend being observed; one T_{reg}-depleted CTL line had a higher frequency of T_{reg} than the T_{reg}-repleted line whereas the inverse

was true of the second set of lines. Similarly, no trend was observed in specific lysis, with both lines having similar cytotoxic profiles at the end point of 8 weeks.

One of the problems with using $CD25^{high}$ expression as a marker for T_{reg} under these experimental conditions is that the CTL lines are restimulated once a week with autologous LCL, and IL-2 is added three times a week, resulting in a highly activated population of cells. In this case, FOXP3, GITR or CTLA-4 expression might be more appropriate as markers of T_{reg}. In addition, the method employed to isolate T_{reg} results in approximately 80% purity, hence not all T_{reg} were removed from the initial PBMC culture. Repeating the enrichment procedure would increase the purity of T_{reg}. However, this was a preliminary experiment only carried out on two donors. For a more accurate representation, more donors would be required.

4.4 In vivo experiments

The current study could not find any *in vitro* evidence that removal of T_{reg} from cultures affects the $CD8^+$ T cell IFN-γ response to stimulation with a variety of EBV-derived peptides. However, as has been the case in a number of previous studies, particularly looking at mechanisms by which T_{reg} carry out their suppressive activity, results observed *in vitro* do not necessarily reflect *in vivo* observations (Read *et al*, 1998). For example, Jonuleit *et al* demonstrated that anti-TGF-β did not alter the anergic state of human $CD4^+CD25^+$ T_{reg} *in vitro* (Jonuleit *et al*, 2001) whereas data published on *in vivo*

studies revealed reduced suppressive activity when blocking anti-TGF-β antibodies were used (Powrie, 1995;Mason & Powrie, 1998;Fuss *et al*, 2002).

Severe combined immunodeficient (SCID) mice lack functional B and T cells and have been used to study EBV-associated B-cell lymphoproliferative disease (BLPD). The introduction of PBMC from healthy EBV seropositive donors to SCID mice gives rise to EBV-positive BLPD, of human B-cell origin, in approximately 50% of SCID mice (Picchio *et al*, 1992a). This model was used to investigate the effect of T_{reg} activity on tumour outgrowth by injecting SCID mice with unmanipulated PBMC, T_{reg}-depleted PBMC or T_{reg}-repleted PBMC. It was plausible that a lack of T_{reg} would allow an unsuppressed T cell response to tumours and hence the group injected with T_{reg}-depleted PBMC would develop tumours more slowly, or not at all. This theory was supported by the publication of a study, with the same experimental design, in which CD4-depleted PBMC significantly reduced tumour incidence (Johannessen *et al*, 2000) and a study by Shimizu *et al*, where T_{reg} depletion resulted in a potent immune response to syngeneic tumours in mice and subsequent tumour eradication (Shimizu *et al*, 2002a).

The results showed that mice injected with unmanipulated PBMC developed tumours at a faster rate than both the T_{reg}-depleted PBMC group and the T_{reg}-repleted PBMC group. PBMC with and without T_{reg} caused similar levels of tumour development although tumours in both these groups took longer to develop than the unmanipulated control

cells. Thus the manipulation of the cells resulted in reduced tumourgenicity, although the mechanism involved it is not clear.

4.5 Working Model

From the results presented in this thesis, we postulate that early during primary infection EBV-infected tonsillar B cells express viral IL-10 and LMP-1, creating an environment conducive to T_{reg} activation at the primary site of infection. This scenario would explain the high level of $FOXP3^+$ cells in the tonsil of IM patients. Whilst T_{reg} induction occurs, the virus infects and becomes latent in B cells as they pass through the tonsil and enter the circulation. In asymptomatic silent converters, these T_{reg} would be responsible for suppressing T cell-mediated immunopathology by limiting the cytotoxic $CD8^+$ T cell response to EBV during early infection. In contrast, if the initial viral dose is high or the frequency of $CD4^+CD25^+$ T cells low then they may not be able to restrict the cytotoxic response to EBV, and acute IM would result. In order to establish this hypothesis and elucidate the role of Treg in EBV infection, a long term study, with periodic blood sampling and larger donor recruitment would be required to carry out comparative experiments on acute IM, silent seroconverters, EBV-negative individuals and convalescent IM patients.

5 References

References

Aandahl,E.M., Michaelsson,J., Moretto,W.J., Hecht,F.A., & Nixon,D.F. (2004b) Human CD4(+) CD25(+) regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *Journal of Virology*, **78**, 2454-2459.

Aandahl,E.M., Michaelsson,J., Moretto,W.J., Hecht,F.A., & Nixon,D.F. (2004a) Human CD4(+) CD25(+) regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *Journal of Virology*, **78**, 2454-2459.

Aandahl,E.M., Michaelsson,J., Moretto,W.J., Hecht,F.A., & Nixon,D.F. (2004c) Human CD4(+) CD25(+) regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *Journal of Virology*, **78**, 2454-2459.

Abbot,S.D., Rowe,M., Cadwallader,K., Ricksten,A., Gordon,J., Wang,F., Rymo,L., & Rickinson,A.B. (1990a) Epstein-Barr virus nuclear antigen 2 induces expression of the virus-encoded latent membrane protein. *The Journal of Virology*, **64**, 2126-2134.

Abbot,S.D., Rowe,M., Cadwallader,K., Ricksten,A., Gordon,J., Wang,F., Rymo,L., & Rickinson,A.B. (1990b) Epstein-Barr virus nuclear antigen 2 induces expression of the virus-encoded latent membrane protein. *The Journal of Virology*, **64**, 2126-2134.

Abbot,S.D., Rowe,M., Cadwallader,K., Ricksten,A., Gordon,J., Wang,F., Rymo,L., & Rickinson,A.B. (1990c) Epstein-Barr virus nuclear antigen 2 induces expression of the virus-encoded latent membrane protein. *The Journal of Virology*, **64**, 2126-2134.

Adhikary,D., Behrends,U., Moosmann,A., Witter,K., Bornkamm,G.W., & Mautner,J. (2006b) Control of Epstein-Barr virus infection in vitro by T helper cells specific for virion glycoproteins. *Journal of Experimental Medicine*, **203**, 995-1006.

Adhikary,D., Behrends,U., Moosmann,A., Witter,K., Bornkamm,G.W., & Mautner,J. (2006a) Control of Epstein-Barr virus infection in vitro by T helper cells specific for virion glycoproteins. *Journal of Experimental Medicine*, **203**, 995-1006.

Adldinger,H.K., Delius,H., Freese,U.K., Clarke,J., & Bornkamm,G.W. (1985) A putative transforming gene of jijoye virus differs from that of Epstein-Barr virus prototypes. *Virology*, **141**, 221-234.

Alfieri,C., Birkenbach,M., & Kieff,E. (1991e) Early events in Epstein-Barr virus infection of human B lymphocytes. *Virology*, **181**, 595-608.

Alfieri,C., Birkenbach,M., & Kieff,E. (1991a) Early events in Epstein-Barr virus infection of human B lymphocytes. *Virology*, **181**, 595-608.

Alfieri,C., Birkenbach,M., & Kieff,E. (1991b) Early events in Epstein-Barr virus infection of human B lymphocytes. *Virology*, **181**, 595-608.

Alfieri,C., Birkenbach,M., & Kieff,E. (1991c) Early events in Epstein-Barr virus infection of human B lymphocytes. *Virology*, **181**, 595-608.

Alfieri,C., Birkenbach,M., & Kieff,E. (1991d) Early events in Epstein-Barr virus infection of human B lymphocytes. *Virology*, **181**, 595-608.

Allan,G.J., Inman,G.J., Parker,B.D., Rowe,D.T., & Farrell,P.J. (1992) Cell growth effects of Epstein--Barr virus leader protein. *Journal of General Virology*, **73**, 1547-1551.

Allday,M.J., Crawford,D.H., & Griffin,B.E. (1989) Epstein-Barr Virus Latent Gene Expression during the Initiation of B Cell Immortalization. *Journal of General Virology*, **70**, 1755-1764.

Allday,M. & Crawford,D. (1988) ROLE OF EPITHELIUM IN EBV PERSISTENCE AND PATHOGENESIS OF B-CELL TUMOURS. *The Lancet*, **331**, 855-857.

Altiok,E., Klein,G., Zech,L., Uno,M., Henriksson,B.E., Battat,S., Ono,Y., & Ernberg,I. (1989) Epstein--Barr Virus-Transformed Pro-B Cells are Prone to Illegitimate Recombination between the Switch Region of the {micro} Chain Gene and Other Chromosomes. *Proceedings of the National Academy of Sciences*, **86**, 6333-6337.

Amyes,E., Hatton,C., Montamat-Sicotte,D., Gudgeon,N., Rickinson,A.B., McMichael,A.J., & Callan,M.F.C. (2003a) Characterization of the CD4+ T Cell Response to Epstein-Barr Virus during Primary and Persistent Infection. *The Journal of Experimental Medicine*, **198**, 903-911.

Amyes,E., Hatton,C., Montamat-Sicotte,D., Gudgeon,N., Rickinson,A.B., McMichael,A.J., & Callan,M.F.C. (2003b) Characterization of the CD4+ T Cell Response to Epstein-Barr Virus during Primary and Persistent Infection. *The Journal of Experimental Medicine*, **198**, 903-911.

Amyes,E., Hatton,C., Montamat-Sicotte,D., Gudgeon,N., Rickinson,A.B., McMichael,A.J., & Callan,M.F.C. (2003c) Characterization of the CD4+ T Cell Response to Epstein-Barr Virus during Primary and Persistent Infection. *The Journal of Experimental Medicine*, **198**, 903-911.

Amyes,E., Hatton,C., Montamat-Sicotte,D., Gudgeon,N., Rickinson,A.B., McMichael,A.J., & Callan,M.F.C. (2003d) Characterization of the CD4+ T Cell Response to Epstein-Barr Virus during Primary and Persistent Infection. *The Journal of Experimental Medicine*, **198**, 903-911.

Andersson,J., Boasso,A., Nilsson,J., Zhang,R., Shire,N.J., Lindback,S., Shearer,G.M., & Chougnet,C.A. (2005a) Cutting edge: The prevalence of regulatory T cells lymphoid tissue is correlated with viral load HIV-infected patients. *Journal of Immunology*, **174**, 3143-3147.

Andersson,J., Boasso,A., Nilsson,J., Zhang,R., Shire,N.J., Lindback,S., Shearer,G.M., & Chougnet,C.A. (2005b) Cutting edge: The prevalence of regulatory T cells lymphoid tissue is correlated with viral load HIV-infected patients. *Journal of Immunology*, **174**, 3143-3147.

Arrand,J.R. & Rymo,L. (1982) Characterization of the major Epstein-Barr virus-specific RNA in Burkitt lymphoma-derived cells. *The Journal of Virology*, **41**, 376-389.

Asano,M., Toda,M., Sakaguchi,N., & Sakaguchi,S. (1996) Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *Journal of Experimental Medicine*, **184**, 387-396.

Asseman,C., Mauze,S., Leach,M.W., Coffman,R.L., & Powrie,F. (1999) An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *Journal of Experimental Medicine*, **190**, 995-1003.

Bacchetta,R., Gregori,S., & Roncarolo,M.G. (2005b) CD4+ regulatory T cells: Mechanisms of induction and effector function. *Autoimmunity Reviews*, **4**, 491-496.

Bacchetta,R., Gregori,S., & Roncarolo,M.G. (2005a) CD4+ regulatory T cells: Mechanisms of induction and effector function. *Autoimmunity Reviews*, **4**, 491-496.

Bacchetta,R., Gregori,S., & Roncarolo,M.G. (2005c) CD4+ regulatory T cells: Mechanisms of induction and effector function. *Autoimmunity Reviews*, **4**, 491-496.

Baecher-Allan,C., Brown,J.A., Freeman,G.J., & Hafler,D.A. (2001b) CD4+CD25high regulatory cells in human peripheral blood. *J Immunol*, **167**, 1245-1253.

Baecher-Allan,C., Brown,J.A., Freeman,G.J., & Hafler,D.A. (2001a) CD4+CD25high regulatory cells in human peripheral blood. *J Immunol*, **167**, 1245-1253.

Baecher-Allan,C., Viglietta,V., & Hafler,D.A. (2002) Inhibition of human CD4(+)CD25(+high) regulatory T cell function. *J Immunol*, **169**, 6210-6217.

Baer,R., Bankier,A.T., Biggin,M.D., Deininger,P.L., Farrell,P.J., Gibson,T.J., Hatfull,G., Hudson,G.S., Satchwell,S.C., Seguin,C., Tuffnell,P.S., & Barrell,B.G. (1984a) DNA sequence and expression of the B95-8 Epstein[mdash]Barr virus genome. *Nature*, **310**, 207-211.

Baer,R., Bankier,A.T., Biggin,M.D., Deininger,P.L., Farrell,P.J., Gibson,T.J., Hatfull,G., Hudson,G.S., Satchwell,S.C., Seguin,C., Tuffnell,P.S., & Barrell,B.G. (1984b) DNA sequence and expression of the B95-8 Epstein[mdash]Barr virus genome. *Nature*, **310**, 207-211.

Balachandran,N., Oba,D.E., & Hutt-Fletcher,L.M. (1987) Antigenic cross-reactions among herpes simplex virus types 1 and 2, Epstein-Barr virus, and cytomegalovirus. *The Journal of Virology*, **61**, 1125-1135.

Battaglia,M., Gregori,S., Bacchetta,R., & Roncarolo,M.G. (2006a) Tr1 cells: From discovery to their clinical application. *Seminars in Immunology*, **18**, 120-127.

Battaglia,M., Gregori,S., Bacchetta,R., & Roncarolo,M.G. (2006b) Tr1 cells: From discovery to their clinical application. *Seminars in Immunology*, **18**, 120-127.

Bayliss,G.J. & Wolf,H. (1980) Epstein-Barr virus-induced cell fusion. *Nature*, **287**, 164-165.

Beilharz,M.W., Sammels,L.M., Paun,A., Shaw,K., van Eeden,P., Watson,M.W., & Ashdown,M.L. (2004) Timed Ablation of Regulatory CD4+ T Cells Can Prevent Murine AIDS Progression. *The Journal of Immunology*, **172**, 4917-4925.

Belkaid,Y., Piccirillo,C.A., Mendez,S., Shevach,E.M., & Sacks,D.L. (2002c) CD4(+)CD25(+) regulatory T cells control Leishmania major persistence and immunity. *Nature*, **420**, 502-507.

Belkaid,Y., Piccirillo,C.A., Mendez,S., Shevach,E.M., & Sacks,D.L. (2002b) CD4(+)CD25(+) regulatory T cells control Leishmania major persistence and immunity. *Nature*, **420**, 502-507.

Belkaid,Y., Piccirillo,C.A., Mendez,S., Shevach,E.M., & Sacks,D.L. (2002a) CD4(+)CD25(+) regulatory T cells control Leishmania major persistence and immunity. *Nature*, **420**, 502-507.

Belkaid,Y. & Rouse,B.T. (2005b) Natural regulatory T cells in infectious disease. *Nature Immunology*, **6**, 353-360.

Belkaid,Y. & Rouse,B.T. (2005a) Natural regulatory T cells in infectious disease. *Nature Immunology*, **6**, 353-360.

Belkaid,Y. & Rouse,B.T. (2005c) Natural regulatory T cells in infectious disease. *Nature Immunology*, **6**, 353-360.

Bennett,C.L., Christie,J., Ramsdell,F., Brunkow,M.E., Ferguson,P.J., Whitesell,L., Kelly,T.E., Saulsbury,F.T., Chance,P.F., & Ochs,H.D. (2001a) The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature Genetics*, **27**, 20-21.

Bennett,C.L., Christie,J., Ramsdell,F., Brunkow,M.E., Ferguson,P.J., Whitesell,L., Kelly,T.E., Saulsbury,F.T., Chance,P.F., & Ochs,H.D. (2001b) The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature Genetics*, **27**, 20-21.

Biglino,A., Sinicco,A., Forno,B., Pollono,A.M., Sciandra,R., Martini,C., Pich,P., & Gioannini,P. (1996) Serum cytokine profiles in acute primary HIV-1 infection and in infectious mononucleosis. *Clinical Immunology and Immunopathology*, **78**, 61-69.

Bodescot,M., Brison,O., & Perricaudet,M. (1986) An Epstein-Barr virus transcription unit is at least 84 kilobases long. *Nucleic Acids Research*, **14**, 2611-2620.

Bodescot,M. & Perricaudet,M. (1987) Clustered alternative splice sites in Epstein-Barr virus RNAs. *Nucleic Acids Research*, **15**, 5887.

Boettler,T., Spangenberg,H.C., Neumann-Haefelin,C., Panther,E., Urbani,S., Ferrari,C., Blum,H.E., von Weizsacker,F., & Thimme,R. (2005a) T Cells with a CD4+CD25+ Regulatory Phenotype Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Chronic Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7860-7867.

Boettler,T., Spangenberg,H.C., Neumann-Haefelin,C., Panther,E., Urbani,S., Ferrari,C., Blum,H.E., von Weizsacker,F., & Thimme,R. (2005d) T Cells with a CD4+CD25+ Regulatory Phenotype Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Chronic Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7860-7867.

Boettler,T., Spangenberg,H.C., Neumann-Haefelin,C., Panther,E., Urbani,S., Ferrari,C., Blum,H.E., von Weizsacker,F., & Thimme,R. (2005e) T Cells with a CD4+CD25+ Regulatory Phenotype Suppress In Vitro Proliferation of Virus-

Specific CD8+ T Cells during Chronic Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7860-7867.

Boettler,T., Spangenberg,H.C., Neumann-Haefelin,C., Panther,E., Urbani,S., Ferrari,C., Blum,H.E., von Weizsacker,F., & Thimme,R. (2005f) T Cells with a CD4+CD25+ Regulatory Phenotype Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Chronic Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7860-7867.

Boettler,T., Spangenberg,H.C., Neumann-Haefelin,C., Panther,E., Urbani,S., Ferrari,C., Blum,H.E., von Weizsacker,F., & Thimme,R. (2005g) T Cells with a CD4+CD25+ Regulatory Phenotype Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Chronic Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7860-7867.

Boettler,T., Spangenberg,H.C., Neumann-Haefelin,C., Panther,E., Urbani,S., Ferrari,C., Blum,H.E., von Weizsacker,F., & Thimme,R. (2005c) T Cells with a CD4+CD25+ Regulatory Phenotype Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Chronic Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7860-7867.

Boettler,T., Spangenberg,H.C., Neumann-Haefelin,C., Panther,E., Urbani,S., Ferrari,C., Blum,H.E., von Weizsacker,F., & Thimme,R. (2005b) T Cells with a CD4+CD25+ Regulatory Phenotype Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Chronic Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7860-7867.

Borza,C.M. & Hutt-Fletcher,L.M. (2002) Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus. *Nature Medicine*, **8**, 594-599.

Borza,C.M. & Hutt-Fletcher,L.M. (1998) Epstein-Barr Virus Recombinant Lacking Expression of Glycoprotein gp150 Infects B Cells Normally but Is Enhanced for Infection of Epithelial Cells. *The Journal of Virology*, **72**, 7577-7582.

Botten,J., Whitton,J.L., Barrowman,P., Sidney,J., Whitmire,J.K., Alexander,J., Ting,J.P.C., Bui,H.H., Sette,A., & Buchmeier,M.J. (2007a) HLA-A2-restricted protection against lethal lymphocytic choriomeningitis. *Journal of Virology*, **81**, 2307-2317.

Botten,J., Whitton,J.L., Barrowman,P., Sidney,J., Whitmire,J.K., Alexander,J., Ting,J.P.C., Bui,H.H., Sette,A., & Buchmeier,M.J. (2007b) HLA-A2-restricted protection against lethal lymphocytic choriomeningitis. *Journal of Virology*, **81**, 2307-2317.

Bovenzi,P., Mirandola,P., Secchiero,P., Strumia,R., Cassai,E., & Di Luca,D. (1993) Human herpesvirus 6 (variant A) in Kaposi's sarcoma. *The Lancet*, **341**, 1288-1289.

Boyer,O., Saadoun,D., Abriol,J., Dodille,M., Piette,J.C., Cacoub,P., & Klatzmann,D. (2004) CD4(+)CD25(+) regulatory T-cell deficiency in patients with hepatitis C-mixed cryoglobulinemia vasculitis. *Blood*, **103**, 3428-3430.

Brunkow,M.E., Jeffery,E.W., Hjerrild,K.A., Paeper,B., Clark,L.B., Yasayko,S.A., Wilkinson,J.E., Galas,D., Ziegler,S.F., & Ramsdell,F. (2001a) Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics*, **27**, 68-73.

Brunkow,M.E., Jeffery,E.W., Hjerrild,K.A., Paeper,B., Clark,L.B., Yasayko,S.A., Wilkinson,J.E., Galas,D., Ziegler,S.F., & Ramsdell,F. (2001b) Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics*, **27**, 68-73.

Burkitt,D.P. (1983) The Discovery of Burkitts-Lymphoma. *Cancer*, **51**, 1777-1786.

Cabrera,R., Tu,Z.K., Xu,Y.L., Firpi,R.J., Rosen,H.R., Liu,C., & Nelson,D.R. (2004a) An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology*, **40**, 1062-1071.

Cabrera,R., Tu,Z.K., Xu,Y.L., Firpi,R.J., Rosen,H.R., Liu,C., & Nelson,D.R. (2004b) An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology*, **40**, 1062-1071.

Cabrera,R., Tu,Z.K., Xu,Y.L., Firpi,R.J., Rosen,H.R., Liu,C., & Nelson,D.R. (2004c) An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology*, **40**, 1062-1071.

Cabrera,R., Tu,Z.K., Xu,Y.L., Firpi,R.J., Rosen,H.R., Liu,C., & Nelson,D.R. (2004d) An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology*, **40**, 1062-1071.

Cabrera,R., Tu,Z.K., Xu,Y.L., Firpi,R.J., Rosen,H.R., Liu,C., & Nelson,D.R. (2004e) An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology*, **40**, 1062-1071.

Cabrera,R., Tu,Z.K., Xu,Y.L., Firpi,R.J., Rosen,H.R., Liu,C., & Nelson,D.R. (2004f) An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology*, **40**, 1062-1071.

Cabrera,R., Tu,Z.K., Xu,Y.L., Firpi,R.J., Rosen,H.R., Liu,C., & Nelson,D.R. (2004g) An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology*, **40**, 1062-1071.

Calender,A., Billaud,M., Aubry,J.P., Banchereau,J., Vuillaume,M., & Lenoir,G.M. (1987) Epstein-Barr Virus (EBV) Induces Expression of B-Cell Activation Markers on in vitro Infection of EBV-Negative B-Lymphoma Cells. *Proceedings of the National Academy of Sciences*, **84**, 8060-8064.

Callan,M.F.C. (2004a) The immune response to Epstein-Barr virus. *Microbes and Infection*, **6**, 937-945.

Callan,M.F.C. (2004b) The immune response to Epstein-Barr virus. *Microbes and Infection*, **6**, 937-945.

Callan,M.F.C. (2004c) The immune response to Epstein-Barr virus. *Microbes and Infection*, **6**, 937-945.

Callan,M.F.C., Fazou,C., Yang,H.B., Rostron,T., Poon,K., Hatton,C., & McMichael,A.J. (2000a) CD8(+) T-cell selection, function, and death in the primary immune response in vivo. *Journal of Clinical Investigation*, **106**, 1251-1261.

Callan,M.F.C., Fazou,C., Yang,H.B., Rostron,T., Poon,K., Hatton,C., & McMichael,A.J. (2000b) CD8(+) T-cell selection, function, and death in the primary immune response in vivo. *Journal of Clinical Investigation*, **106**, 1251-1261.

Callan,M.F.C., Steven,J., Krausa,P., Wilson,J.D.K., Moss,P.A.H., Gillespie,G.M., Bell,J.I., Rickinson,A.B., & McMichael,A.J. (1996) Large clonal expansions of CD8(+) T cells in acute infectious mononucleosis. *Nature Medicine*, **2**, 906-911.

Callan,M.F.C., Tan,L., Annels,N., Ogg,G.S., Wilson,J.D.K., O'Callaghan,C.A., Steven,N., McMichael,A.J., & Rickinson,A.B. (1998a) Direct visualization of antigen-specific CD8(+) T cells during the primary immune response to Epstein-Barr virus in vivo. *Journal of Experimental Medicine*, **187**, 1395-1402.

Callan,M.F.C., Tan,L., Annels,N., Ogg,G.S., Wilson,J.D.K., O'Callaghan,C.A., Steven,N., McMichael,A.J., & Rickinson,A.B. (1998b) Direct visualization of antigen-specific CD8(+) T cells during the primary immune response to Epstein-Barr virus in vivo. *Journal of Experimental Medicine*, **187**, 1395-1402.

Callinan,R.B. & Kefford,B. (1981) Mortalities associated with herpesvirus infection in captive macropods. *Journal of Wildlife Diseases*, **17**, 311-317.

Campbell,D.J. & Ziegler,S.F. (2007a) FOXP3 modifies the phenotypic and functional properties of regulatory T cells. *Nature Reviews Immunology*, **7**, 305-310.

Campbell,D.J. & Ziegler,S.F. (2007b) FOXP3 modifies the phenotypic and functional properties of regulatory T cells. *Nature Reviews Immunology*, **7**, 305-310.

Campbell,D.J. & Ziegler,S.F. (2007c) FOXP3 modifies the phenotypic and functional properties of regulatory T cells. *Nature Reviews Immunology*, **7**, 305-310.

Catalina,M.D., Sullivan,J.L., Bak,K.R., & Luzuriaga,K. (2001a) Differential evolution and stability of epitope-specific CD8(+) T cell responses in EBV infection. *Journal of Immunology*, **167**, 4450-4457.

Catalina,M.D., Sullivan,J.L., Bak,K.R., & Luzuriaga,K. (2001b) Differential evolution and stability of epitope-specific CD8(+) T cell responses in EBV infection. *Journal of Immunology*, **167**, 4450-4457.

Catalina,M.D., Sullivan,J.L., Brody,R.M., & Luzuriaga,K. (2002) Phenotypic and functional heterogeneity of EBV epitope-specific CD8(+) T cells. *Journal of Immunology*, **168**, 4184-4191.

Chang,Y., Cesarman,E., Pessin,M.S., Lee,F., Culpepper,J., Knowles,D.M., & Moore,P.S. (1994) Identification of Herpesvirus-Like DNA Sequences in AIDS-Associated Kaposi's Sarcoma. *Science*, **266**, 1865-1869.

Chatila,T.A., Blaeser,F., Ho,N., Lederman,H.M., Voulgaropoulos,C., Helms,C., & Bowcock,A.M. (2000) JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *Journal of Clinical Investigation*, **106**, R75-R81.

Cordier,M., Calender,A., Billaud,M., Zimber,U., Rousselet,G., Pavlish,O., Banchereau,J., Tursz,T., Bornkamm,G., & Lenoir,G.M. (1990b) Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. *The Journal of Virology*, **64**, 1002-1013.

Cordier,M., Calender,A., Billaud,M., Zimber,U., Rousselet,G., Pavlish,O., Banchereau,J., Tursz,T., Bornkamm,G., & Lenoir,G.M. (1990a) Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. *The Journal of Virology*, **64**, 1002-1013.

Crawford,D.H. (2001a) Biology and disease associations of Epstein-Barr virus. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **356**, 461-473.

Crawford,D.H. (2001c) Biology and disease associations of Epstein-Barr virus. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **356**, 461-473.

Crawford,D.H. (2001e) Biology and disease associations of Epstein-Barr virus. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **356**, 461-473.

Crawford,D.H. (2001b) Biology and disease associations of Epstein-Barr virus. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **356**, 461-473.

Crawford,D.H. (2001d) Biology and disease associations of Epstein-Barr virus. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **356**, 461-473.

Crawford,D.H. & Ando,I. (1986) Eb Virus Induction Is Associated with B-Cell Maturation. *Immunology*, **59**, 405-409.

Crawford,D.H., Macsween,K.F., Higgins,C.D., Thomas,R., McAulay,K., Williams,H., Harrison,N., Reid,S., Conacher,M., Douglas,J., & Swerdlow,A.J. (2006a) A Cohort Study among University Students: Identification of Risk Factors for Epstein-Barr Virus Seroconversion and Infectious Mononucleosis. *Clinical Infectious Diseases*, **43**, 276-282.

Crawford,D.H., Macsween,K.F., Higgins,C.D., Thomas,R., McAulay,K., Williams,H., Harrison,N., Reid,S., Conacher,M., Douglas,J., & Swerdlow,A.J. (2006b) A Cohort Study among University Students: Identification of Risk Factors for Epstein-Barr Virus Seroconversion and Infectious Mononucleosis. *Clinical Infectious Diseases*, **43**, 276-282.

Cupedo,T., Nagasawa,M., Weijer,K., Blom,B., & Spits,H. (2005) Development and activation of regulatory T cells in the human fetus. *European Journal of Immunology*, **35**, 383-390.

Curiel,T.J., Coukos,G., Zou,L.H., Alvarez,X., Cheng,P., Mottram,P., Evdemon-Hogan,M., Conejo-Garcia,J.R., Zhang,L., Burow,M., Zhu,Y., Wei,S., Kryczek,I., Daniel,B., Gordon,A., Myers,L., Lackner,A., Disis,M.L., Knutson,K.L., Chen,L.P., & Zou,W.P. (2004a) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature Medicine*, **10**, 942-949.

Curiel,T.J., Coukos,G., Zou,L.H., Alvarez,X., Cheng,P., Mottram,P., Evdemon-Hogan,M., Conejo-Garcia,J.R., Zhang,L., Burow,M., Zhu,Y., Wei,S., Kryczek,I., Daniel,B., Gordon,A., Myers,L., Lackner,A., Disis,M.L., Knutson,K.L.,

Chen,L.P., & Zou,W.P. (2004c) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature Medicine*, **10**, 942-949.

Curiel,T.J., Coukos,G., Zou,L.H., Alvarez,X., Cheng,P., Mottram,P., Evdemon-Hogan,M., Conejo-Garcia,J.R., Zhang,L., Burow,M., Zhu,Y., Wei,S., Kryczek,I., Daniel,B., Gordon,A., Myers,L., Lackner,A., Disis,M.L., Knutson,K.L., Chen,L.P., & Zou,W.P. (2004b) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature Medicine*, **10**, 942-949.

Dambaugh,T., Hennessy,K., Chamnankit,L., & Kieff,E. (1984) U2 Region of Epstein-Barr Virus DNA May Encode Epstein-Barr Nuclear Antigen 2. *Proceedings of the National Academy of Sciences*, **81**, 7632-7636.

Darrasse-Jeze,G., Marodon,G., Salomon,B.L., Catala,M., & Klatzmann,D. (2005) Ontogeny of CD4(+)CD25(+) regulatory/suppressor T cells in human fetuses. *Blood*, **105**, 4715-4721.

Dieckmann,D., Bruett,C.H., Ploettner,H., Lutz,M.B., & Schuler,G. (2002) Human CD4(+)CD25(+) regulatory, contact-dependent T cells induce interleukin 1-producing, contact-independent type 1-like regulatory T cells. *Journal of Experimental Medicine*, **196**, 247-253.

Diepstra,A., Niens,M., Vellenga,E., van Imhoff,G.W., Nolte,I.M., Schaapveld,M., van der,S.G., van den,B.A., Kibbelaar,R.E., te Meerman,G.J., & Poppema,S. (2005) Association with HLA class I in Epstein-Barr-virus-positive and with HLA class III in Epstein-Barr-virus-negative Hodgkin's lymphoma. *Lancet*, **365**, 2216-2224.

Dittmer,U., He,H., Messer,R.J., Schimmer,S., Olbrich,A.R.M., Ohlen,C., Greenberg,P.D., Stromnes,I.M., Iwashiro,M., Sakaguchi,S., Evans,L.H., Peterson,K.E., Yang,G.J., & Hasenkrug,K.J. (2004a) Functional impairment of CD8(+) T cells by regulatory T cells during persistent retroviral infection. *Immunity*, **20**, 293-303.

Dittmer,U., He,H., Messer,R.J., Schimmer,S., Olbrich,A.R.M., Ohlen,C., Greenberg,P.D., Stromnes,I.M., Iwashiro,M., Sakaguchi,S., Evans,L.H., Peterson,K.E., Yang,G.J., & Hasenkrug,K.J. (2004b) Functional impairment of CD8(+) T cells by regulatory T cells during persistent retroviral infection. *Immunity*, **20**, 293-303.

Dolyniuk,M., Pritchett,R., & Kieff,E. (1976b) Proteins of Epstein-Barr virus. I. Analysis of the polypeptides of purified enveloped Epstein-Barr virus. *The Journal of Virology*, **17**, 935-949.

Dolyniuk,M., Pritchett,R., & Kieff,E. (1976a) Proteins of Epstein-Barr virus. I. Analysis of the polypeptides of purified enveloped Epstein-Barr virus. *The Journal of Virology*, **17**, 935-949.

Dolyniuk,M., Wolff,E., & Kieff,E. (1976c) Proteins of Epstein-Barr Virus. II. Electrophoretic analysis of the polypeptides of the nucleocapsid and the glucosamine- and polysaccharide-containing components of enveloped virus. *The Journal of Virology*, **18**, 289-297.

Douek,D.C., Picker,L.J., & Koup,R.A. (2003) T CELL DYNAMICS IN HIV-1 INFECTION*. *Annual Review of Immunology*, **21**, 265-304.

Dukers,D.F., Meij,P., Vervoort,M.B.H.J., Vos,W., Scheper,R.J., Meijer,C.J.L.M., Bloemena,E., & Middeldorp,J.M. (2000a) Direct immunosuppressive effects of EBV-encoded latent membrane protein 1. *Journal of Immunology*, **165**, 663-670.

Dukers,D.F., Meij,P., Vervoort,M.B.H.J., Vos,W., Scheper,R.J., Meijer,C.J.L.M., Bloemena,E., & Middeldorp,J.M. (2000b) Direct immunosuppressive effects of EBV-encoded latent membrane protein 1. *Journal of Immunology*, **165**, 663-670.

Dunne,P.J., Faint,J.M., Gudgeon,N.H., Fletcher,J.M., Plunkett,F.J., Scares,M.V.D., Hislop,A.D., Annels,N.E., Rickinson,A.B., Salmon,M., & Akbar,A.N. (2002a) Epstein-Barr virus-specific CD8(+) T cells that re-express CD45RA are apoptosis-resistant memory cells that retain replicative potential. *Blood*, **100**, 933-940.

Dunne,P.J., Faint,J.M., Gudgeon,N.H., Fletcher,J.M., Plunkett,F.J., Scares,M.V.D., Hislop,A.D., Annels,N.E., Rickinson,A.B., Salmon,M., & Akbar,A.N. (2002b) Epstein-Barr virus-specific CD8(+) T cells that re-express CD45RA are apoptosis-resistant memory cells that retain replicative potential. *Blood*, **100**, 933-940.

Eggena,M.P., Barugahare,B., Jones,N., Okello,M., Mutalya,S., Kityo,C., Mugyenyi,P., & Cao,H.Y. (2005) Depletion of regulatory T cells in HIV infection is associated with immune activation. *Journal of Immunology*, **174**, 4407-4414.

Epstein,M.A., Achong,B.G., & Barr,Y.M. (1964) VIRUS PARTICLES IN CULTURED LYMPHOBLASTS FROM BURKITT'S LYMPHOMA. *The Lancet*, **283**, 702-703.

Epstein,M.A. & Barr,Y.M. (1964) CULTIVATION IN VITRO OF HUMAN LYMPHOBLASTS FROM BURKITT'S MALIGNANT LYMPHOMA. *The Lancet*, **283**, 252-253.

Epstein,M.A., Henle,G., Achong,B.G., & Barr,Y.M. (1965) MORPHOLOGICAL AND BIOLOGICAL STUDIES ON A VIRUS IN CULTURED LYMPHOBLASTS FROM BURKITT'S LYMPHOMA. *The Journal of Experimental Medicine*, **121**, 761-770.

Fahraeus,R., Jansson,A., Ricksten,A., Sjoblom,A., & Rymo,L. (1990) Epstein-Barr Virus-Encoded Nuclear Antigen 2 Activates the Viral Latent Membrane Protein Promoter by Modulating the Activity of a Negative Regulatory Element. *Proceedings of the National Academy of Sciences*, **87**, 7390-7394.

Fahraeus,R., Jansson,A., Sjoblom,A., Nilsson,T., Klein,G., & Rymo,L. (1993) Cell Phenotype-Dependent Control of Epstein-Barr Virus Latent Membrane Protein 1 Gene Regulatory Sequences. *Virology*, **195**, 71-80.

Filaci,G., Fravega,M., Fenoglio,D., Rizzi,M., Negrini,S., Viggiani,R., & Indiveri,F. (2004) Non-antigen specific CD8+ T suppressor lymphocytes. *Clinical and Experimental Medicine*, **4**, 86-92.

Fingeroth,J.D., Diamond,M.E., Sage,D.R., Hayman,J., & Yates,J.L. (1999) CD21-Dependent Infection of an Epithelial Cell Line, 293,áby Epstein-Barr Virus. *The Journal of Virology*, **73**, 2115-2125.

Fingeroth,J.D., Weis,J.J., Tedder,T.F., Strominger,J.L., Biro,P.A., & Fearon,D.T. (1984) Epstein--Barr Virus Receptor of Human B Lymphocytes is the C3d Receptor CR2. *Proceedings of the National Academy of Sciences*, **81**, 4510-4514.

Foss,H.D., Herbst,H., Hummel,M., Araujo,I., Latza,U., Rancso,C., Dallenbach,F., & Stein,H. (1994a) Patterns of Cytokine Gene-Expression in Infectious-Mononucleosis. *Blood*, **83**, 707-712.

Foss,H.D., Herbst,H., Hummel,M., Araujo,I., Latza,U., Rancso,C., Dallenbach,F., & Stein,H. (1994b) Patterns of Cytokine Gene-Expression in Infectious-Mononucleosis. *Blood*, **83**, 707-712.

Foss,H.D., Herbst,H., Hummel,M., Araujo,I., Latza,U., Rancso,C., Dallenbach,F., & Stein,H. (1994c) Patterns of Cytokine Gene-Expression in Infectious-Mononucleosis. *Blood*, **83**, 707-712.

Foss,H.D., Herbst,H., Hummel,M., Araujo,I., Latza,U., Rancso,C., Dallenbach,F., & Stein,H. (1994d) Patterns of Cytokine Gene-Expression in Infectious-Mononucleosis. *Blood*, **83**, 707-712.

Frade,R., Barel,M., Ehlin-Henriksson,B., & Klein,G. (1985) Gp140, the C3d Receptor of Human B Lymphocytes, is Also the Epstein--Barr Virus Receptor. *Proceedings of the National Academy of Sciences*, **82**, 1490-1493.

Fuss,I.J., Boirivant,M., Lacy,B., & Strober,W. (2002) The interrelated roles of TGF-beta and IL-10 in the regulation of experimental colitis. *Journal of Immunology*, **168**, 900-908.

Gandhi,M.K., Lambley,E., Duraiswamy,J., Dua,U., Smith,C., Elliott,S., Gill,D., Marlton,P., Seymour,J., & Khanna,R. (2006c) Expression of LAG-3 by tumor-infiltrating lymphocytes is co-incident with the suppression of latent membrane antigen-specific CD8+ T-cell function in Hodgkin lymphoma patients. *Blood*.

Gandhi,M.K., Lambley,E., Duraiswamy,J., Dua,U., Smith,C., Elliott,S., Gill,D., Marlton,P., Seymour,J., & Khanna,R. (2006a) Expression of LAG-3 by tumor-infiltrating lymphocytes is co-incident with the suppression of latent membrane antigen-specific CD8+ T-cell function in Hodgkin lymphoma patients. *Blood*.

Gandhi,M.K., Lambley,E., Duraiswamy,J., Dua,U., Smith,C., Elliott,S., Gill,D., Marlton,P., Seymour,J., & Khanna,R. (2006b) Expression of LAG-3 by tumor-infiltrating lymphocytes is co-incident with the suppression of latent membrane antigen-specific CD8+ T-cell function in Hodgkin lymphoma patients. *Blood*.

Gavin,M.A., Torgerson,T.R., Houston,E., deRoos,P., Ho,W.Y., Stray-Pedersen,A., Ocheltree,E.L., Greenberg,P.D., Ochs,H.D., & Rudensky,A.Y. (2006c) Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 6659-6664.

Gavin,M.A., Torgerson,T.R., Houston,E., deRoos,P., Ho,W.Y., Stray-Pedersen,A., Ocheltree,E.L., Greenberg,P.D., Ochs,H.D., & Rudensky,A.Y. (2006b) Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 6659-6664.

Gavin,M.A., Torgerson,T.R., Houston,E., deRoos,P., Ho,W.Y., Stray-Pedersen,A., Ocheltree,E.L., Greenberg,P.D., Ochs,H.D., & Rudensky,A.Y. (2006a) Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 6659-6664.

Gerber,P., Lucas,S., Nonoyama,M., Perlin,E., & Goldstein,L. (1972) ORAL EXCRETION OF EPSTEIN-BARR VIRUS BY HEALTHY SUBJECTS AND PATIENTS WITH INFECTIOUS MONONUCLEOSIS. *The Lancet*, **300**, 988-989.

Gershon,R.K. (1975) Disquisition on Suppressor T-Cells. *Transplantation Reviews*, **26**, 170-185.

Ghosh,D. & Kieff,E. (1990) cis-acting regulatory elements near the Epstein-Barr virus latent-infection membrane protein transcriptional start site. *The Journal of Virology*, **64**, 1855-1858.

Glaser,R., Lang,C.M., Lee,K.J., Schuller,D.E., Jacobs,D., & Mcquattie,C. (1980) Attempt to Infect Nonmalignant Nasopharyngeal Epithelial-Cells from Humans and Squirrel-Monkeys with Epstein-Barr Virus. *Journal of the National Cancer Institute*, **64**, 1085-1090.

Gong,M., Ooka,T., Matsuo,T., & Kieff,E. (1987) Epstein-Barr virus glycoprotein homologous to herpes simplex virus gB. *The Journal of Virology*, **61**, 499-508.

Grazia Roncarolo,M., Gregori,S., Battaglia,M., Bacchetta,R., Fleischhauer,K., & Levings,M.K. (2006) Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological Reviews*, **212**, 28-50.

Grossman,S.R., Johannsen,E., Tong,X., Yalamanchili,R., & Kieff,E. (1994) The Epstein-Barr Virus Nuclear Antigen 2 Transactivator is Directed to Response Elements by the J{kappa} Recombination Signal Binding Protein. *Proceedings of the National Academy of Sciences*, **91**, 7568-7572.

Gutensohn,N. & Cole,P. (1980) Epidemiology of Hodgkins-Disease. *Seminars in Oncology*, **7**, 92-102.

Haan,K.M., Kwok,W.W., Longnecker,R., & Speck,P. (2000b) Epstein-Barr Virus Entry Utilizing HLA-DP or HLA-DQ as a Coreceptor. *The Journal of Virology*, **74**, 2451-2454.

Haan,K.M., Kwok,W.W., Longnecker,R., & Speck,P. (2000a) Epstein-Barr Virus Entry Utilizing HLA-DP or HLA-DQ as a Coreceptor. *The Journal of Virology*, **74**, 2451-2454.

Haan,K.M. & Longnecker,R. (2000) Coreceptor restriction within the HLA-DQ locus for Epstein-Barr virus infection. *Proceedings of the National Academy of Sciences*, **97**, 9252-9257.

Hammerschmidt,W. & Sugden,B. (1989) Genetic analysis of immortalizing functions of Epstein-ûBarr virus in human B lymphocytes. *Nature*, **340**, 393-397.

Hansson,M., Falk,K., & Ernberg,I. (1983) Epstein-Barr virus transformation of human pre-B cells. *The Journal of Experimental Medicine*, **158**, 616-622.

Haque,T., Wilkie,G.M., Jones,M.M., Higgins,C.D., Urquhart,G., Wingate,P., Burns,D., McAulay,K., Turner,M., Bellamy,C., Amlot,P.L., Kelly,D., MacGilchrist,A., Gandhi,M.K., Swerdlow,A.J., & Crawford,D.H. (2007a) Allogeneic cytotoxic T-

cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood*, **110**, 1123-1131.

Haque,T., Wilkie,G.M., Jones,M.M., Higgins,C.D., Urquhart,G., Wingate,P., Burns,D., McAulay,K., Turner,M., Bellamy,C., Amlot,P.L., Kelly,D., MacGilchrist,A., Gandhi,M.K., Swerdlow,A.J., & Crawford,D.H. (2007b) Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood*, **110**, 1123-1131.

Harada,S. & Kieff,E. (1997) Epstein-Barr virus nuclear protein LP stimulates EBNA-2 acidic domain- mediated transcriptional activation. *The Journal of Virology*, **71**, 6611-6618.

Hayday,A. & Tigelaar,R. (2003) Immunoregulation in the tissues by [gamma][delta] T cells. *Nature Reviews Immunology*, **3**, 233-242.

Heineman,T., Gong,M., Sample,J., & Kieff,E. (1988) Identification of the Epstein-Barr virus gp85 gene. *The Journal of Virology*, **62**, 1101-1107.

Henderson,S., Rowe,M., Gregory,C., Croom-Carter,D., Wang,F., Longnecker,R., Kieff,E., & Rickinson,A. (1991a) Induction of bcl-2 expression by epstein-barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell*, **65**, 1107-1115.

Henderson,S., Rowe,M., Gregory,C., Croom-Carter,D., Wang,F., Longnecker,R., Kieff,E., & Rickinson,A. (1991b) Induction of bcl-2 expression by epstein-barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell*, **65**, 1107-1115.

Henle,W., Diehl,V., Kohn,G., Zur Hausen,H., & Henle,G. (1967) Herpes-Type Virus and Chromosome Marker in Normal Leukocytes after Growth with Irradiated Burkitt Cells. *Science*, **157**, 1064-1065.

Hinuma,Y., Konn,M., Yamaguchi,J., Wudarski,D.J., Blakeslee,J.R., Jr., & Grace,J.T., Jr. (1967) Immunofluorescence and Herpes-Type Virus Particles in the P3HR-1 Burkitt Lymphoma Cell Line. *The Journal of Virology*, **1**, 1045-1051.

Hisaeda,H., Maekawa,Y., Iwakawa,D., Okada,H., Himeno,K., Kishihara,K., Tsukumo,S.i., & Yasutomo,K. (2004) Escape of malaria parasites from host immunity requires CD4+CD25+ regulatory T cells. *Nat Med*, **10**, 29-30.

Hislop,A.D., Annels,N.E., Gudgeon,N.H., Leese,A.M., & Rickinson,A.B. (2002) Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. *Journal of Experimental Medicine*, **195**, 893-905.

Hislop,A.D., Kuo,M., Drake-Lee,A.B., Akbar,A.N., Bergler,W., Hammerschmitt,N., Khan,N., Palendira,U., Leese,A.M., Timms,J.M., Bell,A.I., Buckley,C.D., & Rickinson,A.B. (2005a) Tonsillar homing of Epstein-Barr virus-specific CD8(+) T cells and the virus-host balance. *Journal of Clinical Investigation*, **115**, 2546-2555.

Hislop,A.D., Kuo,M., Drake-Lee,A.B., Akbar,A.N., Bergler,W., Hammerschmitt,N., Khan,N., Palendira,U., Leese,A.M., Timms,J.M., Bell,A.I., Buckley,C.D., & Rickinson,A.B. (2005b) Tonsillar homing of Epstein-Barr virus-specific CD8(+) T cells and the virus-host balance. *Journal of Clinical Investigation*, **115**, 2546-2555.

Hislop,A.D., Taylor,G.S., Sauce,D., & Rickinson,A.B. (2007) Cellular responses to viral infection in humans: Lessons from Epstein-Barr virus. *Annual Review of Immunology*, **25**, 587-617.

Hoffmann,P., Eder,R., Kunz-Schughart,L.A., Andreesen,R., & Edinger,M. (2004) Large-scale in vitro expansion of polyclonal human CD4+CD25high regulatory T cells. *Blood*, **104**, 895-903.

Hori,S., Carvalho,T.L., & Demengeot,J. (2002) CD25(+)CD4(+) regulatory T cells suppress CD4(+) T cell-mediated pulmonary hyperinflammation driven by Pneumocystis carinii in immunodeficient mice. *European Journal of Immunology*, **32**, 1282-1291.

Hori,S. & Sakaguchi,S. (2004) Foxp3: a critical regulator of the development and function of regulatory T cells. *Microbes and Infection*, **6**, 745-751.

Horohov,D.W., Moore,R.N., & Rouse,B.T. (1985) Regulation of Herpes-Simplex Virus-Specific Lymphoproliferation by Suppressor Cells. *Journal of Virology*, **56**, 1-6.

Hoshino,Y., Morishima,T., Kimura,H., Nishikawa,K., Tsurumi,T., & Kuzushima,K. (1999) Antigen-driven expansion and contraction of CD8(+)-activated T cells in primary EBV infection. *Journal of Immunology*, **163**, 5735-5740.

Hsu,D.H., Malefyt,R.D., Fiorentino,D.F., Dang,M.N., Vieira,P., Devries,J., Spits,H., Mosmann,T.R., & Moore,K.W. (1990b) Expression of Interleukin-10 Activity by Epstein-Barr-Virus Protein Bcrf1. *Science*, **250**, 830-832.

Hsu,D.H., Malefyt,R.D., Fiorentino,D.F., Dang,M.N., Vieira,P., Devries,J., Spits,H., Mosmann,T.R., & Moore,K.W. (1990a) Expression of Interleukin-10 Activity by Epstein-Barr-Virus Protein Bcrf1. *Science*, **250**, 830-832.

Huang,C.T., Workman,C.J., Flies,D., Pan,X.Y., Marson,A.L., Zhou,G., Hipkiss,E.L., Ravi,S., Kowalski,J., Levitsky,H.I., Powell,J.D., Pardoll,D.M., Drake,C.G., &

Vignali,D.A.A. (2004a) Role of LAG-3 in regulatory T cells. *Immunity*, **21**, 503-513.

Huang,C.T., Workman,C.J., Flies,D., Pan,X.Y., Marson,A.L., Zhou,G., Hipkiss,E.L., Ravi,S., Kowalski,J., Levitsky,H.I., Powell,J.D., Pardoll,D.M., Drake,C.G., & Vignali,D.A.A. (2004b) Role of LAG-3 in regulatory T cells. *Immunity*, **21**, 503-513.

Huen,D.S., Henderson,S.A., Croomcarter,D., & Rowe,M. (1995) The Epstein-Barr-Virus Latent Membrane Protein-1 (Lmp1) Mediates Activation of Nf-Kappa-B and Cell-Surface Phenotype Via 2 Effector Regions in Its Carboxy-Terminal Cytoplasmic Domain. *Oncogene*, **10**, 549-560.

Israele,V., Shirley,P., & Sixbey,J.W. (1991) Excretion of the Epstein-Barr-Virus from the Genital-Tract of Men. *Journal of Infectious Diseases*, **163**, 1341-1343.

Iwashiro,M., Messer,R.J., Peterson,K.E., Stromnes,I.M., Sugie,T., & Hasenkrug,K.J. (2001a) Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection. *Proceedings of the National Academy of Sciences*, **98**, 9226-9230.

Iwashiro,M., Messer,R.J., Peterson,K.E., Stromnes,I.M., Sugie,T., & Hasenkrug,K.J. (2001b) Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection. *Proceedings of the National Academy of Sciences*, **98**, 9226-9230.

Johannessen,I., Asghar,M., & Crawford,D.H. (2000) Essential role for T cells in human B-cell lymphoproliferative disease development in severe combined immunodeficient mice. *British Journal of Haematology*, **109**, 600-610.

Jonuleit,H., Schmitt,E., Stassen,M., Tuettenberg,A., Knop,J., & Enk,A.H. (2001) Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *Journal of Experimental Medicine*, **193**, 1285-1294.

Kanegane,H., Nomura,K., Miyawaki,T., & Tosato,G. (2002) Biological aspects of Epstein-Barr virus (EBV)-infected lymphocytes in chronic active EBV infection and associated malignancies. *Critical Reviews in Oncology/Hematology*, **44**, 239-249.

Kanegane,H., Wado,T., Nunogami,K., Seki,H., Taniguchi,N., & Tosato,G. (1996) Chronic persistent Epstein-Barr virus infection of natural killer cells and B cells associated with granular lymphocytes expansion. *British Journal of Haematology*, **95**, 116-122.

Karandikar,N.J., Vanderlugt,C.L., Walunas,T.L., Miller,S.D., & Bluestone,J.A. (1996) CTLA-4: A negative regulator of autoimmune disease. *Journal of Experimental Medicine*, **184**, 783-788.

Kaye,K.M., Izumi,K.M., & Kieff,E. (1993) Epstein-Barr Virus Latent Membrane Protein 1 is Essential for B-Lymphocyte Growth Transformation. *Proceedings of the National Academy of Sciences*, **90**, 9150-9154.

Khattri,R., Cox,T., Yasayko,S.A., & Ramsdell,F. (2003) An essential role for Scurfin in CD4(+)CD25(+) T regulatory cells. *Nature Immunology*, **4**, 337-342.

Kieff,E. & Rickinson,A. Epstein-Barr Virus and its Replication. In Fields: Virology. Fields B, Knipe D, and Howley P. 4, 2511-2573. 2001. Lippincott Williams & Wilkins: Philadelphia.
Ref Type: Serial (Book,Monograph)

Kim,J.M. & Rudensky,A. (2006) The role of the transcription factor Foxp3 in the development of regulatory T cells. *Immunological Reviews*, **212**, 86-98.

Kinter,A.L., Hennessey,M., Bell,A., Kern,S., Lin,Y., Daucher,M., Planta,M., McGlaughlin,M., Jackson,R., Ziegler,S.E., & Fauci,A.S. (2004c) CD25(+) CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *Journal of Experimental Medicine*, **200**, 331-343.

Kinter,A.L., Hennessey,M., Bell,A., Kern,S., Lin,Y., Daucher,M., Planta,M., McGlaughlin,M., Jackson,R., Ziegler,S.E., & Fauci,A.S. (2004a) CD25(+) CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *Journal of Experimental Medicine*, **200**, 331-343.

Kinter,A.L., Hennessey,M., Bell,A., Kern,S., Lin,Y., Daucher,M., Planta,M., McGlaughlin,M., Jackson,R., Ziegler,S.E., & Fauci,A.S. (2004b) CD25(+) CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *Journal of Experimental Medicine*, **200**, 331-343.

Kitagawa,N., Goto,M., Kurozumi,K., Maruo,S., Fukayama,M., Naoe,E., Yasukawa,M., Hino,K., Suzuki,T., Todo,S., & Takada,K. (2000) Epstein-Barr virus-encoded poly(A-) RNA supports Burkitt's Lymphoma growth through interleukin-10 induction. *Embo Journal*, **19**, 6742-6750.

Kursar,M., Bonhagen,K., Fensterle,J., Kohler,A., Hurwitz,R., Kamradt,T., Kaufmann,S.H.E., & Mittrucker,H.W. (2002) Regulatory CD4+CD25+ T Cells Restrict Memory CD8+ T Cell Responses. *The Journal of Experimental Medicine*, **196**, 1585-1592.

Lam & Crawford,D.H. (1991) The oncogenic potential of Epstein-Barr virus. *Critical Reviews in Oncogenesis*, **2**, 229-245.

Lan,R.Y., Ansari,A.A., Lian,Z.X., & Gershwin,M.E. (2005a) Regulatory T cells: Development, function and role in autoimmunity. *Autoimmunity Reviews*, **4**, 351-363.

Lan,R.Y., Ansari,A.A., Lian,Z.X., & Gershwin,M.E. (2005b) Regulatory T cells: Development, function and role in autoimmunity. *Autoimmunity Reviews*, **4**, 351-363.

Landais,E., Saulquin,X., Scotet,E., Trautmann,L., Peyrat,M.A., Yates,J.L., Kwok,W.W., Bonneville,M., & Houssaint,E. (2004) Direct killing of Epstein-Barr virus (EBV)-infected B cells by CD4 T cells directed against the EBV lytic protein BHRF1. *Blood*, **103**, 1408-1416.

Leach,D.R., Krummel,M.F., & Allison,J.P. (1996) Enhancement of Antitumor Immunity by CTLA-4 Blockade. *Science*, **271**, 1734-1736.

Lemon,S., HUTT,L.M., SHAW,J.E., Li,J.L., & Pagano,J.S. (1977) Replication of EBV in epithelial cells during infectious mononucleosis. *Nature*, **268**, 268-270.

Levine,P.H., Ablashi,D.V., Berard,C.W., Carbone,P.P., Waggoner,D.E., & Malan,L. (1971) Elevated Antibody Titers to Epstein-Barr Virus in Hodgkins Disease. *Cancer*, **27**, 416-&.

Levy,Y., Durier,C., Krzysiek,R., Rabian,C., Capitant,C., Lascaux,A.S., Michon,C., Oksenhendler,E., Weiss,L., Gastaut,J.A., Goujard,C., Rouzioux,C., Maral,J., Delfraissy,J.F., Emilie,D., & Aboulker,J.P. (2003) Effects of interleukin-2 therapy combined with highly active antiretroviral therapy on immune restoration in HIV-1 infection: a randomized controlled trial. *Aids*, **17**, 343-351.

Li,Q., Spriggs,M.K., Kovats,S., Turk,S.M., Comeau,M.R., Nepom,B., & Hutt-Fletcher,L.M. (1997) Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes. *The Journal of Virology*, **71**, 4657-4662.

Li,Q., Turk,S.M., & Hutt-Fletcher,L.M. (1995) The Epstein-Barr virus (EBV) BZLF2 gene product associates with the gH and gL homologs of EBV and carries an epitope critical to infection of B cells but not of epithelial cells. *The Journal of Virology*, **69**, 3987-3994.

Liu,C.Y., Battaglia,M., Lee,S.H., Sun,Q.H., Aster,R.H., & Visentin,G.P. (2005) Platelet factor 4 differentially modulates CD4(+)CD25(+) (regulatory) versus CD4(+)CD25(-) (nonregulatory) T cells. *Journal of Immunology*, **174**, 2680-2686.

Liu,H.Y., Hu,B., Xu,D.M., & Liew,F.Y. (2003) CD4+CD25+ regulatory T cells cure murine colitis: The role of IL-10, TGF-beta, and CTLA4. *Journal of Immunology*, **171**, 5012-5017.

Longnecker,R. (2000) Epstein-Barr virus latency: LMP2, a regulator or means for Epstein-Barr virus persistence? *Advances in Cancer Research, Vol 79*, **79**, 175-200.

Lozzio,C.B. & Lozzio,B.B. (1975) Human Chronic Myelogenous Leukemia Cell-Line with Positive Philadelphia Chromosome. *Blood*, **45**, 321-334.

Lu,L., Werneck,M.B.F., & Cantor,H. (2006) The immunoregulatory effects of Qa-1. *Immunological Reviews*, **212**, 51-59.

Luhder,F., Hoglund,P., Allison,J.P., Benoist,C., & Mathis,D. (1998) Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) regulates the unfolding of autoimmune diabetes. *Journal of Experimental Medicine*, **187**, 427-432.

Lundgren,A., Suri-Payer,E., Enarsson,K., Svennerholm,A.M., & Lundin,B.S. (2003) Helicobacterpylori-Specific CD4+ CD25high Regulatory T Cells Suppress Memory T-Cell Responses to H. pylori in Infected Individuals. *Infection and Immunity*, **71**, 1755-1762.

MacDonald,A.J., Duffy,M., Brady,M.T., McKiernan,S., Hall,W., Hegarty,J., Curry,M., & Mills,K.H.G. (2002b) CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. *Journal of Infectious Diseases*, **185**, 720-727.

MacDonald,A.J., Duffy,M., Brady,M.T., McKiernan,S., Hall,W., Hegarty,J., Curry,M., & Mills,K.H.G. (2002a) CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. *Journal of Infectious Diseases*, **185**, 720-727.

MacMahon,B. (1966) Epidemiology of Hodgkin's Disease. *Cancer Research*, **26**, 1189-1200.

Mahdavi,A. & Monk,B.J. (2005a) Vaccines against human papillomavirus and cervical cancer: Promises and challenges. *Oncologist*, **10**, 528-538.

Mahdavi,A. & Monk,B.J. (2005b) Vaccines against human papillomavirus and cervical cancer: Promises and challenges. *Oncologist*, **10**, 528-538.

Maia,D.M. & Peace-Brewer,A.L. (2000) Chronic, active Epstein-Barr virus infection. *Current Opinion in Hematology*, **7**, 59-63.

Maloy,K.J. & Powrie,F. (2001) Regulatory T cells in the control of immune pathology. *Nature Immunology*, **2**, 816-822.

Maloy,K.J., Salaun,L., Cahill,R., Dougan,G., Saunders,N.J., & Powrie,F. (2003) CD4+CD25+ TR Cells Suppress Innate Immune Pathology Through Cytokine-dependent Mechanisms. *The Journal of Experimental Medicine*, **197**, 111-119.

Mannick,J.B., Cohen,J.I., Birkenbach,M., Marchini,A., & Kieff,E. (1991) The Epstein-Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation. *The Journal of Virology*, **65**, 6826-6837.

Marafioti,T., Hummel,M., Foss,H.D., Laumen,H., Korbjuhn,P., Anagnostopoulos,I., Lammert,H., Demel,G., Theil,J., Wirth,T., & Stein,H. (2000) Hodgkin and Reed-Sternberg cells represent an expansion of a single clone originating from a germinal center B-cell with functional immunoglobulin gene rearrangements but defective immunoglobulin transcription. *Blood*, **95**, 1443-1450.

Marchini,A., Kieff,E., & Longnecker,R. (1993) Marker rescue of a transformation-negative Epstein-Barr virus recombinant from an infected Burkitt lymphoma cell line: a method useful for analysis of genes essential for transformation. *The Journal of Virology*, **67**, 606-609.

Marchini,A., Longnecker,R., & Kieff,E. (1992) Epstein-Barr virus (EBV)-negative B-lymphoma cell lines for clonal isolation and replication of EBV recombinants. *The Journal of Virology*, **66**, 4972-4981.

Marshall,D. & Sample,C. (1995) Epstein-Barr virus nuclear antigen 3C is a transcriptional regulator. *The Journal of Virology*, **69**, 3624-3630.

Marshall,N.A., Christie,L.E., Munro,L.R., Culligan,D.J., Johnston,P.W., Barker,R.N., & Vickers,M.A. (2004c) Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood*, **103**, 1755-1762.

Marshall,N.A., Christie,L.E., Munro,L.R., Culligan,D.J., Johnston,P.W., Barker,R.N., & Vickers,M.A. (2004b) Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood*, **103**, 1755-1762.

Marshall,N.A., Christie,L.E., Munro,L.R., Culligan,D.J., Johnston,P.W., Barker,R.N., & Vickers,M.A. (2004a) Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood*, **103**, 1755-1762.

Marshall,N.A., Vickers,M.A., & Barker,R.N. (2003i) Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. *J Immunol*, **170**, 6183-6189.

Marshall,N.A., Vickers,M.A., & Barker,R.N. (2003d) Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. *J Immunol*, **170**, 6183-6189.

Marshall,N.A., Vickers,M.A., & Barker,R.N. (2003e) Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. *J Immunol*, **170**, 6183-6189.

Marshall,N.A., Vickers,M.A., & Barker,R.N. (2003f) Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. *J Immunol*, **170**, 6183-6189.

Marshall,N.A., Vickers,M.A., & Barker,R.N. (2003g) Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. *J Immunol*, **170**, 6183-6189.

Marshall,N.A., Vickers,M.A., & Barker,R.N. (2003h) Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. *J Immunol*, **170**, 6183-6189.

Marshall,N.A., Vickers,M.A., & Barker,R.N. (2003c) Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. *J Immunol*, **170**, 6183-6189.

Marshall,N.A., Vickers,M.A., & Barker,R.N. (2003b) Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. *J Immunol*, **170**, 6183-6189.

Marshall,N.A., Vickers,M.A., & Barker,R.N. (2003a) Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. *J Immunol*, **170**, 6183-6189.

Marshall,N.A., Culligan,D.J., Tighe,J., Johnston,P.W., Barker,R.N., & Vickers,M.A. (2007b) The relationships between Epstein-Barr virus latent membrane protein 1 and regulatory T cells in Hodgkin's lymphoma. *Experimental Hematology*, **35**, 596-604.

Marshall,N.A., Culligan,D.J., Tighe,J., Johnston,P.W., Barker,R.N., & Vickers,M.A. (2007a) The relationships between Epstein-Barr virus latent membrane protein 1 and regulatory T cells in Hodgkin's lymphoma. *Experimental Hematology*, **35**, 596-604.

Mason,D. & Powrie,F. (1998) Control of immune pathology by regulatory T cells. *Current Opinion in Immunology*, **10**, 649-655.

McGeachy,M.J., Stephens,L.A., & Anderton,S.M. (2005) Natural recovery and protection from autoimmune encephalomyelitis: Contribution of CD4(+)CD25(+) regulatory cells within the central nervous system. *Journal of Immunology*, **175**, 3025-3032.

McGeoch,D.J. & Davison,A.J. (1986) DNA sequence of the herpes simplex virus type 1 gene encoding glycoprotein gH, and identification of homologues in the genomes of varicella-zoster virus and Epstein-Barr virus. *Nucleic Acids Research*, **14**, 4281-4292.

McGuirk,P., McCann,C., & Mills,K.H.G. (2002) Pathogen-specific T Regulatory 1 Cells Induced in the Respiratory Tract by a Bacterial Molecule that Stimulates Interleukin 10 Production by Dendritic Cells: A Novel Strategy for Evasion of Protective T Helper Type 1 Responses by Bordetella pertussis. *The Journal of Experimental Medicine*, **195**, 221-231.

McHugh,R.S., Whitters,M.J., Piccirillo,C.A., Young,D.A., Shevach,E.M., Collins,M., & Byrne,M.C. (2002c) CD4+CD25+ Immunoregulatory T Cells: Gene Expression Analysis Reveals a Functional Role for the Glucocorticoid-Induced TNF Receptor. *Immunity*, **16**, 311-323.

McHugh,R.S., Whitters,M.J., Piccirillo,C.A., Young,D.A., Shevach,E.M., Collins,M., & Byrne,M.C. (2002b) CD4+CD25+ Immunoregulatory T Cells: Gene Expression Analysis Reveals a Functional Role for the Glucocorticoid-Induced TNF Receptor. *Immunity*, **16**, 311-323.

McHugh,R.S., Whitters,M.J., Piccirillo,C.A., Young,D.A., Shevach,E.M., Collins,M., & Byrne,M.C. (2002a) CD4+CD25+ Immunoregulatory T Cells: Gene Expression Analysis Reveals a Functional Role for the Glucocorticoid-Induced TNF Receptor. *Immunity*, **16**, 311-323.

Miller,G., NIEDERMA.JC, & Andrews,L.L. (1973) Prolonged Oropharyngeal Excretion of Epstein-Barr Virus After Infectious Mononucleosis. *New England Journal of Medicine*, **288**, 229-232.

Miller,G., Shope,T., Lisco,H., Stitt,D., & Lipman,M. (1972) Epstein-Barr Virus: Transformation, Cytopathic Changes, and Viral Antigens in Squirrel Monkey

and Marmoset Leukocytes. *Proceedings of the National Academy of Sciences*, **69**, 383-387.

Molesworth,S.J., Lake,C.M., Borza,C.M., Turk,S.M., & Hutt-Fletcher,L.M. (2000) Epstein-Barr Virus gH Is Essential for Penetration of B Cells but Also Plays a Role in Attachment of Virus to Epithelial Cells. *The Journal of Virology*, **74**, 6324-6332.

Montagnoli,C., Bacci,A., Bozza,S., Gaziano,R., Mosci,P., Sharpe,A.H., & Romani,L. (2002) B7/CD28-Dependent CD4+CD25+ Regulatory T Cells Are Essential Components of the Memory-Protective Immunity to Candida albicans. *The Journal of Immunology*, **169**, 6298-6308.

Moss,C., Friedmann,P.S., Shuster,S., & Simpson,J.M. (1985) Susceptibility and Amplification of Sensitivity in Contact-Dermatitis. *Clinical and Experimental Immunology*, **61**, 232-241.

Mueller,N., Evans,A., Harris,N.L., Comstock,G.W., Jellum,E., Magnus,K., Orentreich,N., Polk,B.F., & Vogelman,J. (1989) Hodgkin's disease and Epstein-Barr virus. Altered antibody pattern before diagnosis. *The New England Journal of Medicine*, **320**, 689-695.

Murphy,D.B., Herzenberg,L.A., Okumura,K., Herzenberg,L.A., & McDevitt,H.O. (1976) A new I subregion (I-J) marked by a locus (Ia-4) controlling surface determinants on suppressor T lymphocytes. *The Journal of Experimental Medicine*, **144**, 699-712.

Nemerow,G.R., Mold,C., Schwend,V.K., Tollefson,V., & Cooper,N.R. (1987) Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. *The Journal of Virology*, **61**, 1416-1420.

Nitsche,F., Bell,A., & Rickinson,A. (1997) Epstein-Barr virus leader protein enhances EBNA-2-mediated transactivation of latent membrane protein 1 expression: a role for the W1W2 repeat domain. *The Journal of Virology*, **71**, 6619-6628.

O'Connor,R.A., Malpass,K.H., & Anderton,S.M. (2007) The inflamed central nervous system drives the activation and rapid proliferation of Foxp3(+) regulatory T cells. *Journal of Immunology*, **179**, 958-966.

O'Garra,A. & Vieira,P. (2004) Regulatory T cells and mechanisms of immune system control. *Nat Med*, **10**, 801-805.

Ohga,S., Matsuzaki,A., Nishizaki,M., Nagashima,T., Kai,T., Suda,M., & Ueda,K. (1993) Inflammatory Cytokines in Virus-Associated Hemophagocytic Syndrome - Interferon-Gamma As A Sensitive Indicator of Disease-Activity. *American Journal of Pediatric Hematology Oncology*, **15**, 291-298.

Okumura,K., Herzenberg,L.A., Murphy,D.B., McDevitt,H.O., & Herzenberg,L.A. (1976) Selective expression of H-2 (i-region) loci controlling determinants on helper and suppressor T lymphocytes. *The Journal of Experimental Medicine*, **144**, 685-698.

Oswald-Richter,K., Grill,S.M., Shariat,N., Leelawong,M., Sundrud,M.S., Haas,D.W., & Unutmaz,D. (2004b) HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *Plos Biology*, **2**, 955-966.

Oswald-Richter,K., Grill,S.M., Shariat,N., Leelawong,M., Sundrud,M.S., Haas,D.W., & Unutmaz,D. (2004a) HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *Plos Biology*, **2**, 955-966.

Pallesen,G., Hamiltondutoit,S.J., Rowe,M., & Young,L.S. (1991) Expression of Epstein-Barr-Virus Latent Gene-Products in Tumor-Cells of Hodgkins-Disease. *Lancet*, **337**, 320-322.

Pegtel,D.M., Middeldorp,J., & Thorley-Lawson,D.A. (2004) Epstein-Barr virus infection in ex vivo tonsil epithelial cell cultures of asymptomatic carriers. *Journal of Virology*, **78**, 12613-12624.

Pellett,P.E., Biggin,M.D., Barrell,B., & Roizman,B. (1985) Epstein-Barr virus genome may encode a protein showing significant amino acid and predicted secondary structure homology with glycoprotein B of herpes simplex virus 1. *The Journal of Virology*, **56**, 807-813.

Peng,G., Li,S., Wu,W., Sun,Z., Chen,Y., & Chen,Z. (2008) Circulating CD4+ CD25+ regulatory T cells correlate with chronic hepatitis B infection. *Immunology*, **123**, 57-65

Penman,H.G. (1970) Fatal infectious mononucleosis: a critical review. *Journal of Clinical Pathology*, **23**, 765-771.

Perezblas,M., Regueiro,J.R., Ruizcontreras,J., & Arnaizvillena,A. (1992a) Lymphocyte-T Anergy During Acute Infectious-Mononucleosis Is Restricted to the Clonotypic Receptor Activation Pathway. *Clinical and Experimental Immunology*, **89**, 83-88.

Perezblas,M., Regueiro,J.R., Ruizcontreras,J., & Arnaizvillena,A. (1992b) Lymphocyte-T Anergy During Acute Infectious-Mononucleosis Is Restricted to the

Clonotypic Receptor Activation Pathway. *Clinical and Experimental Immunology*, **89**, 83-88.

Picchio,G.R., Kobayashi,R., Kirven,M., Baird,S.M., Kipps,T.J., & Mosier,D.E. (1992b) Heterogeneity Among Epstein-Barr Virus-Seropositive Donors in the Generation of Immunoblastic B-Cell Lymphomas in Scid Mice Receiving Human Peripheral-Blood Leukocyte Grafts. *Cancer Research*, **52**, 2468-2477.

Picchio,G.R., Kobayashi,R., Kirven,M., Baird,S.M., Kipps,T.J., & Mosier,D.E. (1992a) Heterogeneity Among Epstein-Barr Virus-Seropositive Donors in the Generation of Immunoblastic B-Cell Lymphomas in Scid Mice Receiving Human Peripheral-Blood Leukocyte Grafts. *Cancer Research*, **52**, 2468-2477.

Powrie,F. (1995) T-Cells in Inflammatory Bowel-Disease - Protective and Pathogenic Roles. *Immunity*, **3**, 171-174.

Precopio,M.L., Sullivan,J.L., Willard,C., Somasundaran,M., & Luzuriaga,K. (2003a) Differential kinetics and specificity of EBV-specific CD4(+) and CD8(+) T cells during primary infection. *Journal of Immunology*, **170**, 2590-2598.

Precopio,M.L., Sullivan,J.L., Willard,C., Somasundaran,M., & Luzuriaga,K. (2003b) Differential kinetics and specificity of EBV-specific CD4(+) and CD8(+) T cells during primary infection. *Journal of Immunology*, **170**, 2590-2598.

Precopio,M.L., Sullivan,J.L., Willard,C., Somasundaran,M., & Luzuriaga,K. (2003c) Differential kinetics and specificity of EBV-specific CD4(+) and CD8(+) T cells during primary infection. *Journal of Immunology*, **170**, 2590-2598.

Raab-Traub,N. (2002b) Epstein-Barr virus in the pathogenesis of NPC. *Seminars in Cancer Biology*, **12**, 431-441.

Raab-Traub,N. (2002a) Epstein-Barr virus in the pathogenesis of NPC. *Seminars in Cancer Biology*, **12**, 431-441.

Racanelli,V. & Manigold,T. (2007) Presentation of HCV antigens to naive CD8+T cells: Why the where, when, what and how are important for virus control and infection outcome. *Clinical Immunology*, **124**, 5-12.

Raghavan,S., FREDRIKSSON,M., SVENNERHOLM,A.M., HOLMGREN,J., & Suri-Payer,E. (2003) Absence of CD4+CD25+ regulatory T cells is associated with a loss of regulation leading to increased pathology in Helicobacter pylori-infected mice. *Clinical & Experimental Immunology*, **132**, 393-400.

Read,S., Malmstrom,V., & Powrie,F. (2000a) Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory

cells that control intestinal inflammation. *Journal of Experimental Medicine*, **192**, 295-302.

Read,S., Malmstrom,V., & Powrie,F. (2000b) Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *Journal of Experimental Medicine*, **192**, 295-302.

Read,S., Malmstrom,V., & Powrie,F. (2000c) Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *Journal of Experimental Medicine*, **192**, 295-302.

Read,S., Mauze,S., Asseman,C., Bean,A., Coffman,R., & Powrie,F. (1998) CD38(+) CD45RB(low) CD4(+) T cells: a population of T cells with immune regulatory activities in vitro. *European Journal of Immunology*, **28**, 3435-3447.

Rehermann,B., Chang,K.M., McHutchison,J., Kokka,F., Houghton,M., Rice,C.M., & Chisari,F.V. (1996) Differential cytotoxic T-lymphocyte responsiveness to the hepatitis B and C viruses in chronically infected patients. *Journal of Virology*, **70**, 7092-7102.

Rickinson,A.B. (1986) Chronic, Symptomatic Epstein-Barr Virus-Infections. *Immunology Today*, **7**, 13-14.

Rickinson,A.B., Epstein,M.A., & Crawford,D.H. (1975) Absence of Infectious Epstein-Barr Virus in Blood in Acute Infectious-Mononucleosis. *Nature*, **258**, 236-238.

Rifa'i,M., Kawamoto,Y., Nakashima,I., & Suzuki,H. (2004) Essential Roles of CD8+CD122+ Regulatory T Cells in the Maintenance of T Cell Homeostasis. *The Journal of Experimental Medicine*, **200**, 1123-1134.

Robertson,E.S., Grossman,S., Johannsen,E., Miller,C., Lin,J., Tomkinson,B., & Kieff,E. (1995) Epstein-Barr virus nuclear protein 3C modulates transcription through interaction with the sequence-specific DNA-binding protein J kappa. *The Journal of Virology*, **69**, 3108-3116.

Robertson,E.S., Lin,J., & Kieff,E. (1996) The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBPJ(kappa). *The Journal of Virology*, **70**, 3068-3074.

Roizman,B. & Baines,J. (1991) The diversity and unity of herpesviridae. *Comparative Immunology, Microbiology and Infectious Diseases*, **14**, 63-79.

Roizmann,B., Desrosiers,R.C., Fleckenstein,B., Lopez,C., Minson,A.C., & Studdert,M.J. (1992) The Family Herpesviridae - An Update. *Archives of Virology*, **123**, 425-449.

Roncador,G., Brown,P.J., Maestre,L., Hue,S., Martinez-Torrecuadrada,J.L., Ling,K.L., Pratap,S., Toms,C., Fox,B.C., Cerundolo,V., Powrie,F., & Banham,A.H. (2005) Analysis of FOXP3 protein expression in human CD4(+)CD25(+) regulatory T cells at the single-cell level. *European Journal of Immunology*, **35**, 1681-1691.

Rouse,B.T. & Horohov,D.W. (1986) Immunosuppression in Viral-Infections. *Reviews of Infectious Diseases*, **8**, 850-873.

Rouse,B.T. & Suvas,S. (2004b) Regulatory cells and infectious agents: Detentes cordiale and contraire. *Journal of Immunology*, **173**, 2211-2215.

Rouse,B.T. & Suvas,S. (2004c) Regulatory cells and infectious agents: Detentes cordiale and contraire. *Journal of Immunology*, **173**, 2211-2215.

Rouse,B.T. & Suvas,S. (2004a) Regulatory cells and infectious agents: Detentes cordiale and contraire. *Journal of Immunology*, **173**, 2211-2215.

Rouse,B.T., Sarangi,P.P., & Suvas,S. (2006) Regulatory T cells in virus infections. *Immunological Reviews*, **212**, 272-286.

Rowe,M., Khanna,R., Jacob,C.A., Argaet,V., Kelly,A., Powis,S., Belich,M., Croomcarter,D., Lee,S., Burrows,S.R., Trowsdale,J., Moss,D.J., & Rickinson,A.B. (1995) Restoration of Endogenous Antigen-Processing in Burkitts-Lymphoma Cells by Epstein-Barr-Virus Latent Membrane Protein-1 - Coordinate Up-Regulation of Peptide Transporters and Hla-Class-I Antigen Expression. *European Journal of Immunology*, **25**, 1374-1384.

Rowe,M., Rowe,D.T., Gregory,C.D., Young,L.S., Farrell,P.J., Rupani,H., & Rickinson,A.B. (1987) Differences in B-Cell Growth Phenotype Reflect Novel Patterns of Epstein-Barr-Virus Latent Gene-Expression in Burkitt-Lymphoma Cells. *Embo Journal*, **6**, 2743-2751.

Rubin,A. (1966) Brief Note Lymphocyte RNA Synthesis in Infectious Mononucleosis: The Response to Phytohemagglutinin in Vitro. *Blood*, **28**, 602-605.

Rushbrook,S.M., Ward,S.M., Unitt,E., Vowler,S.L., Lucas,M., Klenerman,P., & Alexander,G.J.M. (2005d) Regulatory T Cells Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Persistent Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7852-7859.

Rushbrook,S.M., Ward,S.M., Unitt,E., Vowler,S.L., Lucas,M., Klenerman,P., & Alexander,G.J.M. (2005e) Regulatory T Cells Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Persistent Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7852-7859.

Rushbrook,S.M., Ward,S.M., Unitt,E., Vowler,S.L., Lucas,M., Klenerman,P., & Alexander,G.J.M. (2005g) Regulatory T Cells Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Persistent Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7852-7859.

Rushbrook,S.M., Ward,S.M., Unitt,E., Vowler,S.L., Lucas,M., Klenerman,P., & Alexander,G.J.M. (2005a) Regulatory T Cells Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Persistent Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7852-7859.

Rushbrook,S.M., Ward,S.M., Unitt,E., Vowler,S.L., Lucas,M., Klenerman,P., & Alexander,G.J.M. (2005f) Regulatory T Cells Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Persistent Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7852-7859.

Rushbrook,S.M., Ward,S.M., Unitt,E., Vowler,S.L., Lucas,M., Klenerman,P., & Alexander,G.J.M. (2005c) Regulatory T Cells Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Persistent Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7852-7859.

Rushbrook,S.M., Ward,S.M., Unitt,E., Vowler,S.L., Lucas,M., Klenerman,P., & Alexander,G.J.M. (2005b) Regulatory T Cells Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Persistent Hepatitis C Virus Infection. *The Journal of Virology*, **79**, 7852-7859.

Sakaguchi,S. (2000) Regulatory T cells: Key controllers of immunologic self-tolerance. *Cell*, **101**, 455-458.

Sakaguchi,S. (2004) Naturally arising CD4(+) regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annual Review of Immunology*, **22**, 531-562.

Sakaguchi,S. (2005) Naturally arising Foxp3-expressing CD25(+) CD4(+) regulatory T cells in immunological tolerance to self and non-self. *Nature Immunology*, **6**, 345-352.

Sakaguchi,S., Sakaguchi,N., Asano,M., Itoh,M., & Toda,M. (1995a) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *The Journal of Immunology*, **155**, 1151-1164.

Sakaguchi,S., Sakaguchi,N., Asano,M., Itoh,M., & Toda,M. (1995b) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *The Journal of Immunology*, **155**, 1151-1164.

Sakaguchi,S., Ono,M., Setoguchi,R., Yagi,H., Hori,S., Fehervari,Z., Shimizu,J., Takahashi,T., & Nomura,T. (2006) Foxp3+CD25+CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunological Reviews*, **212**, 8-27.

Sample,J., Lancz,G., & Nonoyama,M. (1986) Mapping of genes in BamHI fragment M of Epstein-Barr virus DNA that may determine the fate of viral infection. *The Journal of Virology*, **57**, 145-154.

Sereti,I., Imamichi,H., Natarajan,V., Imamichi,T., Ramchandani,M.S., Badralmaa,Y., Berg,S.C., Metcalf,J.A., Hahn,B.K., Shen,J.M., Powers,A., Davey,R.T., Kovacs,J.A., Shevach,E.M., & Lane,H.C. (2005) In vivo expansion of CD4+CD45RO-CD25+ T cells expressing foxP3 in IL-2-treated HIV-infected patients. *Journal of Clinical Investigation*, **115**, 1839-1847.

Setoguchi,R., Hori,S., Takahashi,T., & Sakaguchi,S. (2005) Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *Journal of Experimental Medicine*, **201**, 723-735.

Shapiro,I.M. & Volsky,D.J. (1983) Infection of Normal Human Epithelial Cells by Epstein-Barr Virus. *Science*, **219**, 1225-1228.

Shapiro,I.M., Volsky,D.J., Saemundsen,A.K., Anisimova,E., & Klein,G. (1982) Infection of the human T-cell-derived leukemia line Molt-4 by Epstein-Barr virus (EBV): Induction of EBV-determined antigens and virus reproduction. *Virology*, **120**, 171-181.

Sheldon,P.J., Hemsted,E.H., PAPAMICH.M, & Holborow,E.J. (1973) Thymic Origin of Atypical Lymphoid-Cells in Infectious Mononucleosis. *Lancet*, **1**, 1153-1155.

Shevach,E.M. (2000) Regulatory T cells in autoimmmunity. *Annual Review of Immunology*, **18**, 423-449.

Shevach,E.M. (2002a) CD4+CD25+ SUPPRESSOR T CELLS: MORE QUESTIONS THAN ANSWERS. *Nature Reviews Immunology*, **2**, 389-400.

Shevach,E.M. (2002b) CD4+CD25+ SUPPRESSOR T CELLS: MORE QUESTIONS THAN ANSWERS. *Nature Reviews Immunology*, **2**, 389-400.

Shimizu,J., Yamazaki,S., Takahashi,T., Ishida,Y., & Sakaguchi,S. (2002d) Stimulation of CD25+CD4+regulatory T cells through GITR breaks immunological self-tolerance. *Nature Immunology*, **3**, 135-142.

Shimizu,J., Yamazaki,S., Takahashi,T., Ishida,Y., & Sakaguchi,S. (2002c) Stimulation of CD25+CD4+regulatory T cells through GITR breaks immunological self-tolerance. *Nature Immunology*, **3**, 135-142.

Shimizu,J., Yamazaki,S., Takahashi,T., Ishida,Y., & Sakaguchi,S. (2002a) Stimulation of CD25+CD4+regulatory T cells through GITR breaks immunological self-tolerance. *Nature Immunology*, **3**, 135-142.

Shimizu,J., Yamazaki,S., Takahashi,T., Ishida,Y., & Sakaguchi,S. (2002b) Stimulation of CD25+CD4+regulatory T cells through GITR breaks immunological self-tolerance. *Nature Immunology*, **3**, 135-142.

Siddiqui,A. (1983) Hepatitis B Virus DNA in Kaposi Sarcoma. *Proceedings of the National Academy of Sciences*, **80**, 4861-4864.

Sixbey,J.W., Nedrud,J.G., Raab-Traub,N., Hanes,R.A., & Pagano,J.S. (1984) Epstein-Barr virus replication in oropharyngeal epithelial cells. *The New England Journal of Medicine*, **310**, 1225-1230.

Sixbey,J.W., Davis,D.S., Young,L.S., Hutt-Fletcher,L., Tedder,T.F., & Rickinson,A.B. (1987) Human Epithelial Cell Expression of an Epstein-barr Virus Receptor. *Journal of General Virology*, **68**, 805-811.

Sixbey,J.W., Vesterinen,E.H., Nedrud,J.G., Raab-Traub,N., Walton,L.A., & Pagano,J.S. (1983a) Replication of Epstein-Barr virus in human epithelial cells infected in vitro. *Nature*, **306**, 480-483.

Sixbey,J.W., Vesterinen,E.H., Nedrud,J.G., Raab-Traub,N., Walton,L.A., & Pagano,J.S. (1983b) Replication of Epstein-Barr virus in human epithelial cells infected in vitro. *Nature*, **306**, 480-483.

Sixbey,J., Lemon,S., & Pagano,J. (1986) A SECOND SITE FOR EPSTEIN-BARR VIRUS SHEDDING: THE UTERINE CERVIX. *The Lancet*, **328**, 1122-1124.

Sonoda,K.H., Faunce,D.E., Taniguchi,M., Exley,M., Balk,S., & Stein-Streilein,J. (2001) NK T cell-derived IL-10 is essential for the differentiation of antigen-specific T regulatory cells in systemic tolerance. *Journal of Immunology*, **166**, 42-50.

Spriggs,M.K., Armitage,R.J., Comeau,M.R., Strockbine,L., Farrah,T., Macduff,B., Ulrich,D., Alderson,M.R., Mullberg,J., & Cohen,J.I. (1996) The extracellular

domain of the Epstein-Barr virus BZLF2 protein binds the HLA-DR beta chain and inhibits antigen presentation. *The Journal of Virology*, **70**, 5557-5563.

Stephens,G.L., McHugh,R.S., Whitters,M.J., Young,D.A., Luxenberg,D., Carreno,B.M., Collins,M., & Shevach,E.M. (2004) Engagement of Glucocorticoid-Induced TNFR Family-Related Receptor on Effector T Cells by its Ligand Mediates Resistance to Suppression by CD4+CD25+ T Cells. *The Journal of Immunology*, **173**, 5008-5020.

Steven,N.M., Annels,N.E., Kumar,A., Leese,A.M., Kurilla,M.G., & Rickinson,A.B. (1997b) Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T cell response. *Journal of Experimental Medicine*, **185**, 1605-1617.

Steven,N.M., Annels,N.E., Kumar,A., Leese,A.M., Kurilla,M.G., & Rickinson,A.B. (1997a) Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T cell response. *Journal of Experimental Medicine*, **185**, 1605-1617.

Steven,N.M., Leese,A.M., Annels,N.E., Lee,S.P., & Rickinson,A.B. (1996a) Epitope focusing in the primary cytotoxic T cell response to Epstein-Barr virus and its relationship to T cell memory. *Journal of Experimental Medicine*, **184**, 1801-1813.

Steven,N.M., Leese,A.M., Annels,N.E., Lee,S.P., & Rickinson,A.B. (1996b) Epitope focusing in the primary cytotoxic T cell response to Epstein-Barr virus and its relationship to T cell memory. *Journal of Experimental Medicine*, **184**, 1801-1813.

Straus,S.E. (1988) The Chronic Mononucleosis Syndrome. *Journal of Infectious Diseases*, **157**, 405-412.

Sugimoto,K., Ikeda,F., Stadanlick,J., Nunes,F.A., Alter,H., & Chang,K.M. (2003b) Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology*, **38**, 1437-1448.

Sugimoto,K., Ikeda,F., Stadanlick,J., Nunes,F.A., Alter,H., & Chang,K.M. (2003a) Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology*, **38**, 1437-1448.

Suri-Payer,E., Amar,A.Z., McHugh,R., Natarajan,K., Margulies,D.H., & Shevach,E.M. (1999) Post-thymectomy autoimmune gastritis: fine specificity and pathogenicity of anti-H/K ATPase-reactive T cells. *European Journal of Immunology*, **29**, 669-677.

Suvas,S., Azkur,A.K., Kim,B.S., Kumaraguru,U., & Rouse,B.T. (2004b) CD4(+) CD25(+) regulatory T cells control the severity of viral immunoinflammatory lesions. *Journal of Immunology*, **172**, 4123-4132.

Suvas,S., Azkur,A.K., Kim,B.S., Kumaraguru,U., & Rouse,B.T. (2004c) CD4(+) CD25(+) regulatory T cells control the severity of viral immunoinflammatory lesions. *Journal of Immunology*, **172**, 4123-4132.

Suvas,S., Azkur,A.K., Kim,B.S., Kumaraguru,U., & Rouse,B.T. (2004a) CD4(+) CD25(+) regulatory T cells control the severity of viral immunoinflammatory lesions. *Journal of Immunology*, **172**, 4123-4132.

Suvas,S., Kumaraguru,U., Pack,C.D., Lee,S., & Rouse,B.T. (2003a) CD4+CD25+T cells regulate virus-specific primary and memory CD8+T cell responses. *Journal of Experimental Medicine*, **198**, 889-901.

Suvas,S., Kumaraguru,U., Pack,C.D., Lee,S., & Rouse,B.T. (2003b) CD4+CD25+T cells regulate virus-specific primary and memory CD8+T cell responses. *Journal of Experimental Medicine*, **198**, 889-901.

Suzuki,T., Tahara,H., Narula,S., Moore,K.W., Robbins,P.D., & Lotze,M.T. (1995) Viral interleukin 10 (IL-10), the human herpes virus 4 cellular IL-10 homologue, induces local anergy to allogeneic and syngeneic tumors. *The Journal of Experimental Medicine*, **182**, 477-486.

Swaminathan,S., Tomkinson,B., & Kieff,E. (1991) Recombinant Epstein-Barr Virus with Small RNA (EBER) Genes Deleted Transforms Lymphocytes and Replicates in vitro. *Proceedings of the National Academy of Sciences*, **88**, 1546-1550.

Tada,T., Taniguchi,M., & David,C.S. (1976) Properties of the antigen-specific suppressive T-cell factor in the regulation of antibody response of the mouse. IV. Special subregion assignment of the gene(s) that codes for the suppressive T-cell factor in the H-2 histocompatibility complex. *The Journal of Experimental Medicine*, **144**, 713-725.

Tamaru,Y., Miyawaki,T., Iwai,K., Tsuji,T., Nibu,R., Yachie,A., Koizumi,S., & Taniguchi,N. (1993) Absence of Bcl-2 Expression by Activated Cd45Ro+ T-Lymphocytes in Acute Infectious-Mononucleosis Supporting Their Susceptibility to Programmed Cell-Death. *Blood*, **82**, 521-527.

Tan,L.C., Gudgeon,N., Annels,N.E., Hansasuta,P., O'Callaghan,C.A., Rowland-Jones,S., McMichael,A.J., Rickinson,A.B., & Callan,M.F.C. (1999a) A re-evaluation of the frequency of CD8(+) T cells specific for EBV in healthy virus carriers. *Journal of Immunology*, **162**, 1827-1835.

Tan,L.C., Gudgeon,N., Annels,N.E., Hansasuta,P., O'Callaghan,C.A., Rowland-Jones,S., McMichael,A.J., Rickinson,A.B., & Callan,M.F.C. (1999b) A re-evaluation of the frequency of CD8(+) T cells specific for EBV in healthy virus carriers. *Journal of Immunology*, **162**, 1827-1835.

Terabe,M. & Berzofsky,J.A. (2004) Immunoregulatory T cells in tumor immunity. *Current Opinion in Immunology*, **16**, 157-162.

Terabe,M., Matsui,S., Noben-Trauth,N., Chen,H.J., Watson,C., Donaldson,D.D., Carbone,D.P., Paul,W.E., & Berzofsky,J.A. (2000) NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nature Immunology*, **1**, 515-520.

Thimme,R., Oldach,D., Chang,K.M., Steiger,C., Ray,S.C., & Chisari,F.V. (2001) Determinants of viral clearance and persistence during acute hepatitis C virus infection. *Journal of Experimental Medicine*, **194**, 1395-1406.

Thomas,R., Macsween,K.F., McAulay,K., Clutterbuck,D., Anderson,R., Reid,S., Higgins,C.D., Swerdlow,A.J., Harrison,N., Williams,H., & Crawford,D.H. (2006a) Evidence of shared Epstein-Barr viral isolates between sexual partners, and low level EBV in genital secretions. *J Med.Virol.*, **78**, 1204-1209.

Thomas,R., Macsween,K.F., McAulay,K., Clutterbuck,D., Anderson,R., Reid,S., Higgins,C.D., Swerdlow,A.J., Harrison,N., Williams,H., & Crawford,D.H. (2006b) Evidence of shared Epstein-Barr viral isolates between sexual partners, and low level EBV in genital secretions. *J Med.Virol.*, **78**, 1204-1209.

Thorley-Lawson,D.A. & Geilinger,K. (1980) Monoclonal Antibodies against the Major Glycoprotein (gp350/220) of Epstein-Barr Virus Neutralize Infectivity. *Proceedings of the National Academy of Sciences*, **77**, 5307-5311.

Thornton,A.M. & Shevach,E.M. (1998a) CD4(+)CD25(+) immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *Journal of Experimental Medicine*, **188**, 287-296.

Thornton,A.M. & Shevach,E.M. (1998b) CD4(+)CD25(+) immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *Journal of Experimental Medicine*, **188**, 287-296.

Tomkinson,B.E., Wagner,D.K., Nelson,D.L., & Sullivan,J.L. (1987) Activated lymphocytes during acute Epstein-Barr virus infection. *The Journal of Immunology*, **139**, 3802-3807.

Tortorella,D., Gewurz,B.E., Furman,M.H., Schust,D.J., & Ploegh,H.L. (2000) Viral subversion of the immune system. *Annual Review of Immunology*, **18**, 861-926.

Tugizov,S.M., Berline,J.W., & Palefsky,J.M. (2003a) Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells. *Nat Med*, **9**, 307-314.

Tugizov,S.M., Berline,J.W., & Palefsky,J.M. (2003b) Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells. *Nat Med*, **9**, 307-314.

Twomey,J.J. (1974) Abnormalities in Mixed Leukocyte Reaction During Infectious-Mononucleosis. *Journal of Immunology*, **112**, 2278-2281.

Uehara,T., Miyawaki,T., Ohta,K., Tamaru,Y., Yokoi,T., Nakamura,S., & Taniguchi,N. (1992) Apoptotic Cell-Death of Primed Cd45Ro+ Lymphocytes-T in Epstein-Barr Virus-Induced Infectious-Mononucleosis. *Blood*, **80**, 452-458.

Une,Y., Uemura,K., Nakano,Y., Kamiie,J., Ishibashi,T., & Nomura,Y. (1999) Herpesvirus infection in tortoises (Malacochersus tornieri and Testudo horsfieldii). *Veterinary Pathology*, **36**, 624-627.

Vahlenkamp,T.W., Tompkins,M.B., & Tompkins,W.A.F. (2004) Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4(+)CD25(+) T regulatory cells. *Journal of Immunology*, **172**, 4752-4761.

Vogel,J., Hinrichs,S.H., Reynolds,R.K., Luciw,P.A., & Jay,G. (1988) The HIV tat gene induces dermal lesions resembling Kaposi's sarcoma in transgenic mice. *Nature*, **335**, 606-611.

Voo,K.S., Peng,G., Guo,Z., Fu,T., Li,Y., Frappier,L., & Wang,R.F. (2005d) Functional characterization of EBV-encoded nuclear antigen 1-specific CD4+ helper and regulatory T cells elicited by in vitro peptide stimulation. *Cancer Res.*, **65**, 1577-1586.

Voo,K.S., Peng,G., Guo,Z., Fu,T., Li,Y., Frappier,L., & Wang,R.F. (2005e) Functional characterization of EBV-encoded nuclear antigen 1-specific CD4+ helper and regulatory T cells elicited by in vitro peptide stimulation. *Cancer Res.*, **65**, 1577-1586.

Voo,K.S., Peng,G., Guo,Z., Fu,T., Li,Y., Frappier,L., & Wang,R.F. (2005a) Functional characterization of EBV-encoded nuclear antigen 1-specific CD4+ helper and regulatory T cells elicited by in vitro peptide stimulation. *Cancer Res.*, **65**, 1577-1586.

Voo,K.S., Peng,G., Guo,Z., Fu,T., Li,Y., Frappier,L., & Wang,R.F. (2005b) Functional characterization of EBV-encoded nuclear antigen 1-specific CD4+ helper and regulatory T cells elicited by in vitro peptide stimulation. *Cancer Res.*, **65**, 1577-1586.

Voo,K.S., Peng,G., Guo,Z., Fu,T., Li,Y., Frappier,L., & Wang,R.F. (2005c) Functional characterization of EBV-encoded nuclear antigen 1-specific CD4+ helper and regulatory T cells elicited by in vitro peptide stimulation. *Cancer Res.*, **65**, 1577-1586.

Wallace,L.E., Wright,J., Ulaeto,D.O., Morgan,A.J., & Rickinson,A.B. (1991) Identification of 2 T-Cell Epitopes on the Candidate Epstein-Barr-Virus Vaccine Glycoprotein-Gp340 Recognized by Cd4+ T-Cell Clones. *Journal of Virology*, **65**, 3821-3828.

Wang,D., Liebowitz,D., & Kieff,E. (1985b) An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell*, **43**, 831-840.

Wang,D., Liebowitz,D., & Kieff,E. (1985a) An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell*, **43**, 831-840.

Wang,F., Blaese,R.M., Zoon,K.C., & Tosato,G. (1987) Suppressor T-Cell Clones from Patients with Acute Epstein-Barr Virus-Induced Infectious-Mononucleosis. *Journal of Clinical Investigation*, **79**, 7-14.

Wang,F., Gregory,C., Sample,C., Rowe,M., Liebowitz,D., Murray,R., Rickinson,A., & Kieff,E. (1990a) Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *The Journal of Virology*, **64**, 2309-2318.

Wang,F., Tsang,S.F., Kurilla,M.G., Cohen,J.I., & Kieff,E. (1990b) Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1. *The Journal of Virology*, **64**, 3407-3416.

Wang,F., Tsang,S.F., Kurilla,M.G., Cohen,J.I., & Kieff,E. (1990c) Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1. *The Journal of Virology*, **64**, 3407-3416.

Wang,F., Tsang,S.F., Kurilla,M.G., Cohen,J.I., & Kieff,E. (1990d) Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1. *The Journal of Virology*, **64**, 3407-3416.

Wang,J., Ioan-Facsinay,A., Van der Voort,E.I.H., Huizinga,T.W.J., & Toes,R.E.M. (2007) Transient expression of FOXP3 in human activated nonregulatory CD4(+) T cells. *European Journal of Immunology*, **37**, 129-138.

Wang,X. & Hutt-Fletcher,L.M. (1998) Epstein-Barr Virus Lacking Glycoprotein gp42 Can Bind to B Cells but Is Not Able To Infect. *The Journal of Virology*, **72**, 158-163.

Wang,X., Kenyon,W.J., Li,Q., Mullberg,J., & Hutt-Fletcher,L.M. (1998a) Epstein-Barr Virus Uses Different Complexes of Glycoproteins gH and gL To Infect B Lymphocytes and Epithelial Cells. *The Journal of Virology*, **72**, 5552-5558.

Wang,X., Kenyon,W.J., Li,Q., Mullberg,J., & Hutt-Fletcher,L.M. (1998b) Epstein-Barr Virus Uses Different Complexes of Glycoproteins gH and gL To Infect B Lymphocytes and Epithelial Cells. *The Journal of Virology*, **72**, 5552-5558.

Weiner,H.L. (2001b) Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunological Reviews*, **182**, 207-214.

Weiner,H.L. (2001a) Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunological Reviews*, **182**, 207-214.

Weiss,L., Donkova-Petrini,V., Caccavelli,L., Balbo,M., Carbonneil,C., & Levy,Y. (2004e) Human immunodeficiency virus-driven expansion of CD4(+)CD25(+) regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood*, **104**, 3249-3256.

Weiss,L., Donkova-Petrini,V., Caccavelli,L., Balbo,M., Carbonneil,C., & Levy,Y. (2004d) Human immunodeficiency virus-driven expansion of CD4(+)CD25(+) regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood*, **104**, 3249-3256.

Weiss,L., Donkova-Petrini,V., Caccavelli,L., Balbo,M., Carbonneil,C., & Levy,Y. (2004c) Human immunodeficiency virus-driven expansion of CD4(+)CD25(+) regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood*, **104**, 3249-3256.

Weiss,L., Donkova-Petrini,V., Caccavelli,L., Balbo,M., Carbonneil,C., & Levy,Y. (2004b) Human immunodeficiency virus-driven expansion of CD4(+)CD25(+) regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood*, **104**, 3249-3256.

Weiss,L., Donkova-Petrini,V., Caccavelli,L., Balbo,M., Carbonneil,C., & Levy,Y. (2004a) Human immunodeficiency virus-driven expansion of CD4(+)CD25(+) regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood*, **104**, 3249-3256.

Weiss,L.M., Strickler,J.G., Warnke,R.A., Purtilo,D.T., & Sklar,J. (1987) Epstein-Barr viral DNA in tissues of Hodgkin's disease. *American Journal of Pathology*, **129**, 86-91.

White,C.A., Cross,S.M., Kurilla,M.G., Kerr,B.M., Schmidt,C., Misko,I.S., Khanna,R., & Moss,D.J. (1996a) Recruitment during infectious mononucleosis of

CD3(+)CD4(+)CD8(+) virus-specific cytotoxic T cells which recognise Epstein-Barr virus lytic antigen BHRF1. *Virology*, **219**, 489-492.

White,C.A., Cross,S.M., Kurilla,M.G., Kerr,B.M., Schmidt,C., Misko,I.S., Khanna,R., & Moss,D.J. (1996b) Recruitment during infectious mononucleosis of CD3(+)CD4(+)CD8(+) virus-specific cytotoxic T cells which recognise Epstein-Barr virus lytic antigen BHRF1. *Virology*, **219**, 489-492.

Wick,M.J., Woronzoff-Dashkoff,K.P., & McGlennen,R.C. (2002a) The molecular characterization of fatal infectious mononucleosis. *American Journal of Clinical Pathology*, **117**, 582-588.

Wick,M.J., Woronzoff-Dashkoff,K.P., & McGlennen,R.C. (2002b) The molecular characterization of fatal infectious mononucleosis. *American Journal of Clinical Pathology*, **117**, 582-588.

Wildin,R.S., Ramsdell,F., Peake,J., Faravelli,F., Casanova,J.L., Buist,N., Levy-Lahad,E., Mazzella,M., Goulet,O., Perroni,L., Dagna Bricarelli,F., Byrne,G., McEuen,M., Proll,S., Appleby,M., & Brunkow,M.E. (2001) X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*, **27**, 18-20.

Wilkie,G.M., Taylor,C., Jones,M.M., Burns,D.M., Turner,M., Kilpatrick,D., Amlot,P.L., Crawford,D.H., & Haque,T. (2004) Establishment and characterization of a bank of cytotoxic T lymphocytes for immunotherapy of Epstein-Barr virus-associated diseases. *Journal of Immunotherapy*, **27**, 309-316.

Williams,H., McAulay,K., Macsween,K.F., Gallacher,N.J., Higgins,C.D., Harrison,N., Swerdlow,A.J., & Crawford,D.H. (2005) The immune response to primary EBV infection: a role for natural killer cells. *British Journal of Haematology*, **129**, 266-274.

Wilson,A.D., Hopkins,J.C., & Morgan,A.J. (2001) In vitro cytokine production and growth inhibition of lymphoblastoid cell lines by CD4(+) T cells from Epstein-Barr virus (EBV) seropositive donors. *Clinical and Experimental Immunology*, **126**, 101-110.

Woisetschlaeger,M., Jin,X.W., Yandava,C.N., Furmanski,L.A., Strominger,J.L., & Speck,S.H. (1991) Role for the Epstein-Barr Virus Nuclear Antigen 2 in Viral Promoter Switching During Initial Stages of Infection. *Proceedings of the National Academy of Sciences*, **88**, 3942-3946.

Woisetschlaeger,M., Yandava,C.N., Furmanski,L.A., Strominger,J.L., & Speck,S.H. (1990) Promoter Switching in Epstein-Barr Virus During the Initial Stages of

Infection of B Lymphocytes. *Proceedings of the National Academy of Sciences*, **87**, 1725-1729.

Woisetschlaeger,M., Strominger,J.L., & Speck,S.H. (1989) Mutually Exclusive Use of Viral Promoters in Epstein--Barr Virus Latently Infected Lymphocytes. *Proceedings of the National Academy of Sciences*, **86**, 6498-6502.

Wolf,H., Haus,M., & Wilmes,E. (1984) Persistence of Epstein-Barr virus in the parotid gland. *The Journal of Virology*, **51**, 795-798.

Woodberry,T., Suscovich,T.J., Henry,L.M., Davis,J.K., Frahm,N., Walker,B.D., Scadden,D.T., Wang,F., & Brander,C. (2005a) Differential targeting and shifts in the immunodominance of Epstein-Barr virus-specific CD8 and CD4 T cell responses during acute and persistent infection. *Journal of Infectious Diseases*, **192**, 1513-1524.

Woodberry,T., Suscovich,T.J., Henry,L.M., Davis,J.K., Frahm,N., Walker,B.D., Scadden,D.T., Wang,F., & Brander,C. (2005b) Differential targeting and shifts in the immunodominance of Epstein-Barr virus-specific CD8 and CD4 T cell responses during acute and persistent infection. *Journal of Infectious Diseases*, **192**, 1513-1524.

Wu,L., Borza,C.M., & Hutt-Fletcher,L.M. (2005) Mutations of Epstein-Barr Virus gH That Are Differentially Able To Support Fusion with B Cells or Epithelial Cells. *The Journal of Virology*, **79**, 10923-10930.

Wu,T.C., Mann,R.B., Charache,P., Hayward,S.D., Staal,S., Lambe,B.C., & Ambinder,R.F. (1990) Detection of Ebv Gene-Expression in Reed-Sternberg Cells of Hodgkins-Disease. *International Journal of Cancer*, **46**, 801-804.

Yang,J., Lemas,V.M., Flinn,I.W., Krone,C., & Ambinder,R.F. (2000) Application of the ELISPOT assay to the characterization of CD8(+) responses to Epstein-Barr virus antigens. *Blood*, **95**, 241-248.

Yates,J., Warren,N., Reisman,D., & Sugden,B. (1984) A cis-Acting Element from the Epstein-Barr Viral Genome That Permits Stable Replication of Recombinant Plasmids in Latently Infected Cells. *Proceedings of the National Academy of Sciences*, **81**, 3806-3810.

Yewdell,J.T. & Bennink,J.R. (1999) Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annual Review of Immunology*, **17**, 51-88.

Young,L.S. & Murray,P.G. (2003b) Epstein-Barr virus and oncogenesis: from latent genes to tumours. *Oncogene*, **22**, 5108-5121.

Young,L.S. & Murray,P.G. (2003a) Epstein-Barr virus and oncogenesis: from latent genes to tumours. *Oncogene*, **22**, 5108-5121.

Young,L.S., Sixbey,J.W., Clark,D., & Rickinson,A.B. (1986) EPSTEIN-BARR VIRUS RECEPTORS ON HUMAN PHARYNGEAL EPITHELIA. *The Lancet*, **327**, 240-242.

Young,L.S. & Rickinson,A.B. (2004) Epstein-Barr virus: 40 years on. *Nat Rev Cancer*, **4**, 757-768.

Zhang,Q., Gutsch,D., & Kenney,S. (1994) Functional and Physical Interaction Between P53 and Bzlf1 - Implications for Epstein-Barr-Virus Latency. *Molecular and Cellular Biology*, **14**, 1929-1938.

Zhao,B., Marshall,D.R., & Sample,C.E. (1996) A conserved domain of the Epstein-Barr virus nuclear antigens 3A and 3C binds to a discrete domain of Jkappa. *The Journal of Virology*, **70**, 4228-4236.

Zheng,Y. & Rudensky,A.Y. (2007) Foxp3 in control of the regulatory T cell lineage. *Nat Immunol*, **8**, 457-462.

Zimberstrobl,U., Kremmer,E., Grasser,F., Marschall,G., Laux,G., & Bornkamm,G.W. (1993a) The Epstein-Barr-Virus Nuclear Antigen-2 Interacts with An Ebna2 Responsive Cis-Element of the Terminal Protein-1 Gene Promoter. *Embo Journal*, **12**, 167-175.

Zimberstrobl,U., Kremmer,E., Grasser,F., Marschall,G., Laux,G., & Bornkamm,G.W. (1993b) The Epstein-Barr-Virus Nuclear Antigen-2 Interacts with An Ebna2 Responsive Cis-Element of the Terminal Protein-1 Gene Promoter. *Embo Journal*, **12**, 167-175.

Zimberstrobl,U., Strobl,L.J., Meitinger,C., Hinrichs,R., Sakai,T., Furukawa,T., Honjo,T., & Bornkamm,G.W. (1994) Epstein-Barr-Virus Nuclear Antigen-2 Exerts Its Transactivating Function Through Interaction with Recombination Signal Binding-Protein Rbp-J-Kappa, the Homolog of Drosophila Suppressor of Hairless. *Embo Journal*, **13**, 4973-4982.

Zur Hausen,H., Schulte-Holthausen,H., Klein,G., Henle,W., Henle,G., Clifford,P., Santesson,L. (1970) Epstein-Barr Virus in Burkitt's Lymphoma and Nasopharyngeal Carcinoma: EBV DNA in Biopsies of Burkitt Tumours and Anaplastic Carcinomas of the Nasopharynx. *Nature*, **228**, 1056-1058.