Visualising Antigen-Specific T Cells During Primary & Persistent Infection with Epstein-Barr Virus

LINDA CHENG-CHOO TAN

WOLFSON COLLEGE, OXFORD

&

THE MRC HUMAN IMMUNOLOGY UNIT

INSTITUTE OF MOLECULAR MEDICINE

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A thesis submitted to the University of Oxford in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

To my parents, and to Andrew and Caroline;

thank you for everything.

TABLE OF CONTENTS

Abstract v

Acknowledgements vi

Publications based on results in this thesis vii

Abbreviations viii

Chapter 1 - General Introduction **1**

1.1. Cell-Mediated Immunity **2**
Peptide presentation by class I MHC molecules 2
The class I antigen processing pathway 4
The human T cell antigen receptor 7
TCR recognition of peptide-MHC complexes 9

1.2. The Epstein-Barr Virus **14**
General aspects 14
Gene expression 14
CTL responses in EBV infection 15
Immune evasion 16

1.3. Rheumatoid Arthritis **16**

1.4. Aims of this Thesis **18**

Chapter 2 - Materials and Methods

2.1. General Cell Culture **19**
Tissue culture media
Freezing of cell stocks

2.2. Donor Blood Samples **20**
Preparation of human PBMC from whole blood
Preparation of human lymphocytes from synovial fluid
Isolation of $CD4^+$ or $CD8^+$ T lymphocytes from PBMC
Generation of human B lymphoblastoid cell lines
Tissue typing of blood donors
EBV serological testing

2.3. Bacterial Culture **22**
Recipes for bacterial broth
Antibiotic selection
Making bacterial glycerol stocks

2.4. Single Stranded Sequencing of T Cell Receptor β-Chains **24**
Extraction of mRNA from lymphocytes
Synthesis of cDNA
Poly G tailing
PCR and purification of products
Cloning of PCR products into Phagescript SK M13 vector
Preparation of fresh competent *E. coli* for transformations
Preparation of single stranded templates for sequencing
Sequencing reactions

2.5. Double Stranded Sequencing of BV14 T Cell Receptors **30**
PCR reactions
Ligations and transformations using the pGEM-T Easy kit
Plasmid DNA minipreps
Sequencing

2.6. Synthetic Peptides **31**

2.7. Limiting Dilution Analysis **32**
Preparation of mononuclear feeder cells
Setting up assay cultures (Day 0)
^{51}Cr-release cytotoxicity assay (Day 14)

2.8. IFNγ Enzyme Linked Immunospot (ELISpot) Assays **34**

2.9. Synthesis of Class I MHC-Peptide Tetrameric Complexes **35**
Recombinant protein expression and inclusion body purification
Refolding soluble class I MHC-peptide complexes
Site-specific biotinylation of MHC-peptide complexes
Preparation of fluorescent-labelled tetrameric complexes

2.10. Flow Cytometry **39**
Cell staining with directly-conjugated mAbs or tetrameric complexes
Multicolour cell staining with unconjugated mAbs
Intracellular staining with the Ki67 marker
FACS data acquisition & analysis

2.11. Antibodies and Sources **40**
Antibodies specific for human leukocyte cell surface differentiation markers
Anti-human TCR variable β chain (BV) specific mAbs
Other monoclonal antibodies

Chapter 3 - Molecular Analysis of Nucleotide Bias during VDJ recombination in T Lymphocyte β-Chains

Introduction **42**

3.1. Sequencing the CDR3 regions of TCR β chains **45**

3.2. Analysis of J gene segment usage **46**

3.3. Analysis of nucleotide nibbling from TCRBV 3' termini in coding joints 48

3.4. Evidence for P nucleotide addition 50

Discussion 50

Chapter 4 - CTL Responses in Primary EBV Infection

Introduction 53

4.1. Construction of tetrameric complexes and staining of control samples 54

4.2. Frequency of circulating EBV-specific T cells during primary EBV infection 56

4.3. Frequency of EBV-specific T cells in postconvalescent IM patients 59

4.4. Phenotype of EBV-specific T cells during the primary T cell response 60

4.5. Phenotype of EBV-specific T cells in postconvalescent IM patients 62

4.6. Sequence of TCRs involved in the B8/RAKFKQLL response 64

Discussion 65

Chapter 5 - Long Term Memory Response to EBV Infection in Healthy Seropositives

Introduction 69

5.1. Measurement of T cell frequencies by LDA 71

5.2. Quantitation of T cell responses by ELISpot assay 72

5.3. Enumeration of antigen-specific T cells using MHC-peptide tetrameric complexes 74

5.4. Phenotypic analysis of EBV-specific CTL within peripheral blood from long term virus carriers 76

5.5. Comparison of results obtained using the three methods 79

Discussion 79

Chapter 6 - Enriched Populations of EBV-Specific CTL in Rheumatoid Joints

Introduction 85

6.1. Enumeration of virus-specific T cells within synovial fluid and peripheral blood using HLA-peptide tetrameric complexes 86

6.2. Quantitation of EBV-specific T cells within synovial fluid and peripheral blood using an ELISpot assay for IFNγ secretion....91

6.3. The phenotype of EBV-specific T lymphocytes within peripheral blood and synovial fluid..... 92

6.4. Expression of chemokine receptors and integrins by $CD8^+$ T cells within peripheral blood and synovial fluid..... 96

6.5. Proliferation of lymphocytes within synovial fluid..... 98

Discussion..... 100

Chapter 7 - Concluding Discussion

Introduction..... 104

7.1. The high frequencies of antigen-specific T cells in primary EBV infection..... 105

7.2. Downregulation of the immune response following primary infection..... 112

7.3. Memory responses to persistent EBV infection..... 114

7.4. Final considerations..... 116

Bibliography..... 119

Appendix A. Amino acids and genetic codes..... 140

Appendix B. EBV-encoded CTL epitopes..... 141

Appendix C. Sequences of T cell receptor CDR3 regions..... 142

ABSTRACT

Visualising Antigen-Specific T Cells During Primary & Persistent Infection with Epstein-Barr Virus

Linda Cheng-Choo Tan **D. Phil. Thesis**

Wolfson College & MRC Human Immunology Unit, Oxford **Trinity Term, 1999**

Cytotoxic T lymphocytes play an important role in mediating host immune reactions to viruses and other pathogens. Selective mechanisms operate during V(D)J recombination to enhance the diversity of the T cell repertoire that is generated, particularly in the CDR3 regions of the TCR, which mediate peptide recognition. The influence of V gene 3' sequences on the composition of the CDR3 loop in TCR β chains is analysed; in particular, A/T-rich coding termini are shown to be more susceptible to exonuclease "nibbling" during recombination.

The recent development of peptide-MHC tetrameric complexes has enabled us to detect T lymphocytes according to their antigen specificity. Their use in detection and characterisation of EBV-specific $CD8^+$ T cells during the primary acute phase of infection is described here. In particular, CTL responses to EBV lytic cycle antigens have only recently been reported and this study reveals unexpectedly high frequencies of activated, circulating $CD8^+$ T lymphocytes which are directed towards lytic cycle epitopes, compared to well-characterised latent cycle antigens.

In a second cohort of healthy long term asymptomatic donors, the frequency of $CD8^+$ T cells recognising EBV lytic and latent cycle antigens was analysed by tetramer staining, ELISpot assays and limiting dilution assays; the tetramers detected antigen-specific $CD8^+$ T lymphocytes with greater efficiency than other methods. Lytic cycle antigen-specific T lymphocytes were clearly detectable in all the asymptomatic donors, at higher frequencies than those specific for latent antigens.

The final section of this thesis investigates the existence of enriched populations of EBV-specific T lymphocytes found within synovial joint fluid of rheumatoid arthritis patients. Although these cells do not appear to be directly involved in the initiation of disease, their ability to secrete proinflammatory cytokines within joints probably contributes to the maintenance of chronic inflammation in these patients.

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PUBLICATIONS BASED ON RESULTS IN THIS THESIS

1. **Direct visualization of antigen-specific CD8$^+$ T cells during the primary immune response to Epstein-Barr virus in vivo.** MFC Callan, L Tan, N Annels, GS Ogg, JDK Wilson, CA O'Callaghan, N Steven, AJ McMichael and AB Rickinson. *Journal of Experimental Medicine* 1998, 187: 1395-1402.

2. **A re-evaluation of the frequency of CD8$^+$ T cells specific for EBV in healthy virus carriers.** LC Tan, N Gudgeon, NE Annels, P Hansasuta, CA O'Callaghan, S Rowland-Jones, AJ McMichael, AB Rickinson and MFC Callan. *The Journal of Immunology* 1999, 162: 1827-1835.

3. **Visualising T cells specific for herpesvirus in patients with arthritis: a possible role for virus-specific T cells in the pathogenesis of chronic inflammatory joint disease.** LC Tan, AG Mowat, C. Fazou, T Rostron, PR Dunbar, C. Tournay, F. Romagné, M-A Peyrat, E Houssaint, M Bonneville, AB Rickinson, AJ McMichael and MFC Callan. *(manuscript submitted)*

ABBREVIATIONS

AIDS, acquired immune deficiency syndrome
amp, ampicillin
APC, antigen-presenting cell
ATP, adenosine triphosphate
bp, base pairs
BSA, bovine serum albumin
B-LCL, human B lymphoblastoid cell line
cDNA, complementary DNA
CDR, complementarity-determining regions
CMV, cytomegalovirus
CTL, cytotoxic T lymphocyte
D region, diversity region of T cell receptor for antigen
DEPC, diethyl pyrocarbonate
DMSO, dimethylsulphoxide
DNA, deoxyribonucleic acid
dNTPs, mixture of deoxynucleoside triphosphates: dATP, dGTP, dCTP, and dTTP
ddN, dideoxynucleoside triphosphates; ie. ddATP, ddGTP, ddCTP & ddTTP
DTT, dithiothreitol
EBNA, Epstein-Barr nuclear antigen
EBV, Epstein-Barr virus
EDTA, ethylenediaminetetraacetic acid
ELISPOT, enzyme-linked immunospot assay
Fab, antigen-binding fragment
FACS, fluorescence-activated cell sorter
FCS, foetal calf serum
FITC, fluorescein isothiocyanate
Fmoc, 9-fluorenylmethoxycarbonyl
FPLC, fast protein liquid chromatography
GP, glycoprotein
HIV, human immunodeficiency virus
HLA, human histocompatibility leukocyte antigens
HPLC, high performance liquid chromatography
HSV, herpes simplex virus
ICAM, intercellular adhesion molecule
IFN, interferon (*eg. IFNγ*)
Ig, immunoglobulin
IL, interleukin (*eg. IL-2*)
IM, infectious mononucleosis
IPTG, isopropyl-β-D-galactoside
ITAM, immunoreceptor tyrosine-based activation motif
J region, joining region of T cell receptor for antigen
LB, type of bacterial nutrient broth (*also SOB & 2xYT*)
LCMV, lymphocytic choriomeningitis virus
LDA, limiting dilution analysis
LFA-1, leukocyte function-associated antigen-1
LMP, latent membrane protein
LPS, lipopolysaccharide
mAb, monoclonal antibody
MES, 2-[N-morpholino]ethanesulphonic acid
MHC, major histocompatibility complex
mRNA, messenger RNA
MW, molecular weight
NP, nucleoprotein
OD, optical density
PBMC, peripheral blood mononuclear cells
PBS, phosphate-buffered saline
PCR, polymerase chain reaction
PE, phycoerythrin
PHA, phytohaemagglutinin
R, receptor (*eg. IL-2R*)
RhA, rheumatoid arthritis
RNA, ribonucleic acid
RNase, ribonuclease
SDS, sodium dodecyl sulphate
SFMC, synovial fluid mononuclear cells
SH, src-homology (*eg. SH2*)
SOB, *see LB*
TAP, transporter associated with antigen processing
TCR, T cell receptor for antigen
TdT, terminal deoxynucleotidyl transferase
TE, Tris-EDTA buffer
tet, tetracycline
Th cell, T helper cell (*eg. Th1*)
TNF, tumour necrosis factor
Tris, tris(hydroxymethyl)aminomethane
U, unit
V region, variable region of T cell receptor for antigen
VLA-4, very late antigen-4
X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside
2-ME, 2-mercaptoethanol
2×YT, *see LB*

CHAPTER 1

General Introduction

Adaptive immunity is a specialised ability of vertebrates to protect themselves against infectious agents. In contrast to the non-specific protection provided by innate immunity, adaptive immune responses react to specific molecular determinants through cell-bound or soluble antigen receptors. Furthermore, there is immunological memory of previous encounters with a particular antigen, leading to enhanced responses during subsequent exposure. Specific, adaptive immunity can be further classified into humoral immunity, which involves the production of antibodies (immunoglobulins) by B lymphocytes, and cell-mediated immunity, which involves interactions between T lymphocytes and infected or malignant host cells. The T cell antigen receptor (TCR) recognises short linear peptide fragments in conjunction with major histocompatibility complex (MHC) molecules. Class I MHC molecules bind peptides derived from endogenously synthesised or cytoplasmic proteins, presenting them on the cell surface to T cells expressing the CD8 co-receptor. Instead, class II MHC molecules bind peptides derived from extracellular proteins, presenting them to T cells expressing CD4. $CD8^+$ (cytotoxic) T lymphocytes are most crucial in mediating the host defence against intracellular bacteria and viruses [reviewed in Yewdell & Bennink, 1992; Germain, 1994]. The following sections provide a description of cytotoxic T cell recognition of antigen, as well as a general overview of Epstein-Barr virus and rheumatoid arthritis.

1.1. Cell-Mediated Immunity

Peptide presentation by class I MHC molecules

MHC-restricted T cell recognition

Early work by Zinkernagel and Doherty [1974a & 1974b] first revealed that cytotoxic T lymphocytes (CTL) can only recognise antigen in the context of self- or matched-class I MHC molecules. Later Townsend *et al* and others demonstrated that CTL responses are predominantly directed against short peptide fragments derived from cytoplasmic and nuclear proteins [Bennink *et al*, 1982; Townsend & Skehel, 1982; Townsend *et al*, 1986; Maryanski, 1986]. The ligands for cytotoxic T cell receptors are constitutively expressed on the majority of nucleated cells. Furthermore, their expression is upregulated by CTL-derived cytokines, such as IFNγ [Nakamura *et al*, 1984]. Each class I MHC molecule is a complex consisting of a 44kD heavy chain membrane protein non-covalently associated with 12kD β_2-microglobulin (β_2m) and a short peptide around nine amino acids long. The heavy chain is encoded by the highly polymorphic major histocompatibility gene complex found on chromosome 6 in humans [Abbas *et al*, 1997]. In each individual there are three loci, HLA-A, -B and -C; however both maternal and paternal-derived alleles are co-dominantly expressed, resulting in a maximum of six possible class I molecules.

Structure of the class I MHC molecule

The first X-ray crystal structure of a human class I MHC molecule, HLA-A2, provided a tremendous boost to our understanding of MHC function [Bjorkman *et al*, 1987a & 1987b]. The three-dimensional structure revealed a peptide-binding site formed by the membrane distal α1 and α2 domains of the MHC heavy chain. These were arranged to form two anti-parallel α-helices supported below by a β-sheet floor, creating a central groove 30Å long, 11 Å deep and 10 Å wide. Additional structures later showed the

extended conformation of the peptide, with the side chains of so-called anchor residues buried within the corresponding specificity pockets (named A to F) lining the groove; other peptide residues lay exposed on the surface of the molecule, where they might interact with the T cell receptor. [Garrett *et al*, 1989; Madden *et al*, 1991 & 1992; Fremont *et al*, 1992; Guo *et al*, 1992; Silver *et al*, 1992; Madden, 1995]. It is now clear from all these studies that conserved non-polymorphic residues, especially those located in the A and F pockets at either end of the groove interact with the peptide backbone to ensure a common orientation of all bound peptides, as well as preferential binding of peptides of eight to eleven amino acids in length. Polymorphic residues of the heavy chain are concentrated in pockets B to F in the central portion of the peptide-binding groove; these residues influence the shape and chemical characteristics of the specificity pockets, imposing stringent binding requirements for amino acids at particular positions along the peptide. As a result each MHC molecule binds a distinct set of peptides sharing a common binding motif, but still is able to present a wide array of peptides to the T cell receptor [reviewed in Elliott *et al*, 1993; Heemels & Ploegh, 1995].

Characterisation of peptides bound to class I MHC molecules

Several novel techniques were employed to analyse the peptides presented by MHC molecules. Falk and Rötzschke used Edman degradation to sequence pools of peptides eluted at low pH from large quantities of immunoaffinity-purified MHC molecules [Rötzschke *et al*, 1990; Falk *et al*, 1991]. Since the anchor positions are consistently occupied by one or a few chemically similar amino acids, this method enabled the binding motifs of many class I MHC alleles to be determined [reviewed in Rammensee *et al*, 1995]. Other groups were also able to sequence the individual peptides bound to MHC molecules, utilising HPLC fractionation in combination with Edman sequencing

[Jardetzky *et al*, 1991; Rudensky *et al*, 1991] or tandem mass spectrometry [Hunt *et al*, 1986 & 1992]; the latter technique provides reliable sequence information from as little as 300 fmol of peptide [Engelhard, 1994].

In addition to anchor residues, other peptide positions have been identified where there is a less stringent amino acid preference, and which are categorised as secondary anchor positions [Ruppert *et al*, 1993; Parker *et al*, 1994]. The knowledge of peptide motifs for a variety of alleles has proven invaluable for the prediction and identification of CTL epitopes within proteins of known sequence [Rötzschke *et al*, 1991; Pamer *et al*, 1992; Hill *et al*, 1992].

The class I antigen processing pathway

Protein degradation in the cytosol

Degradation of the endogenous proteins by the cytosolic proteasomes provides the major source of peptides for class I MHC molecules. Experiments have shown that the presence of inhibitors of proteasome function results in retention of peptide-deprived class I MHC molecules in the ER [Rock *et al*, 1994; Fenteany *et al*, 1995; Hughes *et al*, 1996]. Proteasomes are large multicatalytic proteases, consisting of a barrel-shaped 20S core particle associated with additional regulatory subunits. Crystal structures have revealed that the proteolytic active sites are located on the inner surface of the barrel, through which protein substrates must feed in order to be degraded [Lowe *et al*, 1995; Groll *et al*, 1997]. Two subunits of the proteasome have been found to be encoded in the MHC region [Brown *et al*, 1991; Glynne *et al*, 1991; Kelly *et al*, 1991; Matinez & Monaco, 1991; Ortiz-Navarrete *et al*, 1991]; exposure of cells to IFNγ causes substitution of the normal constitutive subunits with MHC-encoded LMP7, LMP2 and a third, non-MHC-encoded subunit, LMP10 (MECL-1) [Belich *et al*, 1994; Nandi *et al*, 1996; Groettrup *et*

al, 1996]. These proteins have been shown to alter the specificity of the proteasome, upregulating preferential cleavage after basic or hydrophobic residues whilst inhibiting cleavage after acidic residues [Driscoll *et al*, 1993; Gaczynska *et al*, 1993 & 1994; Eleuteri *et al*, 1997]. This activity has been postulated to increase the generation of peptides suitable for binding MHC molecules, as these typically require hydrophobic C-terminal anchor residues. IFNγ has also been found to increase expression of the PA28 activator, which similarly appears to enhance the efficiency of the proteasome in generating MHC ligands [Ma *et al*, 1992; Gray *et al*, 1994; Dick *et al*, 1996]. Additional regulatory components are likely to play a role in antigen processing [Ma *et al*, 1994; Deveraux *et al*, 1994; Hoffman & Rechsteiner, 1994].

Other proteases may also contribute peptides for presentation by class I MHC molecules, so long as the appropriate binding motifs are present [Anderson *et al*, 1991]; however these probably only constitute a minor source of ligands. It has been suggested that the relative efficiency of epitope generation by proteasomes may influence the hierarchy of T cell responses to different epitopes [Niedermann *et al*, 1995]. Furthermore, some proteins derived from pathogens have been shown to contain sequences that prevent proteasome-mediated degradation, thereby avoiding detection by cytotoxic T lymphocytes [Levitskaya et al, 1995].

Peptide transport into the endoplasmic reticulum (ER)

The TAP (transporter associated with processing) transporter is a membrane-bound heterodimer of TAP1 and TAP2 subunits and a member of the ATP-binding cassette (ABC) family of membrane transporters. This complex facilitates peptide transport from the cytosol into the ER in an ATP-dependent manner [Kelly *et al*, 1992; Townsend & Trowsdale, 1993]. Studies on murine and human cell lines carrying mutations in their

TAP genes have demonstrated that this peptide transporter is critical for antigen presentation by class I MHC molecules on the cell surface [Salter & Cresswell, 1986; Townsend *et al*, 1989; Cerundolo *et al*, 1990].

Human TAP molecules have been shown in transport assays to be fairly promiscuous, apart from a preference for peptides with hydrophobic and basic C-termini [Androlewicz & Cresswell, 1994; Momburg *et al*, 1994a; van Endert *et al*, 1995]. Peptides between seven to twelve residues are most efficiently transported, although longer peptides are not excluded [Androlewicz *et al*, 1993; Momburg *et al*, 1994b]. There is also some evidence that transported peptides may be subject to further trimming in the ER by resident peptidases, but these have yet to be fully characterised [Elliott *et al*, 1995].

Assembly of the class I MHC-peptide complex

Stable assembly of newly-synthesised class I molecules is closely associated with peptide binding. In TAP deficient cell lines, empty class I molecules are retained in the ER and do not traffick to the cell surface [Townsend *et al*, 1990; Elliott *et al*, 1991]. Evidence for the involvement of chaperone molecules various stages to assist the formation of correctly folded class I MHC-peptide complexes has come from immunoprecipitation experiments done by several groups. The current model suggests that calnexin binds newly synthesised class I heavy chains when they enter the ER [David *et al*, 1993; Ortmann *et al*, 1994], but is exchanged for calreticulin upon formation of heavy chain-β_2m dimers [Sadasivan *et al*, 1996]. Another protein, tapasin, mediates the association of the class I-β_2m-calreticulin complexes to TAP, where they may bind peptide ligands [Sadasivan *et al*, 1996; Grandea *et al*, 1995; Ortmann *et al*, 1997]. Peptide binding induces a conformational change that enhances the stability of the MHC complex and

allows it to be transported from the ER through the Golgi apparatus to the plasma membrane [Elliott *et al*, 1991].

The human T cell antigen receptor

The T cell receptor that interacts with MHC molecules is a disulphide-linked heterodimer composed of two homologous transmembrane polypeptide chains (each about 40-60 kD in size), designated α and β. Its basic framework structure closely resembles that of the antigen-binding fragment (Fab) of an immunoglobulin molecule in having variable (Vα and Vβ) and constant (Cα and Cβ) domains (see below). The TCR is also non-covalently associated with CD3, a membrane-bound signalling complex of non-polymorphic polypeptides which includes δ, γ, ε, and ζ chains [Abbas *et al*, 1997].

TCR genes and VDJ recombination

Similar to B lymphocyte immunoglobulin receptors, the TCR is assembled from a large collection of gene segments by rearrangement of the germline sequence [Davis & Bjorkman, 1988]. A functional α chain exon is produced by association of variable (V) and joining (J) gene segments, whilst β chains include additional diversity (D) gene segments between V and J. It is estimated that at least 42 AV gene segments and about 50 AJ segments are functionally expressed. Similarly β chains are encoded by approximately 47 BV genes, 2 BD genes and 13 BJ genes [Arden *et al*, 1995; Toyonaga *et al*, 1985; Davis & Bjorkman, 1988]. Combinatorial diversity arising from the association of multiple gene segments contributes to the size of the potential T cell repertoire.

Rearrangement of gene segments takes place during T cell maturation in the thymus, and involves the RAG1 and RAG2 recombinases [Schatz *et al*, 1989; Oettinger *et al*, 1990].

The process is directed by recombination signal sequences (RSS) flanking each gene segment, consisting of a palindromic heptamer and an A/T-rich nonamer separated by either a 12- or 23-bp spacer; recombination only takes place between two RSS of different lengths [Hesse *et al*, 1989; reviewed in Lewis, 1994]. Double-strand breaks are first introduced between the coding sequence and RSS. The resulting coding ends are simultaneously sealed in a hairpin structure, whereas signal ends remain blunt and are subsequently ligated to form a circular piece of extrachromosomal DNA. The two DNA hairpin intermediates are then resolved to form a coding joint (junction of coding ends) [van Gent *et al*, 1995; McBlane *et al*, 1995; Ramsden *et al*, 1996]. The joining of coding ends is fairly imprecise; furthermore, addition of nontemplated or palindromic nucleotides, as well as deletion of terminal nucleotides often takes place [Ferguson & Thompson, 1993]. These mechanisms further contribute towards the generation of diversity, particularly at the coding junctions of both α and β chains. It has been estimated that the unselected immature T cell repertoire has the potential to contain up to 10^{15} specificities [Davis & Bjorkman, 1988].

Structure of the $\alpha\beta$ TCR

The structure of the T cell receptor was long predicted to resemble that of an Ig Fab fragment, from comparisons of their amino acid sequences. Thus by analogy, the TCR antigen binding surface was proposed to consist of six hypervariable loops encoded by both α and β chains, corresponding to the complementarity-determining regions (CDRs) of antibodies [Chothia *et al*, 1988; Davis & Bjorkman, 1988; Claverie *et al*, 1989]. The CDR1- and CDR2-equivalent hypervariable regions are contained entirely within the V domains, and are generally less variable than their counterparts in Ig chains. However the CDR3-equivalent region is encoded by V and J gene segments in the α chain, or V, D

and J segments in the β chain, and therefore variablity of TCRs is even more concentrated in the CDR3 loop than it is in antibody molecules. The model proposed by Davis & Bjorkman in 1988 suggested that the CDR1 and CDR2 loops were mainly responsible for contacting the side chains of the MHC α helices, whilst the CDR3 loops mostly interacted with the bound peptide. The importance of the third CDRs in determining peptide specificity was further demonstrated by the results of experiments that probed TCR-MHC interactions [Engel & Hedrick, 1988; Jorgensen *et al*, 1992; Hong *et al*, 1992; Sun *et al*, 1995; Sant'Angelo *et al*, 1996].

The crystal structures of the TCR β chain and Vα domain were initially determined separately [Bentley *et al*, 1995; Fields *et al*, 1995] and showed that they are indeed composed of Ig-like variable and constant domains. Shortly after this, two structures of an αβ TCR heterodimer in complex with a class I MHC molecule bound to peptide were solved by X-ray crystallography [Garcia *et al*, 1996; Gaboczi *et al*, 1996]. The results confirmed that earlier predictions were largely correct, but also revealed important variations between TCR and antibody structures that relate to their different modes of antigen recognition.

TCR recognition of peptide-MHC complexes

The three dimensional structure of a T cell receptor complexed to peptide-MHC answered at least one burning question for immunologists. All the crystal structures analysed to date have revealed a uniform diagonal orientation of the TCR with respect to MHC, with the α chain over the N-terminal half of the peptide and the β chain over the C-terminal half. [Gaboczi *et al*, 1996; Garcia *et al*, 1996 & reviewed in 1999; Davis *et al*, 1998]. This conserved angle of approach is thought to be important for ensuring correct

positioning of the coreceptor and signalling molecules. The α and β chain CDR3 loops are located over the central portion of the peptide; in addition, both CDR1 loops are positioned over the N-terminal and C-terminal peptide residues and may contact both peptide and MHC. The CDR2 regions have exclusive contact with the α helices of the MHC heavy chain and argue that residues in the tips of these loops have greatest influence on MHC-restriction.

Another observation that has emerged from the TCR crystal structure is the poor shape complementarity between peptide and the TCR recognition surface. This presumably explains the characteristic low affinity and short half-lives of TCR binding to peptide-MHC and may be a mechanism to facilitate the ability of the TCR to adapt to different bound ligands. More highly antigenic peptides may have greater contact with the TCR, increasing the half-life and thus bringing about different signalling outcomes.

From biological experiments, multimerisation and clustering appear to be integral aspects of TCR signalling [Weiss & Littman, 1994; Germain, 1997; Reich *et al*, 1997]. In contrast crystal structures to date show no evidence of oligomeric TCR/MHC assemblies. It remains to be seen how these apparent differences will be reconciled. Additional crystal structures must first be solved in order to allow generalisations to be made about the structural basis of T cell recognition.

Activation-induced T cell effector functions

Activation of $CD8^+$ T cells can result in diverse effects, including proliferation and differentiation, T cell cytotoxicity, cytokine production and T cell antagonism [Marx, 1995]. Two independent mechanisms account for T cell-mediated cytotoxicity . The major pathway involves the secretion of pore-forming perforin proteins. An alternative pathway is mediated by the interaction of Fas ligand expressed on activated T cells with

Fas on the surface of target cells [Kägi *et al*, 1996]. In addition, recent studies have indicated that $CD8^+$ cytotoxic T lymphocytes may be stimulated to differentiate into Tc1 or Tc2 effector subsets, secreting distinct Th1-like (IL-2 and IFNγ) or Th2-like (IL-4, IL-5, IL-6 and IL-10) cytokine patterns [Sad *et al*, 1995; Maggi *et al*, 1994].

T cell activation requires signal transduction by the CD3 complex following engagement of the TCR by its ligand. The members of the CD3 signalling complex contain multiple activation motifs, known as immunoreceptor tyrosine-based activation motifs (ITAMs) within their cytoplasmic tails, which become tyrosine-phosphorylated in response to TCR antigen recognition [Romeo *et al*, 1992; Irving *et al*, 1993]. This initiates a cascade of phosphorylation activity by other additional cytosolic tyrosine kinases, eventually transducing the signal into the cell nucleus. [Weiss & Littman, 1994; Wange & Samelson, 1996]

The quality of the transmitted signal and the resultant functional outcome appears to depend on the biophysical properties which affect the binding affinity and kinetics of the interaction between the TCR and the peptide-MHC complex. Antagonist and partial agonist ligands for the TCR have been described which induce different patterns of T cell-mediated signalling [Evavold *et al*, 1993; Madrenas *et al*, 1995; Sloan-Lancaster *et al*, 1996]. In addition, the phenotypic state and expression of accessory molecules such as costimulators (CD28 & CTLA-4) [Krummel & Allison, 1995], integrins (VLA-4, LFA-1 & CD2) [Hynes, 1992] and adhesion molecules (CD44, CD62L, etc.) [Haynes *et al*, 1989; Spertini *et al*, 1991] also play an important role in determining the response of a T lymphocyte to stimuli. The presence of such molecules on antigen-primed cells results in more rapid T cell responses to recall antigens.

Selection of the T cell repertoire

TCR genes are first rearranged and expressed in the thymus during the earliest stages of T-cell differentiation. TCR β chains are rearranged and transcribed prior to rearrangement of the α chain. Expression of the β chain signals subsequent events in T cell maturation. The processes of thymic selection must lead to the generation of T cells that are self-MHC restricted and self tolerant [Robey & Fowlkes, 1994; Zuniga-Pflücker & Lenardo, 1996]. Therefore thymocytes are selected to survive or be eliminated on the basis of their TCR specificity for MHC and peptide antigens. The stages of thymocyte maturation can be mapped according to levels of expression of their TCR and other accessory molecules.

At the earliest so-called double negative stage, cells do not express either CD4 or CD8 molecules. Newly formed β chains are paired with surrogate pre-T α chains (pTα) and expressed on thymocytes together with CD3 proteins. This results in allelic exclusion of the second β chain and signals thymocytes to proliferate and express both CD4 and CD8 coreceptor molecules. The α chain subsequently rearranges and αβ heterodimers are now expressed at low levels on the cell surface. Cells at this double positive stage may proceed towards one of three possible fates depending on their affinity (or avidity) for peptide-MHC complexes expressed on thymic antigen presenting cells. Cells that fail to recognise peptide-MHC complexes altogether will apoptose (death by neglect); conversely cells with high affinity are negatively selected, and also undergo apoptosis. Only cells that exhibit low affinity interactions with peptide-MHC are positively selected and rescued from death. Positively selected cells eventually differentiate into single positive ($CD4^+$ or $CD8^+$) $TCR\alpha\beta^{hi}$ T cells [Petrie *et al*, 1987; Ohashi *et al*, 1990; Sebza *et al*, 1999].

The TCR repertoire in normal individuals

Expression of variable chains on T lymphocytes has been studied using PCR methods and TCR V chain-specific monoclonal antibodies [Posnett *et al*, 1996]. In cord blood or young individuals TCR variable chain usage by T cells is generally consistent; with some chains such as BV14 and BV17 being commonly found and others such as BV7S1 and BV16 only rarely. However, some variations in repertoires do exist between different individuals. In certain individuals, reduced expression of particular alleles can be attributed to polymorphisms in the non-coding regions of the TCR V genes themselves [Luyrink *et al*, 1993; Kay *et al*, 1994; Donahue *et al*, 1994; Posnett *et al*, 1994a; Bour *et al*, 1999]. Further studies of twin pairs and HLA-identical or non-identical siblings from large families have suggested that HLA genes may also influence the expressed repertoire [Gulwani-Akolkar *et al*, 1991; Akolkar *et al*, 1993]. Thus the patterns of V-segment frequencies are most similar between twins and HLA-identical siblings and most dissimilar between totally mismatched siblings. In addition, it was shown that the frequency of T cells expressing particular BV genes was frequently skewed towards either $CD4^+$ or $CD8^+$ cells, arguing in these cases for their preferential selection by class I or class II MHC proteins during maturation in the thymus.

Although clonal and oligoclonal expansions of T cells are never present in cord blood, they become frequently detectable in peripheral blood from normal individuals with increasing age [Hingorani *et al*, 1993; Posnett *et al*, 1994b]. TCRBV expansions were observed in both $CD4^+$ and $CD8^+$ populations and in particular, one study found that more than half of the aged persons had clonal expansions within the BV3, BV14, BV16 and BV23 families [Schwab *et al*, 1997]. It is not clear whether these expansions represent antigen-driven clonal populations that have persisted following clearance of antigen, or whether they are continuously stimulated by persistent antigen. Analysis of

the phenotype of the $CD8^+$ cells reveals that they are often $CD28^-$ and $CD57^+$ [Morley *et al*, 1995], which is thought to indicate that the cells are within a late differentiation compartment.

1.2. The Epstein-Barr Virus

General aspects

Epstein-Barr virus (EBV) is a ubiquitous human γ-herpes virus that productively infects epithelial cells and establishes latency in B cells. Primary infection with EBV often occurs during childhood and is usually asymptomatic. However, instances where primary infection is delayed till adolescence or later [Rickinson & Kieff, 1996] can give rise to a clinical disease known as acute infectious mononucleosis (IM), or glandular fever. Following primary infection, EBV persists for life in a subset of B cells [Masucci & Ernberg, 1994] and is associated with at least four malignancies: endemic Burkitt's lymphoma, nasopharyngeal carcinoma, a proportion of Hodgkin's lymphomas and the immunoblastic B-cell lymphomas seen in some immunocompromised individuals [Epstein & Achong, 1986].

Gene expression

Primary EBV infection is takes place following oral transmission, in the oropharyngeal epithelial cells [Sixbey *et al*, 1984]. Up to 70 lytic cycle genes may be expressed, divided temporally into immediate early, early and late, which direct viral DNA synthesis and replication [Kieff, 1996]. Newly synthesised infectious virions proceed to establish latent infection in circulating mature B lymphocytes, with expression of eight latent proteins, Epstein-Barr nuclear antigens (EBNA) 1, 2, 3A, 3B, 3C, leader protein (LP), latent

membrane proteins (LMP) 1 and 2. This programme of gene expression, as seen in *in vitro* transformed B-LCLs, drives the expansion of the latently-infected B cell pool [Tierney *et al*, 1994]. Following primary infection, a life-long virus carrier state is established whereby the virus is harboured within a subset of resting B cells and viral gene expression is downregulated. Throughout life, intermittent reactivation of latently-infected B cells into lytic cycle at mucosal sites probably underlies the low level shedding of infectious virus detectable in throat washings of asymptomatic virus carriers [Rickinson & Kieff, 1996].

CTL responses in EBV infection

Although there is a strong antibody response to EBV, T cells play the main role in controlling both the primary and persistent phases of infection and in preventing the development of immunoblastic B-cell lymphomas [Rickinson, 1986]. EBV-specific CTL can be readily generated *in vitro* from peripheral blood of IM patients or healthy seropositive donors, using autologous virus-transformed B-LCLs as stimulators [Murray *et al*, 1992; Khanna *et al*, 1992; Steven *et al*, 1996]. A large number of CTL epitopes have been mapped to virus proteins expressed during latency, and more recently also to those expressed during the lytic life cycle, by assaying CTL clones against targets expressing individual proteins from recombinant vaccinia vectors. The majority of dominant CTL reactivities are directed towards the EBNA3A, 3B and 3C latent proteins, with subdominant responses to LMP2, or less frequently EBNA2, EBNA-LP and LMP1. CTL responses to EBNA1 are almost never detected [reviewed in Rickinson & Moss, 1997]. Responses to epitopes from immediate early and early lytic cycle proteins have only recently been documented, most notably the HLA-B8 restricted BZLF1 peptide, RAKFKQLL, and the HLA-A2 restricted BMLF1 peptide, GLCTLVAML [Steven *et al*,

1997; Bogedain *et al*, 1995; Scotet *et al*, 1996]. These studies were initially hampered by the fact that the B-LCLs traditionally used as stimulator cells do not normally undergo active virus replication.

Immune evasion

Despite strong CTL responses capable of eliminating virus-infected cells, EBV continues to persist indefinitely within its host. This is largely due to its successful strategy for avoiding immune detection of the B cells that constitute the latent reservoir. Viral gene expression in resting virus infected B cells (and Burkitt's lymphoma cells) is limited to the EBNA1 protein, which is not recognised by CTL [Masucci & Ernberg, 1994]. This is due to an internal glycine-alanine repeat within the protein sequence that has been shown to protect it from proteasome degradation and consequently from CTL detection [Levitskaya *et al*, 1995; Shapiro *et al*, 1998]. In other EBV-associated malignancies such as nasopharyngeal carcinoma and Hodgkin's disease, limitation of viral gene expression to EBNA1, LMP1 and LMP2 may also serve to avoid the brunt of CTL reactivities, which are predominantly directed against EBNA3A, 3B and 3C.

1.3. Rheumatoid Arthritis

Rheumatoid arthritis is an autoimmune joint disease characterised by destruction of the joint cartilage and inflammation of the synovium. Large numbers of activated macrophages and $CD4^+$ T lymphocytes may be found in rheumatoid synovial membranes, as well as plasma cells, dendritic cells and activated fibroblasts [Janossy *et al*, 1981; Klareskog *et al*, 1982]. The majority of these cells express abundant levels of class II MHC and adhesion molecules, indicative of their activated state [Cush & Lipsky,

1988; Morales-Ducret *et al*, 1992; Johnson *et al*, 1993]. In severe cases, well-formed lymphoid follicles with germinal centres may also be present. The synovial joint fluid, which normally contains few cells, also becomes infiltrated with neutrophils, macrophages, T lymphocytes (mainly $CD8^+$) and dendritic cells. In addition, patients frequently have circulating IgM or IgG autoantibodies, usually reactive with the Fc portion of their own IgG molecules [Posnett & Edinger, 1997]. However these so-called rheumatoid factors do not appear to contribute to to the pathogenesis of the disease.

Numerous cytokines can be detected within synovial fluid, including IL-1, IL-8, TNF and IFNγ. These cytokines are probably produced by activated T cells and macrophages, and are believed to stimulate synoviocytes to release hydrolytic enzymes, such as collagenase and metalloproteinases, that mediate destruction of the cartilage, ligaments and tendons of the joints. Clinical trials using anti-TNF antibody therapy have shown some success [reviewed in Feldmann *et al*, 1996], verifying that this inflammatory cytokine promotes disease progression.

The etiology of rheumatoid arthritis (RhA) is as yet unknown. However, susceptibility to the disease is strongly linked to the presence of the class II MHC haplotype, HLA-DR4, or other similar alleles that share an identical motif sequence at amino acids 70-74 (QKRAA) of the β chain [Gregersen *et al*, 1987]. Such class II molecules may play a role in shaping the TCR repertoire or in presentation of an inducing microbial or autoantigenic peptide. This hypothesis would suggest that T cell recognition is important in the pathogenesis of RhA.

There are several experimental models of arthritis. MRL/*lpr* mice develop spontaneous arthritis and have high serum levels of rheumatoid factors, but the mechanisms that induce joint disease in this case are unknown [Hang *et al*, 1982; Theofilopoulos *et al*, 1983]. Immunisation of susceptible strains of mice and rats with type II collagen can

produce a T cell-mediated arthritis. Furthermore, transfer of collagen-specific T cells to unimmunised animals is sufficient to cause disease. However there is no convincing evidence for collagen-specific autoimmunity in the human disease [Trentham *et al*, 1977 and 1978]. Experimental arthritis can also be brought about by immunisation with various bacterial antigens, including mycobacterial and streptococcal cell wall proteins [Izui *et al*, 1979]. Once again, however, such diseases bear only a superficial resemblance to human rheumatoid arthritis.

1.4. Aims of this Thesis

The first part of this thesis looks at diversity of the T cell receptor β chain CDR3 region as a result of VDJ recombination. The possible influence of V gene 3' sequences on the composition of the T cell repertoire is investigated. Secondly, the recent development of peptide-MHC tetrameric complexes has enabled us to detect T lymphocytes according to their antigen specificity. Their use in detecting EBV-specific $CD8^+$ T cells is described in the second part of this thesis, detailing a reassessment of the frequencies of antigen-specific $CD8^+$ T lymphocytes that arise in the course of primary infection with Epstein-Barr virus, and continue to circulate in peripheral blood during long term viral persistence. In addition, I have estimated frequencies of EBV-specific T cells in asymptomatic donors by tetrameric staining, ELISpot assays and limiting dilution assays and compared the relative values obtained using the different methods. Lastly, this work focuses on CTL responses to latent versus lytic viral proteins, and examines the frequency and phenotype of these cells during the different phases of infection, as well as during chronic inflammatory disease.

CHAPTER 2

Materials and Methods

General laboratory reagents were purchased from Sigma, United States Biochemical Corp., or BDH Merck Ltd, except where otherwise indicated.

2.1. General Cell Culture

Tissue culture media

Cells were grown at 37°C with 5% CO_2, in RPMI 1640 medium (Gibco BRL) supplemented with 10% FCS (Globepharm Ltd, UK), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (R10 medium), unless otherwise stated. Cell lines were regularly screened for mycoplasma infection.

Freezing of cell stocks

Cells were suspended in a small volume of ice-cold freezing mix (90% FCS, 10% DMSO). 1-ml aliquots were frozen down in screwcap vials at -70°C using a *Cryo 1°C* freezing container (Nalgene). Typically, each aliquot contained several million cells. Once frozen the cells were sometimes transferred to liquid nitrogen tanks for long-term storage.

2.2. Donor Blood Samples

Preparation of human peripheral blood mononuclear cells from whole blood

Venous blood from donors was drawn into a syringe containing sodium heparin (≥1 U/ml) as an anticoagulant. Every 15 ml of blood was diluted with an equal volume of sterile, serum-free RPMI 1640 and layered over 10 ml of Lymphoprep (Nycomed Pharma, Norway). PBMC were isolated by density gradient centrifugation at 340 × g for 30 min at room temperature, with no brake. The cloudy white interface layer, which contained mononuclear cells, was drawn off and washed twice in serum-free RPMI 1640. The cells were counted and either used immediately or frozen down.

Preparation of human lymphocytes from synovial fluid

Synovial fluid was aspirated from knee joints of arthritis patients. Lymphocytes were isolated in exactly the same manner as for blood (see above). The collected cells were either used fresh or frozen down.

Isolation of $CD4^+$ or $CD8^+$ T lymphocytes from PBMC

Dynabeads (Dynal, Norway) directly conjugated to either CD4 or CD8 were used to isolate the relevant T cell populations. These were first washed (using a Dynal Magnetic Particle Concentrator) in ice-cold R10 medium to remove the sodium azide preservative, and then resuspended in R10 supplemented with 10% human serum (hR10). PBMC were also washed twice in cold R10 and resuspended in hR10.

In order to obtain $CD4^+$ lymphocytes, the cells were first negatively selected with CD8-conjugated Dynabeads, using 20-40 Dynabeads per expected positive cell (lymphocytes make up roughly two-thirds of PBMC, and the ratio of $CD4^+$ to $CD8^+$ cells is about 2:1). Dynabeads were incubated with the PBMC in 200 µl hR10 on ice for 30 min. After this 5 ml of hR10 was added gently and the supernatant collected. Cells in the supernatant were

washed once before proceeding to positive selection. This time CD4-conjugated, washed Dynabeads (4 per expected positive cell) were added to the cells, in 200 µl hR10 total volume, and left on ice for 30 min. Once again, 5 ml of hR10 was then added gently and the Dynabead-attached $CD4^+$ cells were retained.

A similar procedure was used to isolate $CD8^+$ lymphocytes. Carrying out negative selection followed by positive selection resulted in efficient removal of double negative ($CD4^-CD8^-$) T cells and gave populations that were >95% pure.

Generation of human B lymphoblastoid cell lines

5-10 × 10^6 freshly isolated PBMC were incubated for 1 h in 1 ml of supernatant from the marmoset EBV-producing cell line, B95.8. The cells were then diluted in R10 medium containing 1 µg/ml cyclosporin A and left for 6-8 weeks until the B-LCL was established. Thereafter the lines were maintained in R10 medium alone.

Tissue typing of blood donors

HLA types of some donors were previously identified by serology. This method involved screening the donors PBMC with a panel of HLA haplotype-specific antibodies by complement-mediated lysis.

Donors for whom no HLA type was known were tissue typed by PCR-SSP phototyping [Bunce *et al*, 1995]. Genomic DNA was extracted from whole blood, PBMC or neutrophil fractions (following Lymphoprep separation of mononuclear cells from whole blood), using a Puregene DNA isolation kit (Gentra Systems, USA). 12.5-25 ng of DNA was required for each sample. Sequence-specific primers (SSP) were used to detect all known HLA-A, -B, -C, DRB1, DRB3, DRB4, DRB5 and DQB1 specificities in an allele-specific or group-specific manner. Each DNA sample required 192 simultaneous PCR reactions, carried out in 96-well V-bottom PCR plates (total reaction volume was 25 µl).

Cycling parameters were as follows:

96°C	60s		1 cycle
96°C	50s	}	
70°C	50s	}	5 cycles
72°C	50s	}	
96°C	50s	}	
65°C	50s	}	21 cycles
72°C	50s	}	
96°C	50s	}	
55°C	50s	}	4 cycles
72°C	90s	}	

Loading buffer was added directly to each well following the PCR and 10 µl of each reaction was run on a 2% agarose gel. The resulting gel was photographed and the tissue type determined by the presence or absence of appropriate-sized bands on the gel.

EBV serological testing

For each donor 5 ml of whole blood was left to stand at room temperature for several hours until the blood was completely clotted. Clotted material was then spun out and the serum collected. Samples were sent to the John Radcliffe Hospital Public Health Laboratory's Virology Department to test for IgG antibodies against EBV.

2.3. Bacterial Culture

Recipes for bacterial broth

Bacterial nutrient broth was always made up with deionised water and autoclaved immediately. All Bacto® media reagents were purchased from Difco Laboratories, USA or Gibco BRL, UK.

Luria-Bertani /litre	low salt LB /litre
10.0 g Bacto®-tryptone	10.0 g Bacto®-tryptone
5.0 g Bacto®-yeast extract	5.0 g Bacto®-yeast extract
10.0 g sodium chloride	5.0 g sodium chloride

2×YT /litre

16.0 g Bacto®-tryptone

10.0 g Bacto®-yeast extract

2.5 g sodium chloride

SOB /litre

20.0 g Bacto®-tryptone

5.0 g Bacto®-yeast extract

0.5 g sodium chloride

40 mM magnesium chloride (added after autoclaving)

SOC /100 ml

2.0 g Bacto®-tryptone

0.5 g Bacto®-yeast extract

10 mM sodium chloride

2.5 mM potassium chloride

20 mM magnesium chloride } added after

20 mM glucose } autoclaving

Agar plates were made by adding 15.0 g Bacto®agar per litre of media before autoclaving. Molten agar was poured into 8 cm sterile disposable petri dishes. To make soft topping agar, the amount of bactoagar added was halved (7.0 g bactoagar per litre). Just before use this was melted in a microwave oven on defrost setting and then held in a water bath at 45°C.

Antibiotic selection

Bacteria were cultured, where indicated, under antibiotic selection. Working concentrations were as follows: tetracycline 12.5 μg/ml, chloramphenicol 100 μg/ml and ampicillin 100 μg/ml. Stock solutions were filter-sterilised and added to media just before use.

Making bacterial glycerol stocks

A 50% solution of glycerol in LB was made up and autoclaved. 700 µl of early log phase bacterial culture was added to a clean tube containing 300 µl of the LB-glycerol mixture and mixed thoroughly. Tubes were snap frozen on dry ice and stored at -70°C.

2.4. Single Stranded Sequencing of T Cell Receptor β-Chains

Extraction of mRNA from lymphocytes

Sources of mRNA were T cell clones, Dynabead-sorted $CD4^+/CD8^+$ T lymphocytes or tetramer-sorted $CD8^+$ T lymphocytes. Between 0.5-5 $\times$ 10^6 cells were pelleted in a clean RNase-free eppendorf tube. 1 ml of TRI Reagent™ (Sigma) was added and the cells lysed by pipetting up and down. The tube was left at room temperature for 5 min, then 200 µl of chloroform was added and the mixture vortexed. After a further incubation on ice for 15 min, the sample was centrifuged at 13,000 rpm for 15 min at 4°C. The upper aqueous phase was transferred into a fresh eppendorf tube, carefully avoiding the DNA-containing interface. The mRNA was precipitated in an equal volume (0.5 ml) of isopropanol and washed twice in 70% ethanol. The resultant pellet was resuspended in DEPC-treated water and the yield was calculated by spectrophotometry:

OD_{260} 1 = 40 µg/ml RNA or 50 µg/ml cDNA (dilulte 1 µl sample into 500 µl water)

Synthesis of cDNA

Approximately 5 µg of mRNA and 0.2 U of oligo dT primer (Collaborative Biomedical Products, USA) were mixed in a total volume of 20 µl DEPC-treated water. The sample was heated to 70°C for 3 min and then allowed to cool on ice for another 3 min. The cDNA synthesis reaction was carried out at 42°C for 60-90 min in a 40 µl reaction

mixture containing 20 U ribonuclease inhibitor (rRNasin®, Promega), 1 mM dNTPs (MBI Fermentas, Lithuania), 25 mM Tris-hydrochloride, pH 8.3, 50 mM potassium chloride, 12 mM DTT, 5 mM magnesium chloride and 30 U Avian Myeloblastosis Virus reverse transcriptase (NBL Gene Sciences, UK). The cDNA product was precipitated in one volume of 4M ammonium acetate and 6 volumes of ethanol, washed in 70% ethanol, and finally stored in autoclaved distilled water at -20°C.

In the case of samples where anchored PCR was required, the samples were further purified on Chroma Spin™ -100 disposable gel filtration columns (Clontech Laboratories, Inc) prior to ethanol precipitation.

Poly G tailing (only for anchored PCR)

Terminal transferase (Boehringer Mannheim) was used to catalyze the addition of dGTP nucleotides to the 3'OH end of cDNA strands. A 20 µl reaction mixture contained the cDNA product, 5 µM dGTP, 0.75 mM cobalt chloride, 200 mM potassium cacodylate, 25 mM Tris-hydrochloride, pH 6.6, 0.25 mg/ml BSA and 25 U of terminal transferase enzyme. After incubation for 5 min at 37°C, the tailed product was purified by phenol/chloroform extraction (take upper phase) and used as a template for anchored PCR.

PCR and purification of products

A simultaneous "no DNA" control reaction was always included for all samples. Furthermore, to avoid contamination, only dedicated reagents and Gilson pipettes were used.

β chain V region-specific PCR was carried out using a 5' TCRBV region-specific primer and a 3' TCRBC region-specific primer. Each primer also contained a restriction enzyme

site (*Not* I or *Sal* I) to facilitate later cloning steps. For anchored PCR the V region-specific primer was replaced by a poly C primer (complementary to the incorporated poly G tail).

TCRBC-specific primer:

B-Sal	CGT TTG TCG TCG ACC TGC TTC CCA TTC ACC

TCRBV-specific primers:

BV9S1	ATA AGA ATG CGG CCG CAA TAA GGA GCT CAT TAT AAA TGA AAC AG
BV16S1	ATA AGA ATG CGG CCG CCA TTT TGT GAA AGA GTC TAA ACA GGA T
BV7S1	ATA AGA ATG CGG CCG CGT TTG TCT ACA GCT ATG AGA AAC TCT
BV12S1	ATA AGA ATG CGG CCG CTA TGG TGT TAA AGA TAC TGA CAA AGG A
BV6S2	ATA AGA ATG CGG CCG CAT TTC CAG AAT GAA GCT CAA CTA GAC

Poly C primer

polyC-Not	GCA TTC AGC TGC GGC CGC $(C)_{14}$

The total reaction volume was 50 µl, containing no more than 0.5 µg of cDNA template, 1 µM each of both primers (forward and reverse), 1.25U BIOTAQ™ DNA polymerase (Bioline, UK), 2 mM magnesium chloride, 200 µM dNTPs, 16 mM ammonium sulphate, 67 mM Tris-hydrochloride, pH 8.8, 0.01% Tween-20.

Cycling parameters were as follows:

94°C	4 min	1 cycle
94°C	60s	5 cycles
65°C	60s	
72°C	120s	
94°C	60s	35 cycles
58°C	60s	
72°C	120s	

5 µl of each sample was analysed on a 1% agarose gel and the successful reactions were purified using the Wizard™ PCR Preps DNA Purification System (Promega).

Cloning of PCR products into Phagescript SK M13 vector

Purified PCR products were digested overnight at 37°C, in a total reaction volume of 50 µl, containing 10 U each of *Not*I and *Sal*I (Boehringer Mannheim), 50 mM Tris-hydrochloride, pH 7.5, 10 mM magnesium chloride, 1 mM DTT, 100 mM potassium chloride, 0.01% BSA and 0.01% Triton X-100. 5µg of Phagescript SK M13 plasmid (Stratagene) was similarly digested for 2 hours in a 20 µl reaction volume. Digested products were run on a 1% low melting point-agarose gel. Bands of the appropriate size were then excised and eluted from the agarose using the same Wizard™ kit detailed in the previous section.

Each ligation reaction contained the insert DNA, 0.2 µg of linearised vector, 5 U of T4 DNA ligase (MBI Fermentas, Lithuania), 40 mM Tris-hydrochloride, pH 7.8, 10 mM magnesium chloride, 10 mM DTT and 0.5 mM ATP in a 20 µl reaction volume. Ideally the DNA insert to vector ratio was 3:1. Tubes were incubated overnight at 16°C. A "no DNA" control was always included. In some cases, several ligations were performed for each sample, containing different amounts of DNA, eg. 1 µl, 5 µl or 15 µl, in order to produce an appropriate density of plaques following transformation into bacteria.

Preparation of fresh competent *E. coli* for transformations

An LB-tetracycline agar plate was streaked from a frozen bacterial glycerol stock of XL1-Blue MRF (Stratagene) and left to grow at 37°C overnight. The following day a few bacterial colonies were lifted from the plate and cultured in 50 ml of SOB to an optical density of 0.3-0.4 at 550 nm. 45 ml of the culture was then transferred to an ice-cold 50 ml Falcon® tube, whilst the remaining bacterial culture was given fresh nutrient broth and left at 37°C to continue growing. The chilled bacteria were pelleted in a bench-top centrifuge and resuspended very gently in 15 ml transformation buffer (10 mM MES, pH

6.3, 45 mM manganese chloride, 10 mM calcium chloride, 100 mM potassium chloride, 3 mM hexamminecobalt chloride). The cells were left on ice for 10', then pelleted once again and resuspended in 3.6 ml transformation buffer. The following were added at 10 minute intervals to the suspension, swirling gently to mix: 140 µl of top-grade DMSO, followed by 140 µl 2.2 M DTT in 10 mM potassium acetate, then a further addition of 140 µl DMSO. Cells were thus rendered transformation competent, and used immediately.

200 µl aliquots of competent cells were dispensed into chilled, sterile 15 ml polypropylene tubes (Falcon® 2059, 17 mm x 100 mm, Becton Dickinson Labware) containing 2-10 µl of ligated DNA product (amount of DNA did not exceed 10 ng) and left on ice for 40 min. The cells were then heat-shocked for 2 min at 42°C and replaced on ice. Meanwhile, a topping mixture was prepared containing 2 ml of XL1-Blue MRF bacteria (from the remaining culture kept back earlier), 1 ml of 2% X-Gal and 0.5 ml of 2% IPTG, in 35 ml of molten (~ 40°C) SOB soft agar. Roughly 4 ml of this mixture was added to each sample and poured immediately onto a small 2×YT agar plate. Once set, the plates were inverted and incubated overnight at 37°C. Successful transformants which contained DNA inserts formed clear plaques instead of blue ones.

Preparation of single stranded templates for sequencing

An overnight standing culture of XL1-Blue MRF bacteria was prepared in 5-10 ml 2×YT broth. The following day the culture was diluted 1:100 with 2×YT broth and dispensed in 1.5 ml aliquots into universal tubes. Clear plaques were picked from the transformation plates with white pipette tips and used to innoculate the medium in each universal tube. These were left shaking at 37°C for 6 hours. The cultures were transferred to eppendorf tubes in order to spin out the cells in a microfuge, keeping the supernatant. The single-

stranded DNA was then precipitated with 200 µl of 30% polyethylene glycol in 2.5 M sodium chloride. The resultant DNA pellet was dissolved in 200 µl TE buffer, pH 8.0, and subject to one round of phenol extraction followed by two rounds of chloroform extraction. The purified DNA templates were then ethanol precipitated with 1/10 volume of 3M sodium acetate and 6 volumes of 100% ethanol, washed in 70% ethanol, and finally redissolved in 7 µl sterile distilled water.

Sequencing reactions

All sequencing reactions were performed using reagents from a T7 Sequenase™ version 2.0 DNA sequencing kit (USB), following the manufacturer's supplied protocol for chain-termination sequencing. 0.5 pmol of M13 -20 primer (5' GTAAAACGACGGCC AGT 3') was annealed to each DNA template by heating the mixture to 65°C for 2 minutes, in a 10 µl volume containing 40 mM Tris-hydrochloride, pH 7.5, 20 mM magnesium chloride and 50 mM sodium chloride, and then allowing the tubes to cool slowly on the bench. In the mean time, 2.5 µl of each termination mix (ddG, ddA, ddT and ddC) was dispensed into 96-well Falcon® flexible assay plates; ie. four separate reactions for every template sequenced. This plate was kept at 37°C in readiness for termination reactions.

The specified radiolabelling mixture was prepared and 5.5 µl added to each DNA tube, incorporating [α-^{35}S]dATP into each template in a 5 min reaction at room temperature. The labelling reaction mixture was then divided between the four termination wells (3.5 µl per well), followed by a further incubation at 37°C for 5 min. All T7 DNA polymerase activity was stopped by addition of 4 µl of stop solution to each well. The samples were then separated by denaturing gel electrophoresis on 6% polyacrylamide gels and the resultant products were visualised by autoradiography.

2.5. Double Stranded Sequencing of BV14 T Cell Receptors

cDNA was prepared from mRNA and purified in the same way as for single-stranded sequencing. It was then used directly for PCR amplification.

PCR reactions

Primers to facilitate double-stranded sequencing were used. Otherwise all other parameters remained unchanged from those detailed in the previous section.

C-region specific primer:

CβR CTT CTG ATG GCT CAA ACA C

BV14-specific primer:

BV14 TCT CGA AAA GAG AAG AGG AAT

The reactions were analysed and purified as before.

Ligations and transformations using the pGEM-T Easy kit

Taq polymerase normally creates A-overhangs on the PCR products that enabled direct blunt-ended ligation into the linearised pGEM-T Easy vector supplied with the kit (Promega). 50 ng of linearised vector was mixed with a roughly equal molar quantity of PCR product, 3 U of T4 DNA ligase, 30 mM Tris-hydrochloride, pH 7.8, 10 mM magnesium chloride, 10 mM DTT and 5 mM ATP in a 10 µl reaction volume. Positive (using control insert DNA supplied) and background control (no DNA) ligations were also set up.

Ligation reactions were carried out at 4°C overnight, to optimise blunt-ended ligation. The following day, 2 µl of each ligation reaction was incubated with 50 µl of freshly-thawed, JM109 high efficiency competent *E. coli*, which were provided with the kit. Cells were heat shocked for 45-50 s at 42°C, and then cultured in 1 ml SOC medium for 1.5 h, at 37°C with shaking. Each transformation culture was plated on LB-ampicillin plates

which had been treated earlier with 100 µl of 0.1M IPTG and 20 µl of X-Gal to facilitate blue/white selection. Successful transformations yielded white bacterial colonies.

Plasmid DNA minipreps

Bacterial colonies containing DNA inserts were picked and grown in 4 ml of LB-ampicillin overnight with shaking at 37°C. Plasmid DNA was purified using a QIAprep spin miniprep kit. Typical yield was 20 µg of DNA.

Sequencing

Automatic fluorescent sequencing was carried out by the Oxford University Biochemistry Department DNA sequencing facility. 2.5 µl of miniprep product (approximately 1 µg DNA) was used for each reaction. A TCRBC-specific primer was supplied (cbeta: TGT GCA CCT CCT TCC CAT TCA CC) at 3.2 pmol/µl.

2.6. Synthetic Peptides

Peptides used in CTL assays, limiting dilution analyses and IFNγ ELISpot assays (see below) were synthesised at the laboratory in-house facility on an automated peptide synthesiser (*396 MPS*, Advanced Chemtech) by conventional solid phase Fmoc chemistry. These peptides were all analysed for purity by reverse phase HPLC. Stock solutions were prepared in PBS at 1-5 mg/ml, sterile filtered with low protein-binding 0.2 µm Millex-GV_4 disposable microfilter units (Millipore) and stored in small aliquots at -20°C.

For peptides containing tyrosine or tryptophan residues, the concentration of each stock solution was determined by measuring their absorbance when diluted 1:50 in 6M guanidine hydrochloride (Pierce). The molar concentration of peptide was calculated

from the extinction coefficients of tyrosine (ε_{Tyr} = 1470) at 275.5 nm or tryptophan (ε_{Trp} = 5690) at 280 nm by applying the following formulae [Edelhoch, 1967]:

$$[C_{tyrosine}] = OD_{276} / \varepsilon_{Tyr} \qquad (moles/dm^3)$$

$$[C_{tryptophan}] = OD_{280} / \varepsilon_{Trp}$$

Concentrations of peptides that did not contain either tyrosine or tryptophan residues were estimated from the peak area on the HPLC trace, compared with known standards. Larger preparations of synthetic peptides, for use in making MHC-peptide tetrameric complexes (see below), were obtained from either of two commercial suppliers, Alta Bioscience, UK and Genosys Biotechnologies Inc., USA.

2.7. Limiting Dilution Analysis

Preparation of mononuclear feeder cells

This method was modified from one described by Steven *et al* [1996]. Three buffy coat layers from different individuals were obtained from the Blood Transfusion Service (Bristol, UK). Mononuclear cells were isolated as from whole blood, using Lymphoprep density gradient centrifugation. The recovered cells were PHA-blasted (~5 μg/ml final concentration) for one hour in R10, then washed thoroughly and pooled. Feeder cells were γ-irradiated before being added to assay cultures.

Setting up assay cultures (Day 0)

Freshly isolated donor PBMC were split to provide 5-10 × 10^5 cells as stimulator APCs and 3.1 × 10^6 as responders. Stimulator cells were peptide-pulsed (5 μM final) and PHA-blasted for 1 h at 37°C, then washed and γ-irradiated.

Responder cells were plated out in a range of dilutions, from 40,000 to 2,000 cells per well in 96-well round bottom plates (24 replicates/input no.). In addition, 0.5-1 × 10^4

stimulators and 5×10^4 feeders were also added to each well in a total volume of 100 µl. R10 medium supplemented with 10 U/ml Lymphocult-T (Biotest AG, Germany) and 25 U/ml rIL-2 (kind gift of Cetus Corp.) was used to sustain the cultures. 40 µl of fresh medium was added to each well every 3-4 days, to bring the total culture volume to 220 µl at the end of the 14-day period.

^{51}Cr-release cytotoxicity assay (Day 14)

Epitope-specific responder frequencies were assayed by measuring CTL-mediated cytotoxicity against ^{51}Cr-labelled peptide-pulsed and unpulsed target cells. Autologous or matched B-LCLs or autologous PHA blasts were used as targets in assays for EBV lytic epitopes and T2 transfectant cells for latent EBV epitopes. The culture plates containing the effector cells were first split two ways; one set each for peptide-pulsed or unpulsed targets. Several million target cells were labelled with 150 µCi [^{51}Cr] sodium chromate in 100 µl of assay medium (RPMI with 5% FCS), at 37°C for 1 hour. Half of these were additionally incubated with 5 µg of the cognate peptide. Target cells were then washed 4 times to remove free chromium, and dispensed in 100 µl volumes into each assay well, at 3×10^3 cells/well. Maximum and spontaneous release was calculated from two additional sets of 24 target wells, incubated with only 100 µl of 5% Triton X-100 or medium, respectively. The assay plates were incubated at 37°C for 4-5 hours, after which 20 µl of the supernatant was removed from each well, and spotted onto filtermats (Wallac Oy, Finland). ^{51}Cr was quantified using a 1205 Betaplate® liquid scintillation counter (Wallac) and specific lysis calculated according to the following formula:

$$\text{Specific lysis} = \frac{\text{(experimental release - medium release)}}{\text{(maximum release - medium release)}} \times 100\%$$

For the purpose of statistical analysis, wells were scored as positive if specific lysis exceeded 10%. The CTL precursor frequency was estimated, assuming Poisson distribution and single hit kinetics [Taswell, 1981], using the method of maximum likelihood [Fazekas de St. Groth, 1982] and χ^2 analysis to determine a linear regression curve. Frequency values were estimated at which 37% of the wells were negative for epitope recognition.

2.8. IFNγ Enzyme Linked Immunospot (ELISpot) Assays

This method was originally described by Lalvani *et al* [1997]. 96-well plates backed with polyvinylidene difluoride (Millipore) were pre-coated with 15μg/ml of an IFNγ specific monoclonal antibody, 1-DIK (Mabtech, Sweden). Sample wells were then washed six times with PBS and blocked with 200 μl of R10 medium. Freshly isolated PBMCs were seeded in duplicate wells, usually at 2.5×10^5, 1.25×10^5 and 6.25×10^4 cells/well, in the presence of 2 μM peptide representing known minimal CTL epitopes. The plates were left undisturbed, overnight at 37°C, 5% CO_2.

On the following day the cells were discarded and the wells washed as before. A second biotin-conjugated anti-IFNγ mAb, 7-B6-1 biotin (Mabtech), was added to the wells at 1 μg/ml, and the plates incubated for 3 hours at room temperature, followed by streptavidin-conjugated alkaline phosphatase (Mabtech) for a further 2 hours. The sample wells were washed throughly before proceeding with the colour development step.

A 30-minute reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) using an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories) resulted in the appearance of dark spots representing individual IFNγ producing $CD8^+$ T cells. The colour development reaction was terminated by washing the

plates under running tap water. The spots were counted under a dissection microscope after leaving the plates to dry.

The number of specific T cell responders was calculated after subtracting values obtained from control wells that did not contain any peptide. Furthermore, results were not considered significant unless they were at least twice that of negative control values and greater than $10/10^6$ cells.

2.9. Synthesis of Class I MHC-Peptide Tetrameric Complexes

Recombinant protein expression and inclusion body purification

This method was developed by Altman *et al* [1996]. HLA-A2 and HLA-B8 expression constructs were made, encoding the extracellular domains of class I heavy chain protein, and modified at the C-terminus to include a substrate sequence for BirA biotinylation. were The constructs were cloned into the expression vector pGMT7 (a pET derivative) [Studier *et al*, 1990], transformed into BL21(DE3)pLysS *Escherichia coli* cells (Novagen) and used for expression of soluble HLA-A2 or HLA-B8 heavy chain protein. Soluble β_2m protein was produced in a similar fashion, form the vector pHN1-β_2m. (Transformed bacterial cells expressing HLA-A2 heavy chain protein were a gift from Dr V. Cerundolo. Cells expressing HLA-B8 heavy chain or β_2m proteins were generously provided by Dr C.A. O'Callaghan.)

An LB-ampicillin/chloramphenicol plate was streaked from the relevant frozen glycerol stock or freshly transformed bacteria and left to grow overnight at 37°C. A single bacterial colony was picked with a sterile innoculating loop and resuspended in 10 ml of low salt LB-amp in a clean flask. The culture was incubated with shaking (~240 rpm) at 37°C for 6-9 hours. This was then added to 500 ml of LB-amp and left standing overnight. The following day the overnight bacterial culture was used to innoculate 6

litres of LB-amp. These were grown up to mid-log phase (OD_{600} = 0.3-0.4) over 3-4 hours before protein expression was induced by addition of 0.5 ml of 1M IPTG to each flask. The cultures were left for a further 5 h at 37°C with shaking, after which the cells were harvested by centrifugation and the supernatant discarded.

The bacterial cell pellet was resuspended in ice-cold PBS and sonicated (*XL* series sonicator; Misonix Inc., USA) in a small glass beaker on ice in order to lyse the cells and release the protein inclusion bodies. Once the cells were completely lysed the inclusion bodies were spun down in 35-ml polycarbonate tubes in a Beckman ultracentrifuge (*J2* series) at 15,000 rpm for 10 min. The inclusion bodies were then washed three times in chilled Triton wash buffer (0.5% Triton X-100, 50 mM Tris, pH 8.0, 100 mM sodium chloride, 0.1% sodium azide and freshly added 2 mM DTT), using a hand-held glass homogeniser to resuspend the pellet after each wash. This was followed by a final wash in detergent-free wash buffer.

The resultant purified protein preparation was solubilised in 20-50 ml of 8M urea buffer, containing 50 mM MES, pH 6.5, 0.1 mM EDTA and 1 mM DTT, and left on an end-over-end rotator overnight at 4°C. Insoluble particles were removed by centrifugation before the solubilised protein was dispensed in aliquots and stored at -70°C for future use. The protein yield was determined using a Coomassie dye-binding protein assay reagent (Bio-Rad Laboratories) and by comparison with known standards. The quality of expression and purification was also verified by running samples on a 15% SDS-polyacrylamide gel.

Refolding soluble class I MHC-peptide complexes

A litre flask containing 500 ml of refolding buffer (100 mM Tris, pH 8.0, 400 mM L-arginine hydrochloride, 2 mM EDTA, 5 mM reduced glutathione and 0.5 mM oxidised glutathione) was freshly prepared and chilled, with stirring, at 4°C. The components of

the class I MHC protein complex were added in the following order: peptide, β_2m, and class I heavy chain. Approximately 10 mg of synthetic peptide, 25 mg of β_2m and 30 mg of heavy chain were required for each refold. Lyophilised peptide was dissolved in 0.5 ml of DMSO and added directly to the refold buffer. The urea-solubilised β_2m and heavy chain proteins (prepared in the previous section) were thawed out and used immediately to avoid oxidation of cysteine residues. These were injected with a syringe into the refolding mixture through a #25 needle, as near as possible to the vigorously rotating magnetic stir bar; this was to ensure rapid and efficient dilution. The amount of heavy chain protein to be added was divided into several separate dilutions (eg. two or three additions over 24 hours), which resulted in higher yields of folded complex. The mixture was left continuously stirring at 4°C for 40-60 hours.

Site-specific biotinylation of MHC-peptide complexes

The refolding mixture by centrifuged and filtered to remove protein precipitates, then concentrated down to less than 10 ml by ultrafiltration, using a large pressurised stirred cell (Amicon, Millipore) and then Centripreps® (Amicon, Millipore) (MW cutoff: 10,000). The protein was then buffer-exchanged into BirA reaction buffer (20 mM Tris, pH 7.5, 25 mM sodium chloride and 7.5 mM magnesium chloride), using disposable PD-10 gel filtration columns (Pharmacia Biotech). Recombinant BirA enzyme (10 μM final concentration, kind gift of Dr CA O'Callaghan) was added, in the presence of 10 mM ATP, pH 7.0, and 0.5 mM biotin, to facilitate incorporation of biotin at the enzyme recognition sequence on the C-terminus of the heavy chain protein. A selection of protease inhibitors was also added to preserve the proteins: 0.2 mM PMSF, 2 μg/ml pepstatin and 2 μg/ml leupeptin. The reaction was left overnight at room temperature.

Folded, biotinylated MHC-peptide complexes were recovered by FPLC size-exclusion gel filtration on a Superdex 75 column (Pharmacia Biotech), using 20 mM Tris, pH 8.0, 50 mM sodium chloride buffer. The protein was further purified by anion-exchange chromatography using a BioCAD®*SPRINT™* perfusion chromatography system (PerSeptive Biosystems Inc., USA) over a 0-500 mM sodium chloride gradient.

Biotinylation was verified by direct blotting of the purified protein onto a piece of nitrocellulose membrane, using a commercial biotinylated antibody as a positive control. The membrane was then stained with ExtrAvidin®-horseradish peroxidase (Sigma). The presence of biotinylated protein on the membrane was detected using chemiluminescent reagents supplied in an ECL kit (Amersham, UK).

Preparation of fluorescent-labelled tetrameric complexes

The amount of biotinylated protein was quantified using the same Bio-Rad protein assay reagent as before. Phycoerythrin-labelled streptavidin (Sigma) was added in small doses, always maintaining a fourfold molar excess of MHC protein (since streptavidin has four biotin binding sites). The resultant tetrameric complexes could be stored for over a year in light-shielded containers at 4 °C.

Table 2.1. List of MHC-peptide tetrameric complexes used

Name	MHC molecule	Peptide	Epitope
A2/GLC	HLA-A2	GLCTLVAML	EBV BMLF1 280-288
A2/FluM[a]	HLA-A2	GILGFVFTL	Influenza matrix 58-66
A11/IVT[b]	HLA-A11	IVTDFSVIK	EBV EBNA3B 416-424
B8/FLR	HLA-B8	FLRGRAYGL	EBV EBNA3A 325-333
B8/RAK	HLA-B8	RAKFKQLL	EBV BZLF1 190-197

[a] *gift of Dr V Cerundolo;* [b] *gift of Dr P Hansasuta*

2.10. Flow Cytometry

Cell staining with directly-conjugated monoclonal antibodies or tetrameric complexes

PBMC or SFMC (either fresh or thawed from frozen) were washed and resuspended in a small volume of FACSwash (0.1% sodium azide, 0.16% BSA in PBS). Where required the cells were blocked in 10% pooled human serum for 10-15 min on ice. They were then divided into sample tubes containing at least 2×10^5 cells each, although larger numbers of cells were required for tetramer staining (up to 10^6 per sample). About 0.1-0.5 μg (usually a couple of microlitres) of each mAb (or tetramer) directly conjugated to FITC, PE or Tricolor was added to each sample, in 50 μl of FACSwash, and the samples incubated for 30 min on ice, in the dark. The cells were then washed twice and fixed in FACSfix (1% FCS, 2.5% formaldehyde in PBS).

Multicolour cell staining with unconjugated monoclonal antibodies

PBMC or SFMC were prepared as before and divided into sample tubes. Cells were first resuspended with the primary unconjugated (mouse) antibody and incubated for 30 min at 4°C. Samples were then washed and stained with a PE- or FITC-labelled secondary (anti-mouse Ig) antibody. The samples were washed again and any directly-conjugated antibodies or tetrameric complexes were added at this stage, in the presence of 1% serum (mouse) matching the specificity of the secondary antibody. Cells were incubated as before and then finally washed and fixed.

Intracellular staining with the Ki67 marker

Samples were first stained with any mAb specific for extracellular epitopes, as described above. They were then washed in FACSwash and fixed in 4% paraformaldehyde for 20 min on ice. This was followed by two washes and resuspension in permeabilisation buffer

(0.1% saponin, 1% FCS, 0.1% sodium azide in PBS). FITC-conjugated Ki67 (DAKO, Denmark) [Gerdes *et al*, 1984] was added to the samples, diluted in permeabilisation buffer, and left for 30 minutes. The samples were washed twice again in permeabilisation buffer, then in FACSwash and finally fixed in FACSfix.

FACS data acquisition & analysis

Samples were analysed on a Becton Dickinson *FACScan* instrument using CellQuest software (Becton Dickinson). In all cases the lymphocyte population was gated by forward and side angle scatter. Markers were set to allow analysis of the $CD8^{hi}$ (and $CD3^{+}$) subset of T lymphocytes.

2.11. Antibodies and Sources

Antibodies specific for human leukocyte cell surface differentiation markers

FITC conjugated; anti-

CD3 (UCHT1)	DAKO
CD25 (Act-1)	DAKO
CD28 (CD28.2)	Immunotech, France
CD38 (AT13/5)	DAKO
CD45RA (ALB11)	Immunotech
CD45RO (UCHL1)	DAKO
CD57 (Leu-7)	Becton Dickinson
CD62L (DREG-56)	PharMingen
CD69 (FN50)	DAKO
HLA-DR, DQ, DP (CR3/43)	DAKO

PE conjugated; anti-

CD4 (MT310)	DAKO
CD19 (HD37)	DAKO

Tricolor conjugated; anti-

CD4 (S3.5) Tricolor	Caltag Laboratories, USA

CD8 (3B5) Tricolor	Caltag

Unconjugated; anti-

CxCR3 (49801.111)	R&D Systems, UK
CxCR4 (12G5)	R&D Systems
CCR2 (48607.121)	R&D Systems
CCR3 (LS63 7B11)	gift of Leukosite
CCR5 (45549.111)	R&D Systems
LFA-1 (αCD11a, DF1524)	Sigma
VLA-4 (2B4)	R&D Systems
$\alpha_4\beta_7$ integrin (ActI)	gift of Leukosite

Anti-human TCR variable β chain (BV) specific mAbs

This antibody panel formed part of the TCR monoclonal antibody workshop. [Posnett *et al*, 1996]

BV1 (BL37.2)	BV13.1 (H131)
BV2 (MPB2)	BV13.2 (H132)
BV3 (JOVI-3)	BV13.6 (JU74.3)
BV5.2/5.3 (1CI)	BV14 (CAS1.1.3)
BV6.7 (QT145)	BV16 (Tamaya1.2)
BV7.1 (3G5)	BV17 (E17.5)
BV8 (JR2)	BV18 (BA62.6)
BV9 (MKB1 P2-10)	BV20 (ELL1.4)
BV11 (C21)	BV21.3 (IM1347)
BV12 (S511)	BV22 (IMMU546)

Other monoclonal antibodies

Secondary antibodies:

rabbit anti-mouse Ig FITC	DAKO
rabbit anti-mouse Ig PE	DAKO

Antibodies against MHC molecules:

W6/32 (folded class I HLA specific)	gift of Dr J Haurum [Parham & Brodsky, 1981]
BB7.2 (HLA-A2 specific)	gift of Dr J Haurum [Barnstable *et al*, 1978]

CHAPTER 3

Molecular Analysis of Nucleotide Bias during VDJ Recombination in T Lymphocyte β Chains

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Recognition of antigen by αβ T lymphocytes is mediated by interaction of the TCR with foreign peptides bound to class I MHC. Structural alignments with immunoglobulin (Ig) [Chothia *et al*, 1988] as well as recent TCR crystal structures [Garcia *et al*, 1996; Garboczi *et al*, 1996] have indicated that the third complementarity-determining (CDR3) loops play a major role in contacting the peptide antigen that lies within the MHC antigen binding groove. This loop corresponds to the hyperviable region of the TCR β chain due to the junctional diversity created during V(D)J recombination. As a result, the events that influence variability in the CDR3 region have a profound effect on the fine specificity of peptide recognition by T lymphocytes.

As with antibody immunoglobulin proteins, each functional T cell receptor is produced by site-specific recombination of variable (V), joining (J) and constant (C) region gene segments. T cell β chains also include additional diversity (D) gene segments. [Tonegawa, 1983; Davis & Bjorkman, 1988]. During maturation of T cells in the thymus, the TCR gene segments are rearranged in a defined order, resulting in the formation of functional TCR α and β exons in which V, D, and J segments are in close proximity to one another. This process of somatic rearrangement is a prerequisite to TCR gene expression and is important for the generation of TCR diversity. Recombination signal sequences, which consist of a palindromic heptamer and an A/T-rich nonamer, separated

by a spacer of 12 or 23 base pairs, direct the rearrangement process. A mature mRNA is produced by splicing to bring V(D)J and C segments together and allows expression of the α and β chain proteins.

Potential for diversity of the TCR is estimated to be greater than that for Ig, largely because of greater potential for junctional diversity, particularly within the β chain. A striking concentration of sequence polymorphism is located in the CDR3 region, which is responsible for contacting peptide antigen. In addition to combinatorial diversity created from recombination of multiple genes, junctional diversity at coding junctions of VJ, VD and DJ also contributes significantly to formation of the TCR repertoire. Imprecise joining of V-DJ and V-J and translation of D region sequences in all three reading frames is commonly found, in addition to frequent deletion and addition of nontemplated nucleotides at coding junctions. Deletions are thought to result from exonuclease nibbling of the free terminal nucleotides prior to ligation with adjacent gene segments. Random addition of (N) nucleotides to free ends is associated with terminal deoxynucleotidyl transferase (TdT) activity. It is also believed that insertion of non-random P (palindromic) nucleotides occurs, which are inversely complementary to the coding ends from which they are derived (see Figure 3.1.). Thus V(D)J recombination enables an extremely diverse T cell repertoire to be generated. Estimates of the potential size of the unselected immature T cell repertoire range from 10^{10} to 10^{15} specificities [Davis & Bjorkman, 1988].

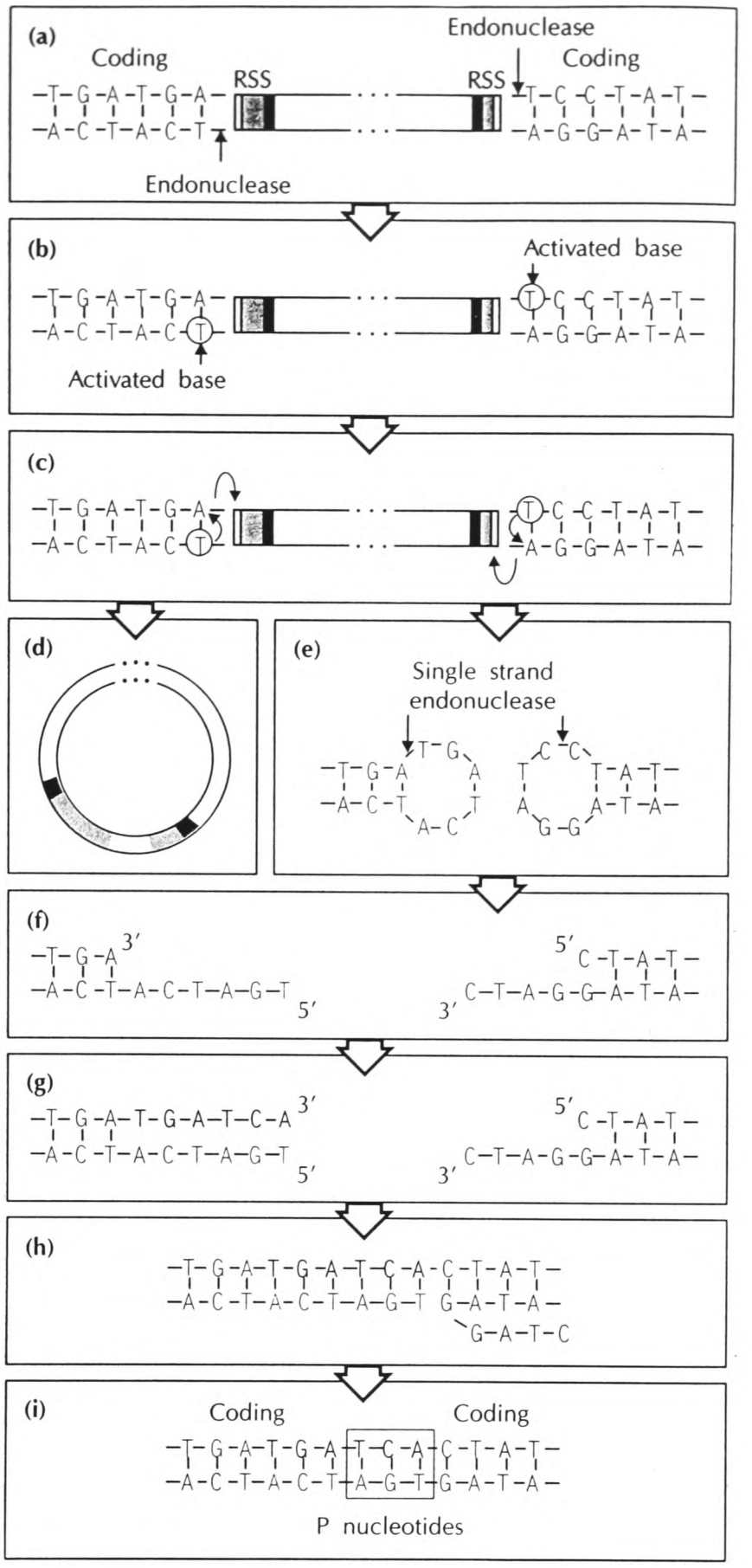


Figure 3.1. A model for V(D)J recombination. (*a*) The recombinase introduces a single-strand nick between the RSS heptamer and the coding region. (*b*) The nucleotide bound to the recombinase is activated and (*c*) attacks its complementary base. This results in a hairpin structure formation at the coding ends, generating double strand breaks between the RSS elements and the coding gene segments. (*d*) The RSS elements are ligated, generating a DNA circle which is lost. (*e*) A single-strand endonuclease nicks between any two bases exposed as a result of hairpin formation. (*f*) Bases are filled in, after which (*g*) the base-paired ends are ligated and (*h*) unpaired nucleotides are removed. (*I*) The end result in this example is the presence of three pairs of P nucleotides and a loss of two base pairs from the end of coding region 2. *Reprinted from Ferguson & Thompson, 1993.*

Individual TCRBV gene segments may influence the generation of diversity during VDJ recombination. For example, through preferential selection of particular D or J gene segments. Furthermore, in experiments with synthetic plasmid substrates, the sequence of flanking coding regions has been shown to have some effect on differential processing of recombination products [Boubnov *et al*, 1993]. This implies that a particular TCRBV gene can impose restrictions on the potential diversity of the primary T cell repertoire and has important repercussions on the fine specificity of peptide recognition.

The influence of TCRBV gene segment 3' terminal sequences on the creation of junctional diversity has not been addressed in a normal unstimulated human repertoire. In this study a large number of unique sequences were sequenced from a limited selection of TCRBV segments that had different terminal sequences, in order to find evidence for skewing of junctional diversity during CDR3 formation. I looked at bias in selection of particular J regions by V genes, also extent of nucleotide deletion, and evidence for P addition.

3.1. Sequencing the CDR3 regions of TCR β chains

Five representative TCRBV genes were selected in order to compare their influence on the generation of junctional diversity during TCR VDJ recombination. These were chosen on the basis of sequence variation and nucleotide base composition of the 3' terminal nucleotides in their germline sequences, as shown in Table 3.1. The major difference between each BV gene was in the last six nucleotides flanking the recombination signal sequence. Furthermore, BV7S1 and BV6S3 were relatively G/C rich in comparison to the other sequences.

Table 3.1. Selected TCR BV gene sequences

TCR gene	3' coding sequence[a]	% base composition A/T	G/C
BV7S1	C**TCTGTGCCAGCAGCCAAGA**-RSS[b]	40	60
BV9S1	T**TCTGTGCCAGCAGCCAAGA**-RSS	45	55
BV6S3	C**TCTGTGCCAGCAGCTTAGC**-RSS	40	60
BV8S1	T**TCTGTGCCAGCAGTTTAGC**-RSS	50	50
BV12S2	T**TCTGTGCCATCAGTGAGTC**-RSS	50	50

[a]nucleotide differences highlighted in bold; [b]RSS, recombination signal sequence.

PBMC were isolated from two healthy donors and sorted into $CD4^+$ or $CD8^+$ lymphocytes by magnetic beading. The HLA type of the two donors was as follows: donor VB - A2, B44, B65; donor SM - A2, A24, B14, B44. cDNA was prepared from donor lymphocytes and used as templates for BV gene-specific PCR and subsequent sequencing of the TCR variable region. The number of sequences analysed for each BV group was as follows[1]: BV6S3 $CD4^+$, 36 clones; BV6S3 $CD8^+$, 37 clones; BV7S1 $CD4^+$, 14 clones; BV7S1 $CD8^+$, 55 clones; BV8S1 $CD4^+$, 50 clones; BV8S1 $CD8^+$, 34 clones; BV9S1 $CD4^+$, 26 clones; BV9S1 $CD8^+$, 31 clones; and BV12S2 $CD4^+$, 30 clones. Where more than one identical TCRB sequence was obtained, these were treated as having arisen from the same clone and therefore only counted once in this analysis.

3.2. Analysis of J gene segment usage

There are 13 TCRBJ segments available which can combine with any one TCRBV segment; the first six are found in one cluster (TCRBJ1) and the remaining seven in a

[1] Sequences of CDR3 coding joints are listed in Appendix C.

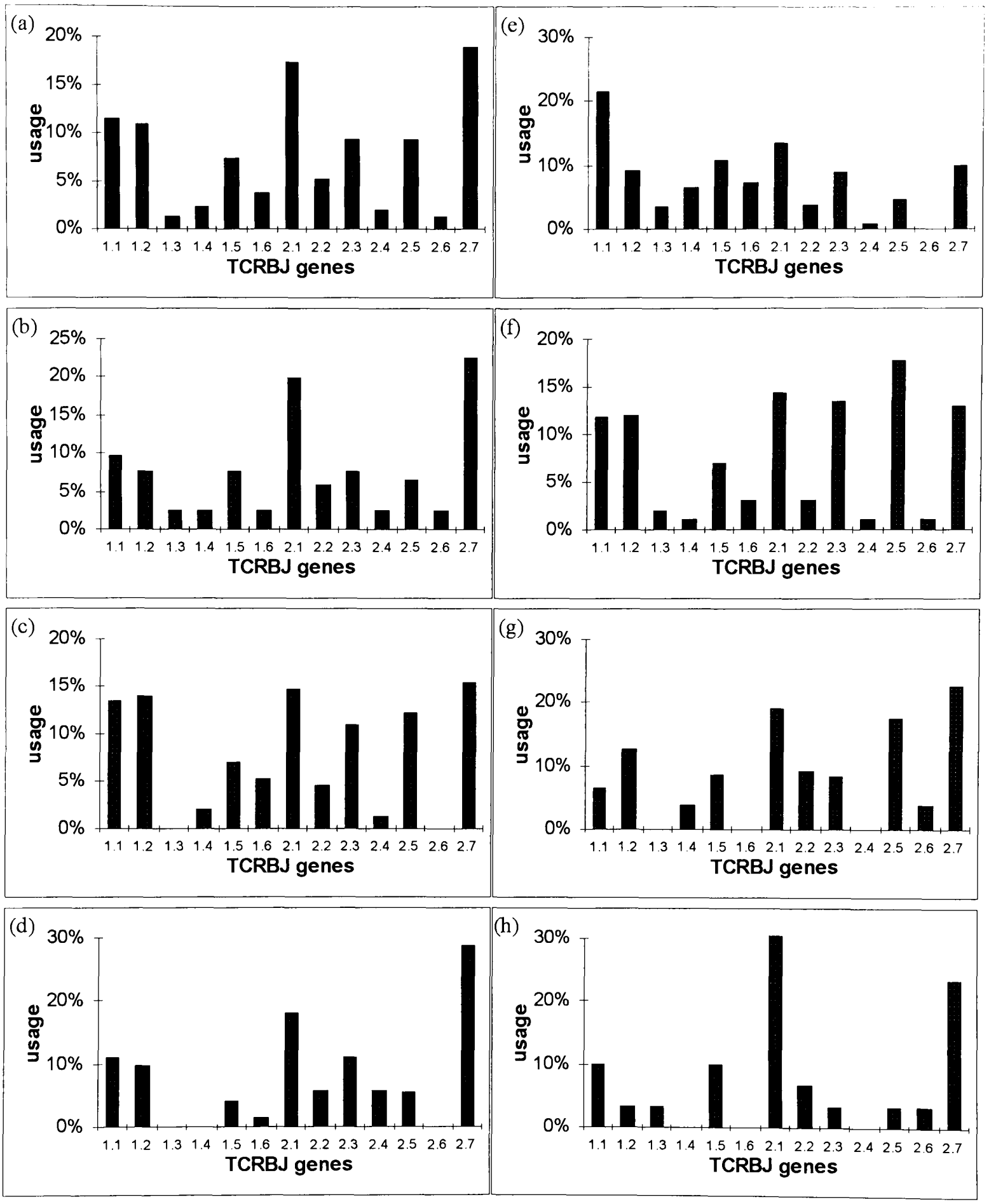


Figure 3.2. Selection of TCRBJ gene segments in each group of TCRBV sequences. (*a*) overall usage, compared to (*b*) the CD4 population and (*c*) CD8 population. Preferences are shown for individual groups of (*d*) BV6S3, (*e*) BV7S1, (*f*) BV8S1, (*g*) BV9S1 and (*h*) BV12S2 sequences.

second cluster (TCRBJ2) [Toyonaga *et al*, 1985]. Segments from the TCRBJ2 cluster were more commonly found in sequences overall (37.1% vs. 62.9%), and also in CD4- (32.7% vs. 67.3%) or CD8-sorted (41.4% vs. 58.6%) populations. In addition, certain individual J gene segments were more widely used than others. Thus 58.5% of all sequences expressed BJ1S1, BJ1S2, BJ2S1 or BJ2S7, whilst BJ1S3, BJ1S4, BJ2S4 and BJ2S6 occurred rarely (Fig 3.2.*a*). This bias in J segment usage was similarly maintained in the CD4 population, but was less marked among the CD8 sequences (Fig 3.2.*b* and *c*), which also included common use of BJ2S3 and BJ2S5. There were some differences within each group of TCRBV sequences. In particular, the preference for the BJ2 cluster was reversed amongst BV7S1 sequences; instead almost 60% of sequences utilised BJ1 gene segments (Fig 3.2.*e*). Also, pairing of BV9S1 to BJ1S1 appeared to be unfavourable, contrary to the general trend (Figure 3.2.*g*).

3.3. Analysis of nucleotide nibbling from TCRBV 3' termini in coding joints

During recombination nucleotides are often deleted or "nibbled" from coding ends by exonucleases. Sequences were analysed to see whether the extent of nucleotide loss differed significantly between TCRBV groups. Consistent with an earlier report [Callan *et al*, 1995], the 3' germline-encoded nucleotides were found to be significantly well-preserved in the coding joints of BV7S1 sequences (Willcoxon test, $P < 0.001$) compared to other sequences. The mean value of terminal nucleotides lost was 2.2 nt in BV7S1 sequences; furthermore, in 31.9% of sequences the V segment remained intact after recombination (Table 3.2.). BV9S1 sequences, which shared identical termini to BV7S1 were also relatively less nibbled, although this comparison was less significant ($P = 0.052$). By comparison, the other sequences showed greater amounts of nucleotide deletion, as summarised in Table 3.2 and Figure 3.3. The highest number of nucleotides

were removed from BV8S1 sequences, which on average lost 4.1 nucleotides. Over 50% of BV8S1 sequences lost the last 5 nucleotides of their V segment during recombination. The average values were very similar between the CD4 and CD8 populations (not shown).

Table 3.2. Comparison of deletion of 3' nucleotides from BV coding regions during VDJ recombination

	BV6S3	BV7S1	BV8S1	BV9S1	BV12S2
0 nucleotides deleted	13.9 %	31.9 %	9.5 %	25.6 %	20.0 %
5 nt or more deleted	36.1 %	20.3 %	52.4 %	19.3 %	33.3 %
ave. nucleotides lost	3.6 nt	2.2 nt	4.1 nt	2.9 nt	3.6 nt

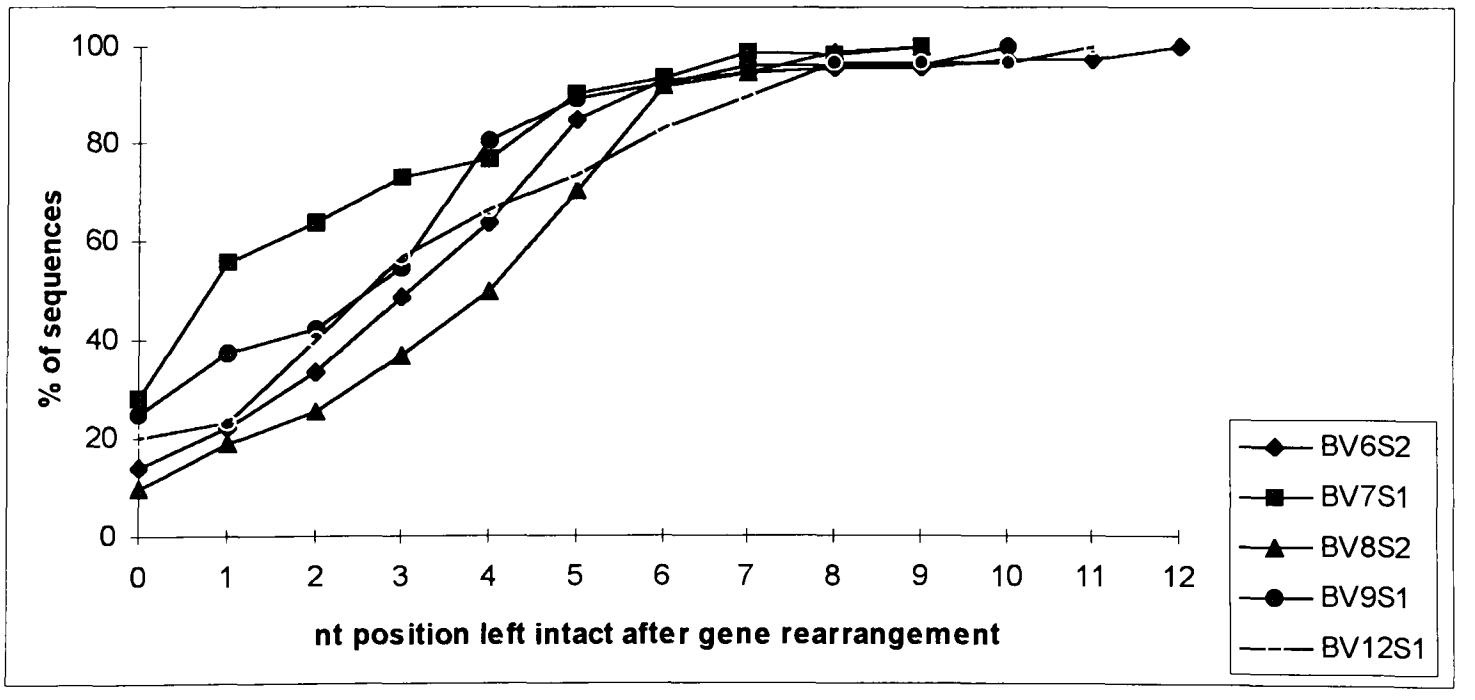


Figure 3.3. Cumulative graph showing exonucleolytic nibbling from the 3' termini of TCRBV genes during V(D)J recombination.

3.4. Evidence for P nucleotide addition

Recombination of V, D and J segments is believed to proceed via a hairpin intermediate structure [Lieber, 1991]. P nucleotide addition has been proposed to occur during the resolution of intermediates, when a nick is introduced into only one strand of the hairpin loop, resulting in a palindromic nucleotide sequence across the coding junction [Lafaille *et al*, 1989]. Examples of P addition could only be observed where the coding nucleotides were not nibbled back prior to junction formation. P nucleotides were identified in about 50% of junctions containing full length BV coding segments.

Discussion

The results of this study show that biased patterns of VDJ rearrangement exist in CDR3 regions belonging to different groups of αβ TCR BV genes. These may be due to cellular selection events or limitations of the rearrangement process, such as the accessibility of DNA to bending and unwinding by recombinases, or the formation and resolution of hairpin loops. In agreement with previous findings of Rosenberg *et al* [1992], there was a marked preference for BJ2 segments over BJ1. It is possible that this was simply due to the fact that pairing of a particular BV segment to a BD1-BJ1 unit does not exclude subsequent rearrangement and pairing with a BD2-BJ2 unit. Therefore there are likely to be twice as many receptors containing gene segments from the BJ2 cluster. Skewing of the repertoire towards particular BJ gene segments may be attributed to the accessibility of the DNA structure to recognition and binding by recombinases. Furthermore, the minor inconsistencies between families and also between CD4 and CD8 lymphocytes indicate that some bias, at least, could be the result of selection events.

Earlier work using extrachromosomal recombination substrates revealed that coding ends consisting of G/C homopolymers showed reduced nucleotide deletion [Boubnov *et al*,

1993]. Another study in mice reported a strong correlation between internal stretches of A/T-rich sequence and high average nucleotide deletion [Nadel & Feeney, 1995] in Ig and TCR repertoires. Similarly, non-random patterns of coding-end processing were found in this study. In particular, coding ends of BV7S1 and BV9S1 sequences were frequently preserved from exonuclease nibbling. A previous report had already demonstrated that BV7S1 sequences were relatively resistent to nibbling [Callan *et al*, 1995]; the observation here of a similar effect in BV9S1 sequences, which carries identical nucleotides in the last five positions, suggests that motifs in the 3' sequence may be involved in directing the common presence of particular residues in the VDJ junction. A/T base composition may also have contributed to levels of nucleotide deletion, as BV7S1 and BV9S1 sequences contained relatively lower proportions of A/T bases (40% and 45%, respectively). Although the nucleotide composition of the BV6S3 was no different from BV7S1, these sequences still underwent higher average nucleotide deletion. The presence of three consecutive pairs A.T nucleotides near the 3' terminus of this gene segment could probably have been a contributing factor in this case, as the weaker pairings in A.T stretches enable more efficient nibbling by exonucleases. Likewise a stretch of four A.T nucleotide pairs in BV8S1 coding ends might have accounted for the observation of greater nucleotide loss compared to the BV12S2 group, although the nucleotide composition of both was identical.

Until recently it was thought that inserted nucleotides at coding junctions was essentially the result of random addition by the TdT enzyme, being both variable in length and unpredictable in sequence. Non-random addition of residues at coding junctions that are inversely complementary to the adjoining full-length coding segment support the model that resolution of hairpin intermediates occurs with the introduction of nick(s) in only one of the two strands. It is not known whether hairpins are obligate intermediates that

occasionally give rise to P nucleotide inserts, or whether there are two pathways for forming coding ends, only one of which generates hairpins. P nucleotides were detected in about 50% of all sequences that retained intact coding ends. They were present in every group of sequences, although it was not possible to compare their frequency between BV groups as there were too few sequences.

Certain mutated versions of RAG1 have been shown to be inhibited by coding sequence variations that normally do not affect recombination with wild-type RAG1 [Sadofsky *et al*, 1995], suggesting that RAG-1 may make direct contact with the DNA at the coding-signal heptamer border. The influence of coding flank sequence on RAG-1 recognition of recombination signals is a possible mechanism by which different BV gene sequences can shape the primary T cell repertoire.

CHAPTER 4

CTL Responses in Primary EBV Infection

Primary infection with virus can stimulate a vigorous cytotoxic T cell response, with activation and proliferation of lymphocytes at the site of infection [Deckhut *et al*, 1993], within draining lymph nodes [McHeyzer-Williams *et al*, 1995], and sometimes also within peripheral blood [Callan *et al*, 1996; Pantaleo *et al*, 1994]. The extent to which these lymphocytes have been specifically activated by antigen or nonspecifically activated, perhaps by cytokines (bystander activation) has long been a matter of debate. In previous studies, measurement of CTL frequencies by LDA indicated that antigen-specific responses consituted a minor fraction of the virus-activated $CD8^+$ T cells. In contrast later studies analysing TCR gene usage during acute viral infections reported the predominance of clonal or oligoclonal $CD8^+$ expansions, implying that the proportion of T cells directly activated by antigen might be greater than originally suggested [Callan *et al*, 1996; Pantaleo *et al*, 1994]. However such expansions were only found in some individuals and their significance was unclear in the absence of more direct means of identifying antigen-specific T cells *in vivo*.

The development of novel techniques for detecting antigen-specific T cells has enabled us to address this apparent discrepancy. Two recent articles have reported that 50% to 70% of activated $CD8^+$ T cells may be LCMV-specific, during primary infection in mice, based on binding to peptide-MHC tetrameric complexes and single-cell analysis of IFNγ production [Murali-Krishna *et al*, 1998; Butz and Bevan, 1998]. In particular, Murali-

Krishna *et al* found that LDA underestimated the frequency of LCMV-specific cells by 20-100 fold. In this chapter I sought to reassess the frequency of antigen-specific cells during primary infection with a naturally-occurring virus in humans, using peptide-MHC tetramers to both quantify and characterise antigen-specific T cells directly. Primary EBV infection in adolescence or young adulthood may be clinically manifest as acute infectious mononucleosis (IM), a disease characterised by a striking expansion of $CD8^+$ T cells in peripheral blood. This highly immunogenic virus provides an ideal natural situation in which to study the development of a primary $CD8^+$ immune response in humans. I constructed tetramers of MHC molecules complexed to defined EBV peptide epitopes, and used these to identify directly and to characterise epitope-specific T cells within the primary T cell response in IM patients. In selected individuals it was possible to study responses in the same patients between 6 and 37 months later, after establishment of the long-term virus carrier state. This study focuses on responses to two immunodominant epitopes from lytic cycle antigens (an HLA-A2 restricted epitope in BMLF1 and an HLA-B8 restricted epitope in BZLF1), as these have been less well characterised, and compares them to a well-known immunodominant epitope from an EBV latent cycle protein (an HLA-B8 restricted epitope in EBNA3A).

4.1. Construction of tetrameric complexes and staining of control samples

The class I MHC-peptide tetrameric complexes developed by Altman *et al* [1996] consist of four biotinylated class I complexes associated with streptavidin. Each class I complex is constructed by refolding a recombinant truncated version of the relevant heavy chain protein (which incorporates a biotinylation substrate sequence tag at the C-terminus), recombinant β_2m and synthetic peptide representing a minimal CTL epitope. Complexes are then biotinylated and purified before being combined with streptavidin in a four-fold

molar excess. Incorporation of streptavidin that has been conjugated to fluorochromes enables such "tetramers" to be used as reagents in FACS analysis to detect antigen-specific $CD8^+$ T lymphocytes. Since streptavidin has a tetrahedral symmetry and the most commonly used fluorochrome, phycoerythrin is quite large, ligand binding is probably trimeric [Davis *et al*, 1998; McMichael & O'Callaghan, 1998].

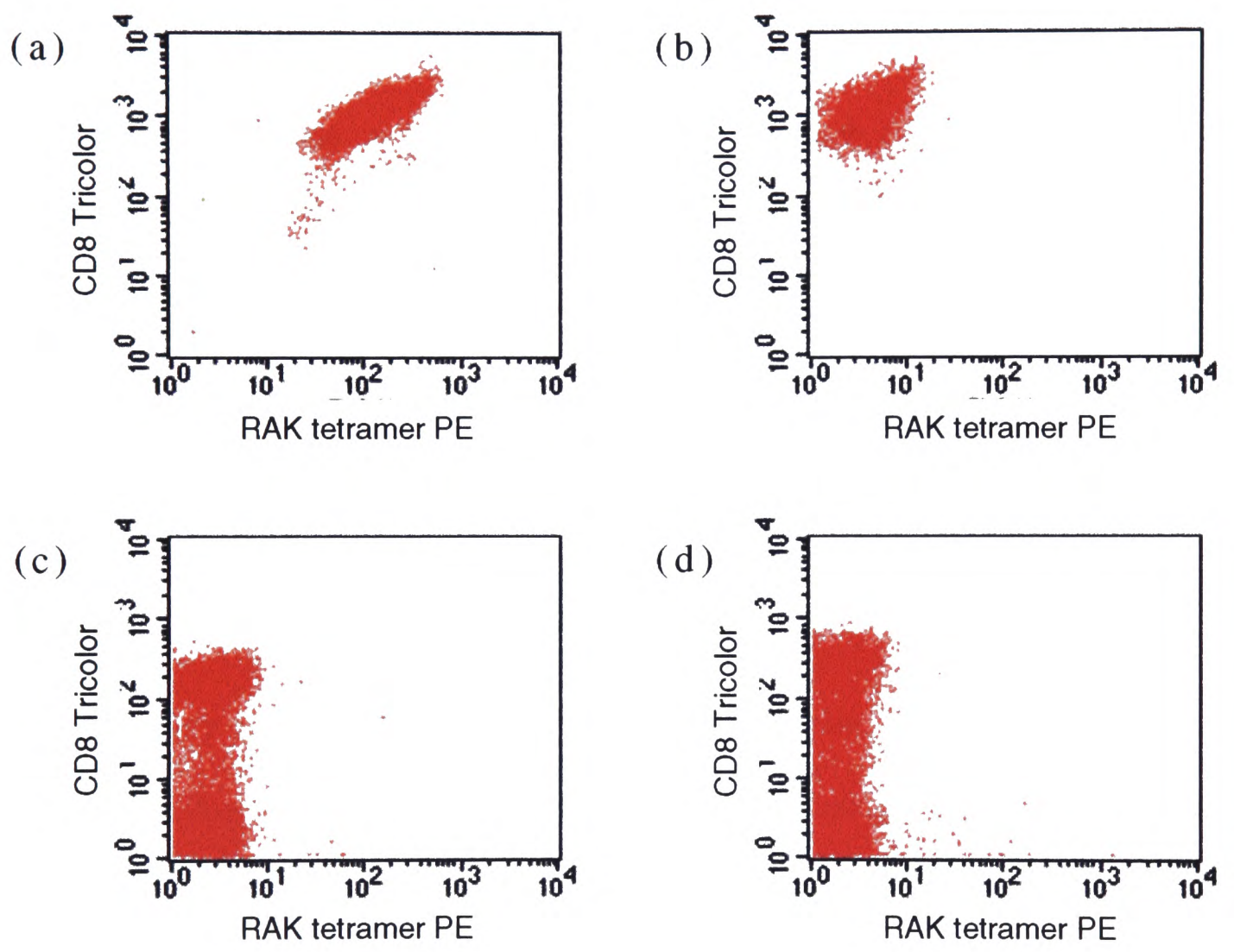


Figure 4.1. Specificity of the HLA-B8/RAK tetrameric complex for HLA-B8 restricted, RAKFKQLL-specific T cells. Samples were double stained with the PE-conjugated B8/RAK tetrameric complex and Tricolor-conjugated anti-CD8. The tetrameric complex stained (*a*) an HLA-B8 restricted, RAKFKQLL-specific T cell clone, but did not stain (*b*) an HLA-B8 restricted, FLRGRAYGL-specific clone. The complex also did not stain $CD8^+$ T cells in PBMC from (*c*) an HLA-B8 negative, EBV-seropositive individual or (*d*) an HLA-B8 positive but EBV-seronegative individual.

Three tetramers were constructed to analyse the frequency of circulating $CD8^+$ T cells specific for two Epstein-Barr virus lytic cycle antigens, HLA-A2 restricted BMLF1 GLCTLVAML epitope, HLA-B8 restricted BZLF1 RAKFKQLL epitope, and one latent cycle antigen, the HLA-B8 restricted EBNA3A FLRGRAYGL epitope, during primary EBV infection. The specificity of these tetramers was first confirmed by staining control samples. Thus, as shown in Figure 4.1., the HLA-B8/RAK tetramer stained an HLA-B8 restricted, RAKFKQLL-specific T cell clone (Fig 4.1.*a*), but did not stain an HLA-B8 restricted T cell clone specific for a different epitope (FLRGRAYGL; Fig 4.1.*b*). Furthermore, the HLA-B8/RAK tetramer did not stain $CD8^+$ T cells in peripheral blood taken from HLA-B8 negative, EBV seropositive individuals (Fig 4.1.*c*) or from HLA-B8 positive, EBV seronegative individuals (Fig 4.1.*d*). The HLA-A2/GLC and HLA-B8/FLR tetramers were also tested in a similar fashion, and their specificity was confirmed (data not shown).

4.2. Frequency of circulating EBV-specific T cells during primary EBV infection

PBMC taken from 10 HLA-A2 positive individuals suffering from recent onset acute IM were stained with the PE-conjugated A2/GLC tetrameric complex and Tricolor-conjugated anti-CD8. In all HLA-A2 positive individuals, a population of $CD8^+$ T cells clearly stained with the A2/GLC tetrameric complex. The frequency of tetramer-reactive T cells in PBMC ranged from 0.5-6.6% of the $CD8^+$ T cells in blood in the 10 donors with AIM (Table 4.1.). In 4 of the 10 donors, the frequency was >5%. Fig 4.2.*a* illustrates the data obtained from patients IM83 and IM74, in whom 5.6% and 6.6% respectively, of circulating $CD8^+$ T cells stained with the HLA-A2/GLC complex.

Table 4.1 Percentage of $CD8^+$ T cells stained with the A2/GLC tetrameric complex in HLA-$A2^+$ IM patients

Patient	% Staining
IM 59	1.0
IM 61	3.0
IM 69	5.5
IM 72	1.0
IM 73	1.8
IM 74	5.3
IM 78	5.6
IM 83	6.6
IM 84	2.8
IM 80	0.5

In a second series, PBMC taken from three HLA-B8 positive individuals suffering from recent onset acute IM were stained with the PE-conjugated HLA-B8/RAK or HLA-B8/FLR tetrameric complex and with Tricolor-conjugated anti-CD8. The frequency of HLA-B8/RAK reactive $CD8^+$ T cells was very high in these donors. Fig 4.2.*b* shows that 44% and 40% of $CD8^+$ T cells in PBMC from patients IM59 and IM70, respectively, stained with this tetrameric complex. In the third patient, IM63, the level of staining was 29%. In contrast, the frequency of $CD8^+$ T cells reactive with the HLA-B8/FLR tetrameric complex was relatively low. Fig 4.2.*c* shows that 1.2% and 2.2% of $CD8^+$ T cells in PBMC from patients IM63 and IM59, respectively, stained with this complex. Only 0.3% of $CD8^+$ T cells from patient IM70 were identified in this way. FLRGRAYGL is known to be a dominant EBV latent cycle epitope and in donor IM63 it was previously shown that the T cell response to this epitope dominates the T cell response to EBV latent

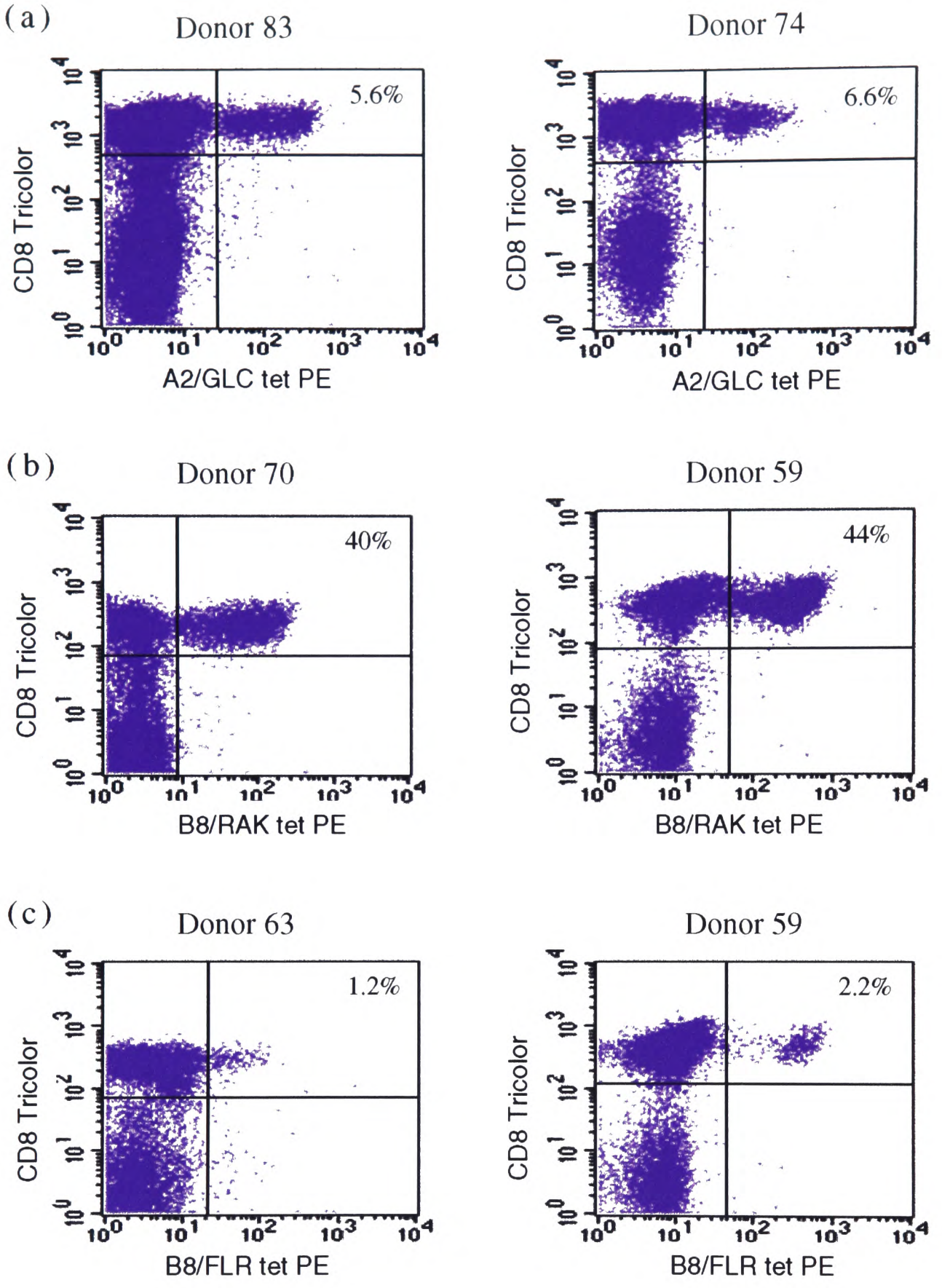


Figure 4.2. The frequency of $CD8^+$ T cells specific for three EBV epitopes in patients with IM. (*a*) PBMC from HLA-$A2^+$ donors, IM74 and IM83, stained with the A2/GLC tetrameric complex and Tricolor-conjugated anti-CD8. (*b*) PBMC from HLA-$B8^+$ donors, IM70 and IM59, stained with the B8/RAK tetrameric complex and Tricolor-conjugated anti-CD8. (*c*) PBMC from HLA-$B8^+$ donors, IM63 and IM59, were stained with the B8/FLR tetrameric complex and Tricolor-conjugated anti-CD8. In each case, the frequency of $CD8^+$ T cells that stain with the relevant tetrameric complex is expressed as the percentage of total $CD8^+$ cells.

cycle antigens [Steven *et al*, 1996]. Nevertheless in all three individuals in this study, the frequency of $CD8^+$ T cells specific for the HLA-B8 restricted EBV lytic cycle epitope (RAKFKQLL) was at least 10-fold greater than the frequency of cells specific for the HLA-B8 restricted latent cycle epitope (FLRGRAYGL).

4.3. Frequency of EBV-specific T cells in postconvalescent IM patients

We had access to samples of peripheral blood taken from two of the HLA-A2 positive patients and all three of the HLA-B8 positive patients between 6 and 37 months after primary infection with EBV. At this time all donors had fully recovered from clinical illness. The frequency of $CD8^+$ T cells reactive with the EBV lytic cycle epitopes (GLCTLVAML and RAKFKQLL) fell in all donors studied, although populations of tetramer-reactive cells remained easily detectable (Fig 4.3.*a* and *b*). Interestingly, the frequency of $CD8^+$ T cells reactive with the EBV latent cycle epitope (FLRGRAYGL) was similar in the acute and follow-up samples (Fig 4.3.*c*).

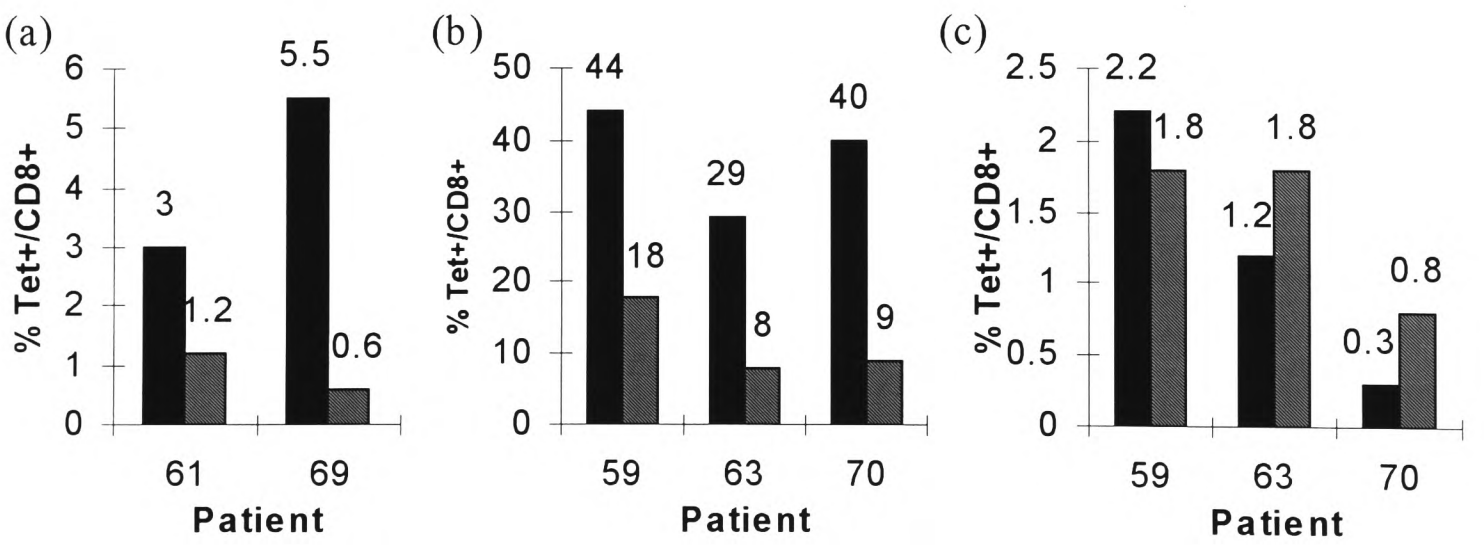


Figure 4.3. The frequency of $CD8^+$ T cells specific for three EBV epitopes in donors during IM (primary infection) and at least 6 months later. PBMC taken from (*a*) HLA-$A2^+$ donors were stained with the A2/GLC tetrameric complex; PBMC from HLA-$B8^+$ donors were stained with the (*b*) B8/RAK, or (*c*) B8/FLR tetrameric complexes. The percentage of tetramer-reactive $CD8^+$ cells in samples taken from donors during IM is shown in solid bars and samples taken at least 6 months later in shaded bars.

4.4. Phenotype of EBV-specific T cells during the primary T cell response

The phenotype of the tetramer-reactive cells was analysed in six IM patients using three-colour FACS analysis. The results of such an analysis, in this case involving the HLA-A2/GLC tetramer-reactive cells in patient IM73, are shown in Figure 4.4. The epitope-specific cells had an activated/memory phenotype; thus the majority expressed the activation markers HLA-DR (Fig 4.4.*b*), and CD38 (Fig 4.4.*c*) [Jackson *et al*, 1990], but showed down-regulation of the lymph node homing receptor CD62L (Fig 4.4.*d*) [Spertini *et al*, 1991], consistent with the finding that the molecule is downregulated after lymphocyte activation. Note also that the majority of the tetramer-reactive cells expressed the CD45RO isoform of CD45 and not the CD45RA isoform (Fig 4.4.*e* and *f*) [Akbar *et al*, 1988]; in this context, CD45RO has been proposed as a marker both for recently activated and for "memory" T cells. CD28, the ligand for B7, is an important costimulatory molecule that is normally expressed on the majority of $CD4^+$ T cells, but only ~50% of $CD8^+$ T cells [Hara *et al*, 1985]. This molecule was expressed on 48% of the epitope-specific cells in patient IM73 (Fig 4.4.*g*), whereas CD57 , which has been postulated to be a marker of terminal T cell differentiation [D'Angeac *et al*, 1994], was expressed on only 2% of that population (Fig 4.4.*h*). Complete results of the phenotypic analysis of the tetramer-reactive T cells taken from four HLA-A2 positive and two HLA-B8 positive patients with acute IM are shown in Table 4.2. Although there was some variation between patients, the tetramer-reactive cells were generally $CD38^+$, $HLA\text{-}DR^+$, $CD45RO^+$, $CD45RA^-$, levels of CD28 positivity were the most variable.

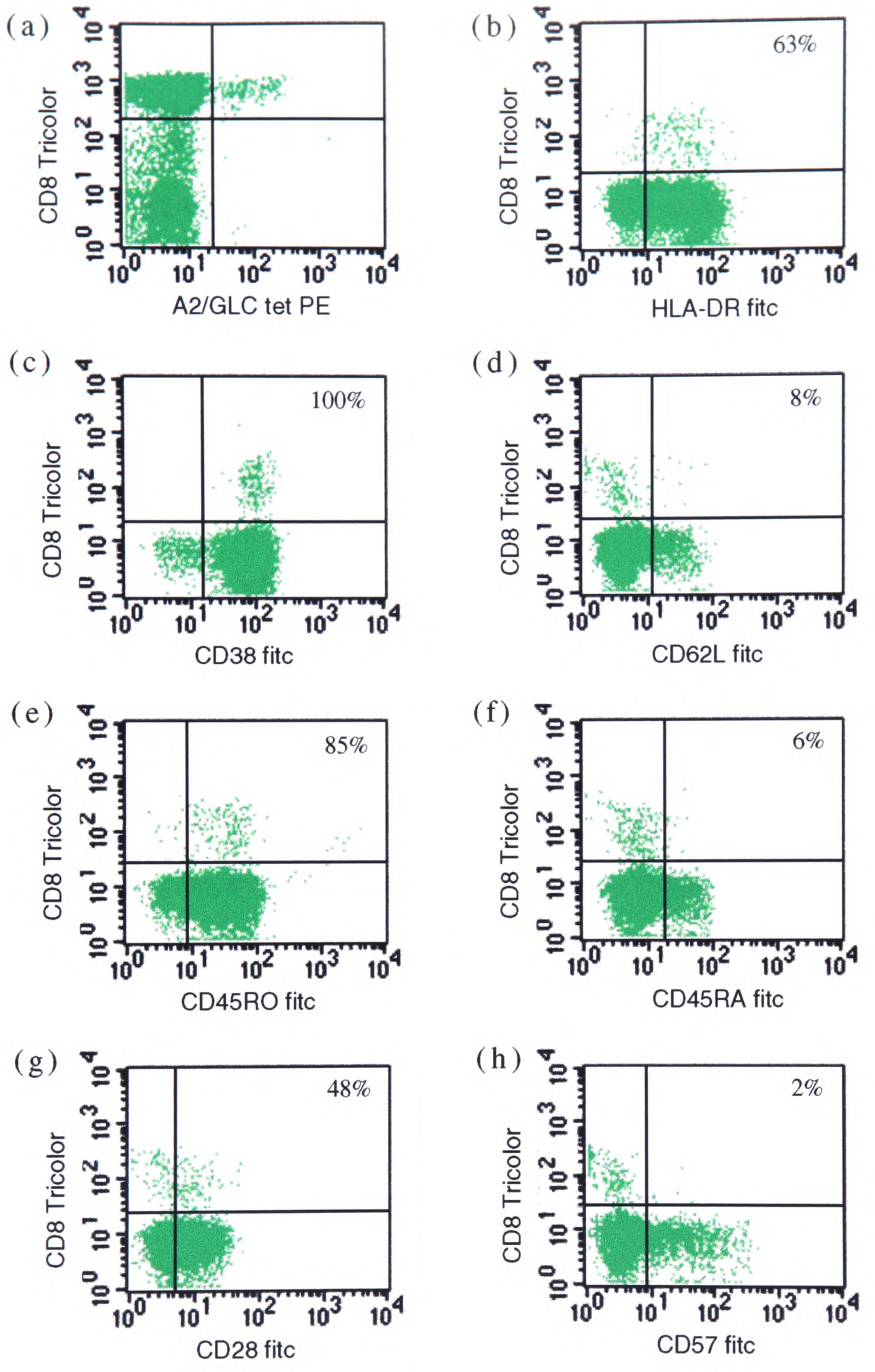


Figure 4.4. Phenotypic analysis of antigen-specific $CD8^+$ T cells in patient IM73 during acute IM. PBMC were stained with the PE-conjugated A2/GLC tetrameric complex, Tricolor-conjugated anti-CD8 and one of a panel of FITC-conjugated antibodies recognising surface glycoproteins. *a* shows staining of PBMC with the A2/GLC tetrameric complex on the x-axis and anti-CD8 on the y-axis. *b-h* are gated on $CD8^+$ cells and show staining with the A2/GLC tetrameric complex on the y-axis and anti- (*b*) HLA-DR, (*c*) CD38, (*d*) CD62L, (*e*) CD45RO, (*f*) CD45RA, (*g*) CD28, (*h*) CD57 on the x-axis. The frequency of cells that stain with a particular phenotypic marker is expressed as the percentage of the tetramer-reactive cells.

Table 4.2. The expression of cell surface phenotypic markers on tetramer-reactive T cells during acute IM

	A2/GLC-specific cells				B8/RAK-specific cells		B8/FLR-specific cells	
Donors	**IM74**	**IM73**	**IM83**	**IM72**	**IM59**	**IM63**	**IM59**	**IM63**
HLA-DR	11[a]	63	91	94	78	56	98	80
CD38	98	100	100	58	ND[b]	99	ND	99
CD62L	2	8	5	43	ND	23	ND	58
CD45RO	64	85	98	100	ND	78	ND	97
CD45RA	14	6	5	8	ND	5	ND	11
CD28	11	48	85	76	9	14	86	67
CD57	37	2	2	23	ND	25	ND	5

[a] *expressed as % of $CD8^+$ tetramer-reactive cells;* [b] *ND, not determined.*

4.5. Phenotype of EBV-specific T cells in postconvalescent IM patients

We were able to compare the phenotype of the HLA-B8 restricted FLRGRAYGL- and RAKFKQLL-specific T cells present within peripheral blood taken from donor IM63 during primary infection and 37 months later. During primary infection 29% of $CD8^+$ T cells reacted with the HLA-B8/RAKFKQLL tetrameric complex (Fig 4.5.*a*). At follow up, still 8% of $CD8^+$ T cells reacted with this complex (Fig 4.5.*f*), though by this time the patient was completely well and the circulating blood picture was normal. Compared with the phenotype of HLA-B8/RAKFKQLL tetramer-reactive cells during the primary infection, those cells present in the follow-up blood showed lower levels of expression of the activation markers CD38 (Fig 4.5.*b* and *g*) and HLA-DR (data not shown) and higher levels of the lymph node homing receptor CD62L (data not shown). There was also an increase in the percentage of tetramer-reactive cells expressing CD57 (data not shown). Significantly, there was a clear reduction in the frequency of expression of CD45RO (Fig

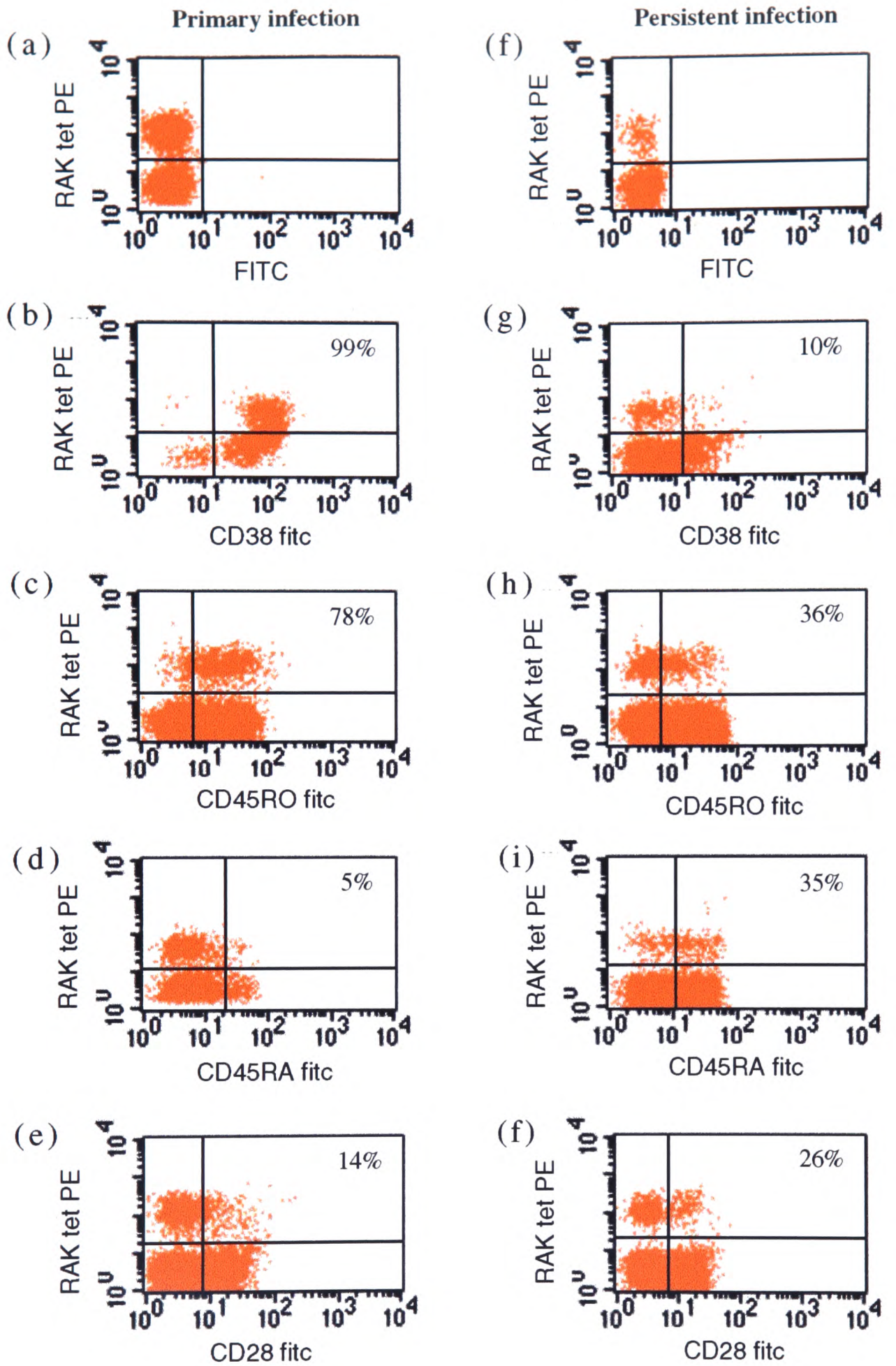


Figure 4.5. Comparison of the frequency and phenotype of antigen-specific $CD8^+$ T cells within PBMC taken from a donor during IM (*a-e*) and 37 months later (*f-j*). Cells were stained with the PE-conjugated B8/RAK tetrameric complex, Tricolor-conjugated anti-CD8 and one of a panel of FITC-conjugated antibodies recognising surface glycoproteins. Each analysis is gated on $CD8^+$ T cells only. Staining with the B8/RAK tetrameric complex is shown on the y-axis and with (*a* and *f*) normal mouse serum - negative control; (*b* and *g*) anti-CD38, (*c* and *h*) anti-CD45RO, (*d* and *i*) anti-CD45RA, or (*e* and *j*) anti-CD28 on the x-axis. The frequency of cells that stain with a particular phenotypic marker is expressed as the percentage of the tetramer-reactive cells.

4.5.*c* and *h*) by the antigen-specific cells, and an increase in the frequency of expression of CD45RA (Fig 4.5.*d* and *i*). Analysis of the HLA-B8 restricted FLRGRAYGL-specific T cells in the same patient showed a similar fall in the proportion of cells expression CD38, HLA-DR and CD45RO and increase in the proportion of cells expressing CD62L, CD57 and CD45RA over the 3 years since primary infection.

4.6. Sequence of TCRs involved in the B8/RAKFKQLL response

PBMC from patient IM59 were stained with the B8/RAK tetramer and reactive cells were sorted by FACS. mRNA extracted from the B8/RAK-specific T cells was reverse transcribed into cDNA and used for anchored PCR and sequencing of the CDR3 region in the TCR β chains. The eleven resultant sequences obtained are listed below in Table 4.3.

Table 4.3. CDR3 regions of B8/RAKFKQLL-specific TCR β chains

BV segment	CDR3 region	BJ segment
BV7S1 (x 3)	TGC GCC AGC **C A S**	GAA AGA CTA GCG GGA GAG GCA GAT ACG **E R L A G E A D T**	CAG TAT TTT **BJ2S3** **Q Y F**
BV6S3 (x2)	TGT GCC AGC **C A S**	AGC TTA GTA GGG AAG GGT ACT GAG **S L V G K G T E**	CAG TTC TTC **BJ2S1** **Q F F**
BV9S1 (x1)	TGT GCC AGC **C A S**	AGC CAA GAA CGG CAC AGG AAC ACT GAA **S Q E R H R N T E**	GCT TTC TTT **BJ1S1** **A F F**
BV9S1 (x1)	TGT GCC AGC **C A S**	AGC CAG GAC GGA CAG ACT AAC TAT GGC **S Q D G Q T N Y G**	TAC ACC TTC **BJ1S2** **Y T F**
BV3S1 (x1)	TGT GCC AGC **C A S**	AGT TTC GTA GTT AGC CCA GAT ACG **S F V V S P D T**	CAG TAT TTT **BJ2S3** **Q Y F**
BV21S3 (x1)	TGT GCC AGC **C A S**	AGC TTA GAA GGG GTA GCC GCT TTG GAT GAG **S L E G V A A L D E**	CAG TTC TTC **BJ2S1** **Q F F**
BV13S6 (x1)	TGT GCC AGC **C A S**	AGT TAC TCG AGA GGA GGC GAA AAT TCA CCC **S Y S R G G E N S P**	CTC CAC TTT **BJ1S6** **L H F**
BV5S1 (x1)	TGT GCC AGC **C A S**	AGC TTG GGA CAG GGT AGC TAC GAG **S L G Q G S Y E**	CAG TAC TCC **BJ2S7** **Q Y F**

This revealed a relatively polyclonal response to this epitope, without any obvious domination of a particular clonotype. However there were 3/11 sequences encoded by BV7S1-BJ2S3 and 2/11 encoded by BV6S3-BJ2S1. In addition two sequences shared BV9S1 but used different BJ chains. Similarly, another group has reported that the B8/RAKFKQLL-specific response results in selection of a diverse TCR repertoire, during both primary and persistent infection [Silins *et al*, 1997].

Discussion

This study looks at primary EBV infection in humans as the model in which to ask what fraction of a primary virus-induced T cell response is actually made up of virus-specific (as opposed to coincidentally activated bystander) cells. Earlier attempts to address this question using two different approaches gave somewhat conflicting results. Cloning experiments *in vitro* suggested that the frequency of T cells specific for a single EBV epitope was no more than 1:100 [Steven *et al*, 1996], whereas a study of TCR usage suggested that the frequency of antigen-specific cells might be as high as 1:3 [Callan *et al*, 1996]. In this study, I used peptide-MHC complexes to directly quantify and characterise epitope-specific T cells during the primary and early persistent phases of EBV infection. The tetrameric complexes, refolded with an HLA-A2 or HLA-B8 restricted EBV lytic cycle epitope, and an HLA-B8 restricted EBV latent cycle epitope, were used to study circulating T cells in HLA-$A2^+$ or HLA-$B8^+$ IM donors.

A very high frequency of EBV epitope-specific T cells was present in peripheral blood in such individuals. Thus, T cells reactive with the HLA-A2 restricted GLCTLVAML peptide from the EBV lytic cycle antigen BMLF1 were detected in PBMC from all 10 HLA-$A2^+$ IM donors tested, at levels between 0.5% and 6.6% of $CD8^+$ T cells. Even

higher frequencies of T cells reactive with the HLA-B8 restricted RAKFKQLL peptide from the EBV lytic cycle antigen BZLF1 were observed in all three HLA-B8$^+$ donors studied; in one individual, 44% of circulating CD8$^+$ T cells reacted with the HLA-B8/RAK tetrameric complex. This represents, in peripheral blood alone, a population of $\sim 3 \times 10^9$ T cells. In these same three patients, the frequency of T cells specific for the HLA-B8 restricted FLRGRAYGL peptide from the EBV latent protein EBNA3A was lower. The relative immunodominance of the RAKFKQLL (BZLF1) epitope over the FLRGRAYGL (EBNA3A) epitope during the primary immune response to EBV in these donors is consistent with the results of previously published functional studies [Steven *et al*, 1996 & 1997].

These numbers of EBV antigen-specific cells, measured directly using tetrameric MHC-peptide complexes, are much greater than the previously reported precursor frequencies, estimated using limiting dilution analysis, in a similar group of patients [Steven *et al*, 1996]. In donor 59, we can compare the frequency of FLRGRAYGL-specific CTLs previously estimated by *in vitro* outgrowth in a limiting dilution assay (frequency of 1:358) with that estimated in this study by tetramer staining (frequency of 1:50). The comparison shows that *in vitro* outgrowth underestimates the true magnitude of this latent antigen-induced primary response by almost a factor of six.

In this cohort, there was some phenotypic heterogeneity within cell populations, both within an individual patient and between patients, perhaps reflecting differences in the duration of antigen exposure. However, certain common themes emerge. The majority of tetramer-reactive cells had an activated/memory phenotype with high levels of expression of CD38, HLA-DR and CD45RO; and relatively low levels of expression of CD62L and CD45RA. By comparison, expression of CD28, the ligand for B7, was extremely variable. In patients IM59 and IM63, it was possible to compare CD28 expression on the

relatively small population of HLA-B8/FLR tetramer reactive cells with that of the greatly expanded population of HLA-B8/RAK tetramer-reactive cells. In both donors, the proportion of the FLRGRAYGL-specific T cells expressing CD28 was much higher than the proportion of the RAKFKQLL-specific T cells expressing this molecule. This would be consistent with the idea that CD28 is expressed by naive T cells [Azuma *et al*, 1993] and T cells in an early differentiation compartment, but that expression is lost after multiple rounds of cell division *in vivo*. It has been suggested that CD57 expression may also be a marker for late or terminal differentiation [D'Angeac *et al*, 1994]. Consistent with this, analysis of the HLA-B8/RAK and HLA-B8/FLR tetramer-reactive cells in donor IM59 showed that expression of CD57 was more frequent on the greatly expanded population of HLA-B8 RAKFKQLL-specific cells.

Although the results clearly show that high numbers of activated, epitope-specific cells are present in peripheral blood during the primary response to EBV, the role of these cells in the control of EBV infection requires further study. A recent study has found that a proportion of the activated T cells specific for one epitope, as detected by tetrameric complexes in primary LCMV infection, are nonfunctional [Zajac *et al*, 1998]. It is important to investigate whether all of these EBV-specific cells are able to kill target cells and by what mechanisms of cytotoxicity.

Following recovery from IM, the numbers of epitope-specific T cells estimated by staining with the HLA-peptide tetrameric complexes fell in most patients studied. Here it is interesting to note that the highly expanded lytic cycle epitope-specific CTLs were more heavily culled with transition to the virus-carrier state than the less expanded latent cycle epitope-specific CTLs; this concept of heavier culling of the more abundant components of the primary response was also suggested by the work with functional assays [Steven *et al*, 1996]. Nevertheless, populations of both lytic cycle and latent cycle

epitope-specific CTLs were still detectable in all the individuals studied at up to 37 months after primary EBV infection. Comparison of the phenotype of the tetramer-reactive T cells in samples of blood taken from donor IM63 during primary infection and 37 months later revealed interesting changes in cell surface marker expression. The epitope-specific T cells in the follow-up samples were unexpectedly heterogenous with respect to expression of markers of activation and differentiation. Most significantly, CD45RA was expressed on a substantial subset of tetramer-reactive $CD8^+$ T cells in the follow-up samples, clearly showing that this CD45 isoform is not a specific marker for "naive" T cells within the $CD8^+$ population. This finding is consistent with the results of a recently published functional study of the characteristics of a population of $CD27^-$ $CD8^+$ T cells that expressed CD45RA [Hamann *et al*, 1997].

In conclusion, this study demonstrates directly the magnitude of the specific primary T cell response induced *in vivo* by a natural virus infection in humans. The results show that massive expansion of activated epitope-specific $CD8^+$ T cells can occur during the primary immune response to EBV and that a population of epitope-specific $CD8^+$ T cells persists, in peripheral blood, at a relatively high frequency, for at least 3 years after primary infection.

CHAPTER 5

The Long Term Memory Response To EBV Infection In Healthy Seropositives

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The existence of T cell responses to EBV latent cycle proteins has long been recognised from work on healthy virus carriers [Murray *et al, 1992;* Khanna *et al*, 1992]. Their ease of experimental detection in T cell memory owes much to the availability of EBV-transformed lymphoblastoid cell lines, expressing the full range of latent antigens, as a ready source of appropriate stimulator cells [Rickinson and Kieff, 1996]. Limiting dilution analysis suggests that the frequency of T cells specific for EBV latent antigens is of the order of 20 to $100/10^6$ PBMC in healthy virus carriers [Steven *et al*, 1996].

T cell reactivities against EBV lytic cycle proteins are much less well characterised, to some extent because the virus lytic cycle is more difficult to reproduce *in vitro*, and work in this area has lagged behind parallel studies in α- and β-herpes viral systems [Tigges *et al*, 1992; Borysiewicz *et al*, 1988]. Recent studies have now identified CTL responses directed against EBV immediate early and early lytic cycle proteins in IM patients [Steven *et al*, 1997]. However relatively little is known about the importance of T cells specific for lytic cycle epitopes in long term virus carriers. In this context, Bogedain *et al* [1995] first demonstrated existence of CTL memory to an HLA-B8 restricted epitope, RAKFKQLL, from the immediate early BZLF1 protein, and subsequently Elliott *et al* [1997] used LDA to show that T cells reactive with the HLA-B8 restricted RAKFKQLL

epitope are also present in long term virus carriers at frequencies comparable to those of T cells reactive with HLA-B8 restricted epitopes from EBV latent proteins.

However new evidence has suggested that LDA may underestimate T cell frequencies by as much as 100-fold [Murali-Krishna *et al*, 1998] and hence novel methods of estimating frequencies of antigen-specific T cells have been developed. These include assays to detect IFNγ release from epitope-specific $CD8^+$ T cells following peptide stimulation [Lalvani *et al*, 1997] and the use of tetrameric MHC-peptide complexes to directly stain T cells of the appropriate specificity [Altman *et al*, 1996]. Results obtained using these techniques have led to a re-evaluation of the nature of both primary and memory responses to viruses [Murali-Krishna *et al*, 1998; Butz & Bevan, 1998; reviewed in McMichael & O'Callaghan, 1998]. Together these studies indicate that the majority of expanded, activated $CD8^+$ T cells that arise during virus infections are indeed antigen-specific. How long antigen-specific cells persist at these high levels remains to be determined. Furthermore it is not known how the situation post-IM compares to that in the majority of EBV carriers who experience clinically silent primary infection, mainly during childhood, and then carry the virus for life.

This chapter describes a study of the frequency and specificity of EBV-reactive T cells in eleven long term EBV carriers with no history of IM, as well as two individuals who suffered IM 10 and 15 years ago (donors VN and RO respectively). Three different methods were used to analyse T cell frequency: limiting dilution analysis, ELISpot assays to detect IFNγ release, and direct staining of antigen-specific T cells with MHC-peptide tetrameric complexes. The main focus was on the responses to two immunodominant epitopes from EBV lytic proteins BZLF1 (HLA-B8 restricted RAKFKQLL) and BMLF1 (HLA-A2 restricted GLCTLVAML), compared with responses to epitopes from EBV latent proteins.

5.1. Measurement of T cell frequencies by LDA

Initially I used limiting dilution analysis to estimate the frequency of T cells reactive with the two immunodominant lytic protein epitopes (RAKFKQLL and GLCTLVAML), in eight EBV-seropositive individuals with one or both of the appropriate HLA restricting alleles (HLA-B8 and HLA-A2). T cells reactive with these epitopes were detected in all eight donors studied, and the data are summarised in the relevant section of Table 5.1. The frequency of T cells specific for the HLA-B8 restricted BZLF1 epitope (RAKFKQLL) ranged from 60-725/10^6 PBMC (donors CH, PA, CM and LU), while the frequency of T cells specific for the HLA-A2 restricted BMLF1 epitope (GLCTLVAML) ranged from 10-380/10^6 PBMC (donors LU, JS, VN, RO and LC).

For comparison, the frequency of T cells reactive with immunodominant epitopes from EBV latent antigens was also evaluated in six of these eight donors, and in two additional individuals (DM and PB). This involved the HLA-B8 restricted epitope, FLRGRAYGL [Burrows *et al*, 1992], the HLA-B7 restricted epitope, RPPIFIRRL [Hill *et al*, 1995], and the HLA-A11 restricted epitope, IVTDFSVIK [Gavioli *et al*, 1993][2]. The frequency of T cells reactive with these epitopes ranged from undetectable levels to 240/10^6 PBMC in our donors. The T cell response to another HLA-A11-restricted epitope AVFDRKSDAK is often subdominant, and consistent with this, low frequencies of T cells specific for this epitope were detectable in the three HLA-A11 positive donors studied (CM, DM and PB).

In all four HLA-B8 positive donors parallel LDAs showed that the response to the immunodominant lytic protein epitope RAKFKQLL was stronger than the response to the immunodominant latent protein epitope FLRGRAYGL.

[2] See Appendix B for a listing of EBV-specific CTL epitopes.

5.2. Quantitation of T cell responses by ELISpot assay

The frequency of T cells reactive with these same lytic and latent cycle epitopes was then analysed by ELISpot assays, now in an extended range of 13 healthy EBV carriers. In control experiments there were no false positive results observed using EBV-immune donors who did not have the relevant HLA restricting allele for the epitope in question or uninfected seronegative individuals who did have the relevant HLA alleles (data not shown).

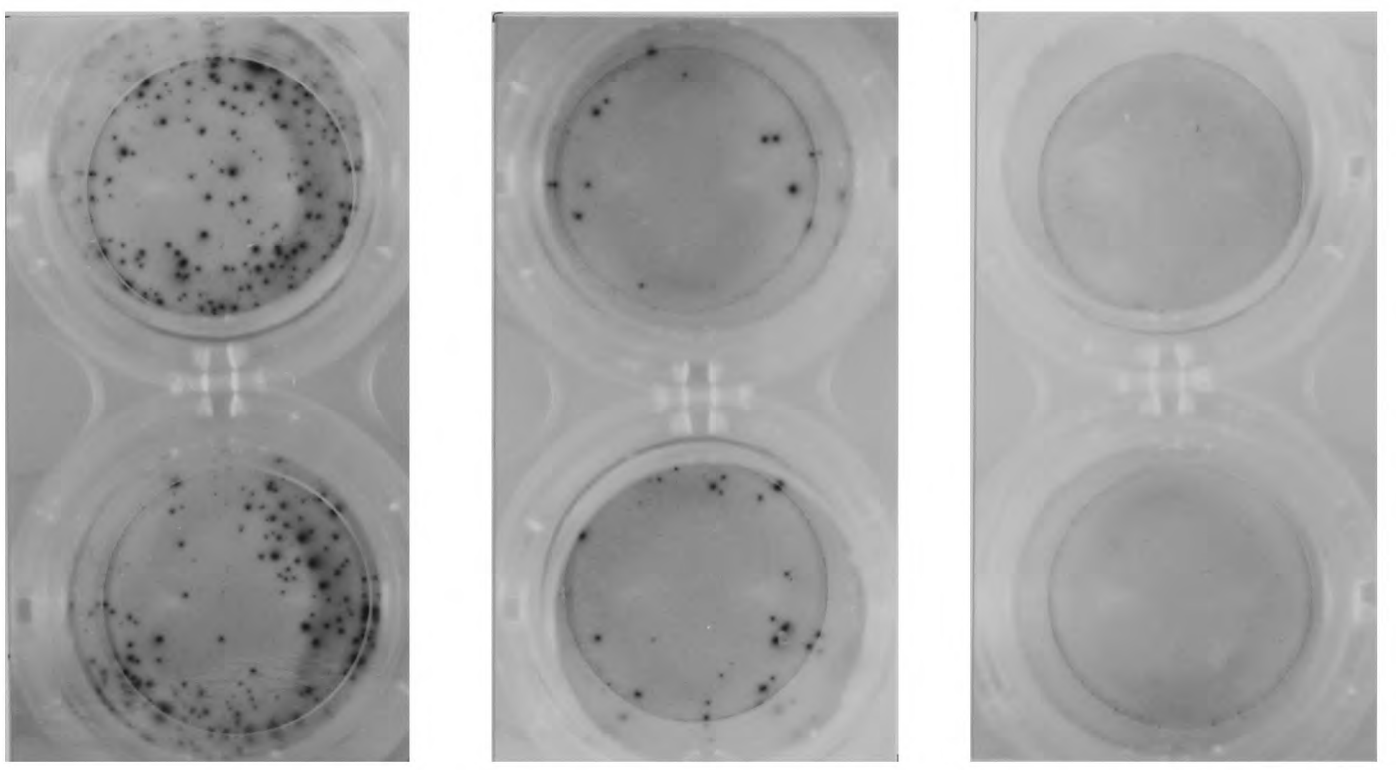


Figure 5.1. ELISpot assay on donor CH, comparing the frequency of (*a*) HLA-B8 restricted RAKFKQLL-reactive T cells ($1{,}020/10^6$ PBMC), and (*b*) HLA-B8 restricted FLRGRAYGL-reactive T cells ($120/10^6$ PBMC). (*c*) Negative control, no peptide added. Wells in the first row were seeded with 1.25×10^5 cells and wells in the second row with 6.25×10^4 cells.

Table 5.1. The frequency of EBV epitope-specific CTL in peripheral blood

Donor	EBV Epitope	LDA[a]	ELISpot[a]	Tetramer[a,b]
CH	B8 RAKFKQLL	60	1,020	2,605 (1.0%)
	B8 FLRGRAYGL	20	120	230 (0.1%)
PA	B8 RAKFKQLL	125	295	175 (0.1%)
	B8 FLRGRAYGL	undetectable	undetectable	undetectable
	B7 RPPIFIRRL	20	165	ND
JB	B8 RAKFKQLL	ND[c]	750	7,000 (5.0%)
	B8 FLRGRAYGL	ND	480	2,900 (2.0%)
AR	B8 RAKFKQLL	ND	250	1,600 (0.5%)
	B8 FLRGRAYGL	ND	460	2,100 (0.7%)
CM	B8 RAKFKQLL	725	2,300	6,200 (4.3%)
	B8 FLRGRAYGL	undetectable	10	undetectable
	A11 IVTDFSVIK	240	810	4,760 (3.3%)
	A11 AVFDRKSDAK	25	400	ND
DM	B8 RAKFKQLL	ND	2,500	11,400 (5.5%)
	B8 FLRGRAYGL	ND	20	undetectable
	A11 IVTDFSVIK	170	1,000	5,000 (3.8%)
	A11 AVFDRKSDAK	10	120	ND
LU	A2 GLCTLVAML	15	25	ND
	B8 RAKFKQLL	60	350	ND
	B8 FLRGRAYGL	40	165	ND
JS	A2 GLCTLVAML	130	710	2,900 (0.8%)
VN	A2 GLCTLVAML	380	710	2,600 (1.1%)
	A2 SVRDRLARL	ND	95	ND
RO	A2 GLCTLVAML	10	15	undetectable
	A2 SVRDRLARL	ND	10	ND
	B7 RPPIFIRRL	175	430	ND
LC	A2 GLCTLVAML	135	360	920 (0.4%)
	A11 IVTDFSVIK	undetectable	undetectable	ND
DA	A11 IVTDFSVIK	ND	500	2,000 (1.6%)
	A11 AVFDRKSDAK	ND	50	ND
PB	A11 IVTDFSVIK	40	300	1,800 (0.7%)
	A11 AVFDRKSDAK	undetectable	70	ND

[a] values are given per 10^6 PBMC; [b] values in parenthesis are given as % of $CD8^+$ cells; [c] ND, not determined.

As summarised in Table 5.1., ELISpot assays using lytic epitopes indicated that the frequency of HLA-B8 restricted, RAKFKQLL-reactive T cells ranged from 250-2,500/10^6 PBMC, and the frequency of HLA-A2 restricted, GLCTLVAML-reactive T cells ranged from 15-710/10^6 PBMC. T cells reactive with dominant and subdominant epitopes from the EBV latent proteins were also detected at frequencies up to 1,000/10^6 PBMC, with responses to the HLA-A11 restricted epitope IVTDFSVIK being particularly strong. Most importantly, the estimates of T cell frequency using ELISpots were higher than those obtained using LDAs, although the relative hierarchy of responses in any one individual was not altered.

5.3. Enumeration of antigen-specific T cells using MHC-peptide tetrameric complexes

Next I analysed the frequency of T cells specific for two lytic cycle epitopes (RAKFKQLL and GLCTLVAML) and two latent epitopes (FLRGRAYGL and IVTDFSVIK) using MHC-peptide tetrameric complexes. In all these experiments PBMC were stained with PE-labelled tetrameric complexes in combination with Tricolor-conjugated anti-CD8, and assayed by two-colour FACS analysis.

In eleven of the twelve individuals studied specific staining with one or more of the relevant tetramers was clearly detectable (Table 5.1. and Figure 5.2.). Thus, using the HLA-B8/RAK tetramer, epitope-specific T cells were detected in all six HLA-B8 positive individuals tested, and their frequencies were unusually high. Fig 5.2.*a* and *b*, show staining of PBMC from donor CM, in whom 6,200/10^6 PBMC (4.3% $CD8^+$ T cells) reacted with the HLA-B8/RAK tetramer, and from donor JB, in whom 7,000/10^6 PBMC (5.0% $CD8^+$ T cells) reacted with this tetramer. A third HLA-B8 positive donor DM, gave 11,400 reactive cells/10^6 PBMC with this reagent, equivalent to 5.5% $CD8^+$ T cells

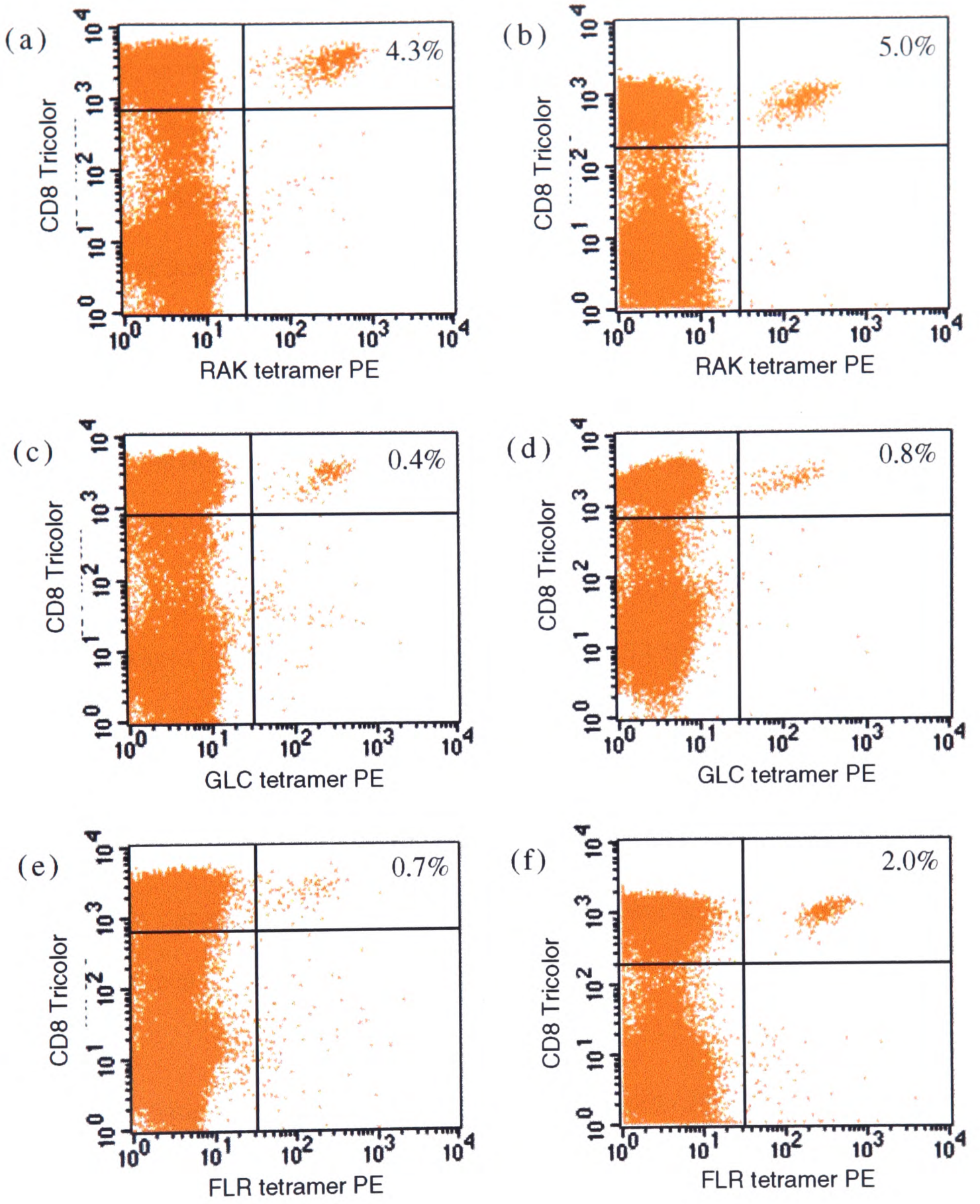


Figure 5.2. Frequency of tetramer-reactive $CD8^+$ T cells in PBMC. PBMC from healthy EBV-seropositive donors were stained with Tricolor-conjugated anti-CD8 and PE-conjugated (*a* and *b*) HLA-B8/RAK tetrameric complexes, (*c* and *d*) HLA-A2/GLC tetrameric complexes, or (*e* and *f*) HLA-B8/FLR tetrameric complexes. Approximately 30,000 events are shown in each plot. The frequency of $CD8^+$ T cells that stain with the relevant tetrameric complex is expressed as the percentage of total $CD8^+$ cells.

(data not shown). The frequency of HLA-A2/GLC-reactive T cells was also high. Fig 5.2.*c* and *d*, show staining of PBMC from donors LC and JS, in whom 920/10^6 PBMC (0.4% $CD8^+$ T cells) and 2,900/10^6 PBMC (0.8% $CD8^+$ T cells), respectively, reacted with the HLA-A2/GLC tetramer.

Significant populations of FLRGRAYGL-specific T cells were only detectable in three of the six HLA-B8 positive donors studied using tetrameric complexes. The frequency of T cells in these three donors ranged from 230-2,900/10^6 PBMC. Fig 5.2.*e* and *f*, show staining of PBMC from donors AT and JB, in whom 2,100/10^6 PBMC (0.7% $CD8^+$ T cells) and 2,900/10^6 PBMC (2.0% $CD8^+$ T cells), respectively, reacted with the HLA-B8/FLR tetramer. The frequency of T cells reactive with the HLA-A11/IVT tetrameric complex ranged from 1,800-5,000/10^6 PBMC (or 0.7-3.8% $CD8^+$ T cells; staining not shown).

In all but one of the 12 donors analysed (the exception being PA), we detected greater numbers of peptide-specific T cells by tetramer staining than by the ELISpot technique or LDA. Once again, however, the hierarchy of responses to different epitopes within a given individual remained consistent.

5.4. Phenotypic analysis of EBV-specific CTL within peripheral blood from long term virus carriers

Identification of EBV-specific memory T cells by tetramer staining enabled co-analysis of expression of other surface markers of cell activation and differentiation. The phenotypic profiles of HLA-B8/RAK tetramer-reactive cells in donor DM are shown in Figure 5.3., and the overall results obtained from five individuals are summarised in Table 5.2. CD25, a marker for early T cell activation [Waldmann, 1991] was not up-regulated on the tetramer-reactive cells, whereas another activation marker, HLA-DR

[Hara *et al*, 1985], was expressed at a high level on a small proportion (up to 11%) of cells. Expression of CD62L was variable, ranging from 8-72% of the antigen-specific T cells in different donors. Spertini *et al* [1991] have shown that this molecule is down-regulated on activated T cells, with expression being regained in the stable memory state. CD45RO is conventionally known to be a marker for activated and memory T cells [Akbar *et al*, 1988]. While it was expressed on the majority (64-82%) of the tetramer population, it was not found on all the cells. Conversely, CD45RA, a marker for "antigen-inexperienced cells", was in fact clearly present on some, albeit usually a minority, of the antigen-specific cells. The tetramer-reactive cells were divided between the $CD28^+$ and $CD28^-$ T cell compartments. Finally, CD57, first proposed by d'Angeac *et al* [1994] as a marker for cells in a state of late or terminal differentiation, was expressed on 69% of tetramer-reactive cells in donor LC and was present on smaller proportions of tetramer-reactive cells in the other donors.

Table 5.2. Phenotypic analysis of tetramer-reactive cells[a]

	CM B8/RAK	DM B8/RAK	VN A2/GLC	LC A2/GLC	CH B8/FLR
CD25	ND[b]	ND	0	0	ND
HLA-DR	7	4	2	11	3
CD62L	17	8	72	23	41
CD45RO	82	71	64	78	79
CD45RA	46	23	24	19	20
CD28	68	57	80	41	77
CD57	33	16	17	69	11

[a] *expressed as % of $CD8^+$ tetramer-reactive cells;* [b] *ND, not determined.*

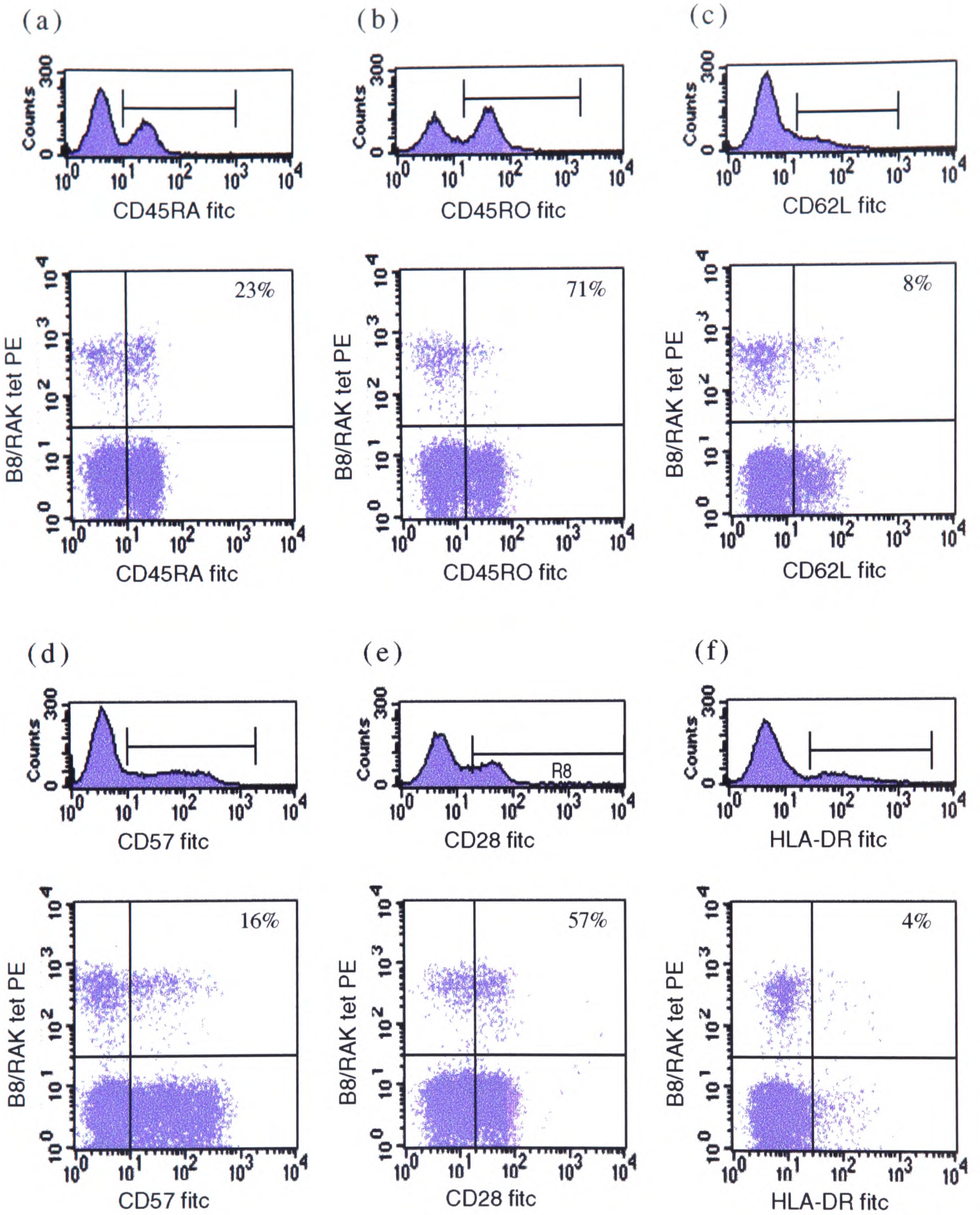


Figure 5.3. Phenotypic analysis of HLA-B8 RAKFKQLL-specific $CD8^+$ T cells in PBMC from donor DM. Samples were stained with the PE-conjugated HLA-B8/RAK tetramer, Tricolor-conjugated anti-CD8, and a FITC-conjugated mAb specific for (*a*) CD45RA, (*b*) CD45RO, (*c*) CD62L, (*d*) CD57, (*e*) CD28, or (*f*) HLA-DR. Expression of phenotypic markers in total PBMC was used to set the vertical markers and is shown in the *upper panels*. Profiles of tetramer staining vs. expression of phenotypic markers within the $CD8^{high}$ ($CD3^+$) subset is shown in the *lower panels*. Approximately 5,000 events are shown in each dot plot. The percentage of $CD8^+$ tetramer-reactive cells expressing a particular phenotypic marker is shown.

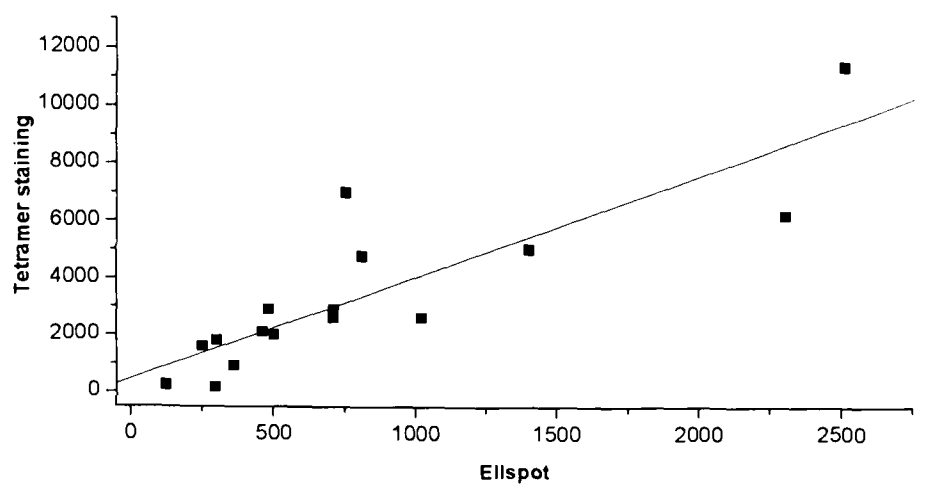
5.5. Comparison of results obtained using the three methods

Estimates of T cell frequency varied substantially according to the method used, although the hierarchy of dominance of the different responses was always similar. The lowest frequencies were obtained from LDAs. By comparison ELISpot assays gave values that were, on average, 5.3-fold higher than LDA values. Nevertheless there was a close correlation between results obtained from ELISpot assays and those obtained from LDAs (Fig 5.4.*b*; $r = 0.88$, $p < 0.0001$). The highest estimates of frequencies were obtained from direct staining with tetrameric complexes. Thus the average values from tetramer staining were 4.4-fold higher than those from ELISpots. The correlation between results obtained from ELISpot assays and staining with tetrameric complexes was also good (Fig 5.4.*a*; $r = 0.86$, $p < 0.0001$). However that between LDA results and staining with tetrameric complexes was less close (Fig. 5.4.*c*; $r = 0.69$, $p = 0.026$).

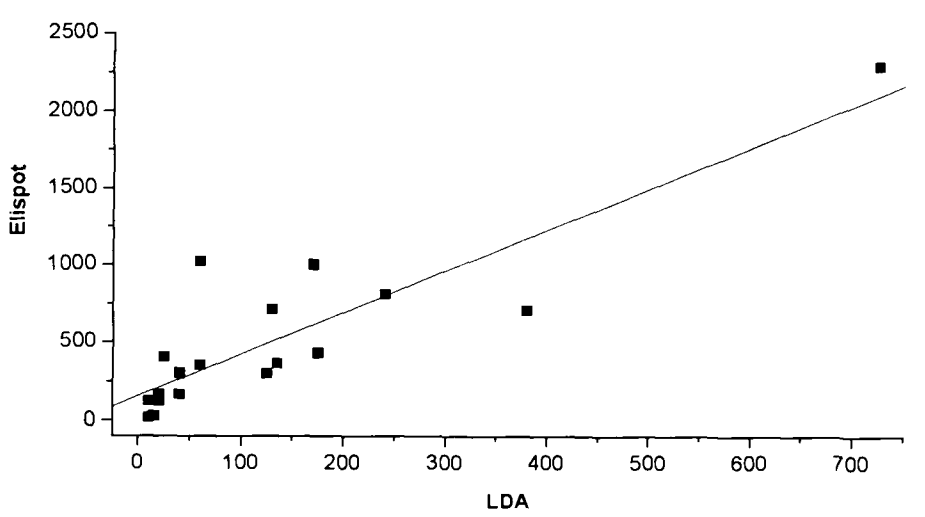
Discussion

Historically, identification of antigen-specific T cells in humans has been accomplished through indirect functional assays. This is largely due to the fact that individual TCRs interact with peptide-MHC complexes with low affinity and fast off-rates [Matsui *et al*, 1994], making direct quantitation of antigen-specific T cells difficult. The traditional LDA relies on the ability of cytotoxic precursor cells to survive, proliferate and express cytolytic activity after two weeks in culture [reviewed in Tough & Sprent, 1998]. Activated $CD8^+$ T cells prone to undergo apoptosis [Akbar *et al*, 1993] are therefore unlikely to be detected in this assay. Furthermore, $CD8^+$ T cells may not always have cytolytic capacity, or they may be in a late differentiation compartment and consequently

(a)



(b)



(c)

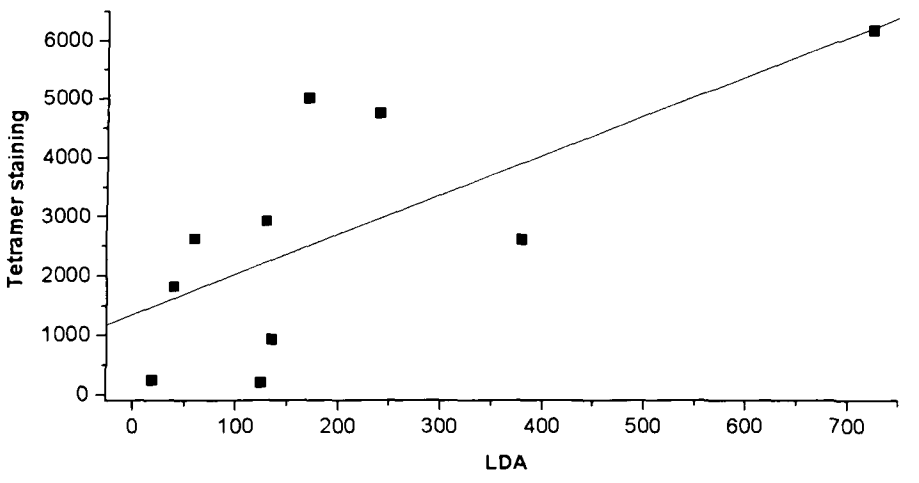


Figure 5.4. Comparison of estimates of CTL frequencies obtained using three different methods. (*a*) Comparison of CTL frequencies obtained from ELISpot assays with those from LDAs ($r = 0.88$, $p < 0.0001$). (*b*) Comparison of CTL frequencies obtained from direct staining with tetrameric complexes with those obtained from ELISpot assays ($r = 0.86$, $p < 0.0001$). (*c*) Comparison of CTL frequencies obtained from tetramer staining with those obtained from LDAs ($r = 0.69$, $p = 0.026$).

lack adequate proliferative capacity. It is not surprising, therefore that this method underestimates the actual frequency of antigen-specific cells. Likewise ELISpot assays measure effector function (IFNγ release) of antigen-specific T cells in response to antigen stimulation. However with this approach there is no requirement for expansion or long-term survival of the cells and it is not surprising that higher frequencies were obtained. Nevertheless, not all cells that respond in an ELISpot assay are necessarily cytotoxic; neither are all antigen-specific T cells capable of secreting IFNγ upon peptide stimulation [Maggi *et al*, 1994]. Direct staining with fluorochrome-labelled tetrameric complexes has the advantage of detecting antigen-specific T cells according to their expression of a specific TCR on the cell surface, and independent of their effector function or proliferative potential. Following stimulation with antigen, however, TCRs may be down-regulated [Viola & Lanzavecchia, 1996], and thus some acutely stimulated T cells may not be detected using this method.

The differences in the estimates of T cells frequencies obtained using the three methods and the imperfect correlation among these estimates suggests that the "memory pool" of antigen-specific T cells within PBMC is functionally heterogenous. Double staining of T cells with tetramers and antibodies against cell surface markers also showed evidence of phenotypic heterogeneity within the epitope-specific population. Markers of activation, memory and late differentiation, as well as costimulatory molecules were expressed on variable proportions of EBV-specific $CD8^+$ T cells within PBMC. While up-regulation of both CD25 and HLA-DR is a feature of antigen-specific $CD8^+$ T cells grown *in vitro*, these were not features of the EBV tetramer-reactive cells stained immediately *ex vivo*. Indeed, CD25 up-regulation was not detected, and HLA-DR was only expressed at high levels on a minor subpopulation of the tetramer-reactive cells, suggesting that only a small proportion of these cells had been recently activated *in vivo*. It is important to note,

however, that recently activated cells may have down-regulated their TCRs and not reacted with the tetrameric complexes. Not all the tetramer reactive T cells were $CD45RO^{bright}$, the conventional marker for antigen-experienced cells. Conversely CD45RA was expressed on a significant minority of the tetramer-reactive cells in many donors studied; in this context it has recently been reported that a subpopulation of $CD45RA^{+}$ cells have high cytolytic activity and may be an important effector population [Hamann *et al*, 1997]. This calls into question the validity of using these two markers to distinguish antigen-primed and antigen-naive cells, at least within the $CD8^{+}$ T cell compartment [Akbar *et al*, 1988]. Lastly up-regulation of CD57 and down-regulation of CD28 seen on some of the antigen-specific cells may reflect recurrent restimulation and perhaps terminal differentiation of subsets of CTL *in vivo* [Azuma *et al*, 1993]. It would be interesting to investigate the functional properties of the phenotypically different subpopulations of antigen-specific cells, especially in relation to IFNγ release and *in vitro* cloning assays.

T cell responses to the two lytic cycle protein epitopes were found in all the donors expressing the appropriate HLA type. T cells specific for the HLA-B8 restricted RAKFKQLL epitope from the lytic cycle protein BZLF1 outnumbered those specific for the HLA-B8 restricted FLRGRAYGL epitope from the EBV latent protein EBNA3A, and accounted for up to 5.5% of the circulating $CD8^{+}$ population. T cells reactive with another epitope, GLCTLVAML, from the lytic protein BMLF1 were also easily detectable in the memory of HLA-$A2^{+}$ virus carriers. BZLF1 is the first immediate early protein to be expressed during lytic replication [Biggin *et al*, 1987] and initiates the expression of early genes, to which group BMLF1 belongs [Kieff & Liebowitz, 1990]. BMLF1, in turn, functions as a *trans*-activator of other lytic cycle genes [Lieberman *et al*, 1986]. T cell recognition of epitopes from these immediate early and early proteins

should enable effective elimination of virus-producing cells at an early stage, perhaps before the formation and release of mature virions. Such T cells would, therefore, be expected to control foci of virus replication within the oropharynx [Falk *et al*, 1997] as well as regulate spontaneous reactivation from latency into lytic cycle, within infected B cells generally [Rowe *et al*, 1992; Prang *et al*, 1997]. The control of EBV lytic infection within B cells in peripheral blood may also have other advantages. EBV-transformed B cells that have been induced into lytic cycle have been shown *in vitro* to have superantigen-like activity and stimulate T cells expressing TCR BV13 chains [Sutkowski *et al*, 1996]. Were this reproduced *in vivo*, persistent superantigenic stimulation of a major subset of T cells could be detrimental to the host. In addition, the lytic cycle gene BCRF1, expressed late in the lytic cycle, encodes an IL-10 homologue, which may be involved in general suppression of T cell responses [Hsu *et al*, 1990].

T cells reactive with epitopes from EBV latent proteins were also easily detectable by tetramer staining in this cohort of individuals; particularly strong responses to the HLA-A11 restricted IVTDFSVIK epitope were noted in four of the five $A11^+$ donors. It is interesting that we were unable to detect a response to this epitope in the fifth donor, LC, who is Asian, consistent with previous reports of a mutation in this epitope in EBV isolates from South-East Asia [de Campos-Lima *et al*, 1993 & 1994]. The magnitude of the latent antigen-specific response in long term virus carriers likewise reflects the fact that the immune system is continually being challenged by EBV-infected B cells that are reactivating *in vivo* from the resting state into virus-driven lymphoproliferation [Rowe *et al*, 1992]. This recrudescence of EBV-driven lymphoproliferative lesions and their control by EBV latent antigen-specific T cells appear to be central features of the healthy carrier state. Thus, when T cell control is ablated by immunosuppresive therapy, there is a significant incidence of lymphoproliferative disease, representing the opportunistic *in*

vivo outgrowth of latently infected cells [Ho *et al*, 1988]. Restoration of EBV latent antigen-specific responses with CTL preparations is, in fact, sufficient to reverse this otherwise fatal condition [Rooney *et al*, 1995; Papadopoulos *et al*, 1994]. Therapies aimed at boosting T cell responses to EBV antigens may also be useful in the management of other EBV-associated malignancies, such as nasopharyngeal carcinomas and Hodgkin's disease [Cochet *et al*, 1993; Brooks *et al*, 1992; Deacon *et al*, 1993]. The present methodologies, particularly ELISpot and tetramer assays, open up the possibility of rapidly screening patients for their resident level of EBV-specific T cell immunity and also of monitoring the frequency of T cells with the appropriate specificities in *in vitro* reactivated populations destined for therapeutic use.

CHAPTER 6

Enriched Populations of EBV-Specific CTL in Synovial Fluid from Patients with Inflammatory Arthritis

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An association between EBV and rheumatoid arthritis (RhA) was first proposed on the basis of high titres of EBV-specific antibodies found in some patients with rheumatoid arthritis [Catalano *et al*, 1979; Yao *et al*, 1986]. The observation that some of the anti-EBV antibodies were cross-reactive with autoantigens such as collagen was put forward as a further argument in support of the link [Baboonian *et al*, 1991]. However, the importance of these findings in the pathophysiology of rheumatoid arthritis has never been established. More recently, work published by Bonneville and colleagues has reopened the debate about the importance of this virus in the aetiology of arthritis. David-Ameline *et al* [1996] showed that a large proportion of T cell clones derived from synovial fluid taken from one individual with rheumatoid arthritis, under polyclonal activation conditions, recognised EBV-transformed lymphoblastoid cell lines in an HLA restricted manner. Subsequent work revealed that these T cell clones recognised epitopes from EBV lytic cycle proteins [Scotet *et al*, 1996]. Analysis of the TCR use of the EBV-specific T cell clones and of the TCR repertoire of synovial fluid lymphocytes suggested that the EBV-specific T cells were clonally expanded within the synovial fluid from this donor [David-Ameline *et al*, 1996; Scotet *et al*, 1996]. In some other donors, short-term T cell lines derived from synovial fluid lymphocytes, but not those derived from peripheral blood lymphocytes, secreted low levels of TNF in response to stimulation with an EBV

antigen [Scotet *et al*, 1996 & 1999]. These results suggest that EBV-specific T cells might form a component of joint infiltrating lymphocytes in patients with RhA.

$CD8^+$ T cells dominate the lymphocyte population in synovial fluid from individuals with chronic inflammatory arthritis. Whilst it is known that these $CD8^+$ T cells are often clonally or oligoclonally expanded, the specificity of the T cells and their relevance to the pathogenesis of joint disease has remained unclear. In this study, I have used tetrameric HLA-peptide complexes to investigate the T cell response to EBV and influenza in individuals with inflammatory arthritis. The tetramers were complexed to peptide epitopes from EBV latent and lytic proteins, and to an epitope from influenza A matrix protein. Together with ELISpot assays for IFNγ release, these were used to quantify and characterise virus-specific T cells within paired samples of peripheral blood and synovial fluid taken from patients with inflammatory arthritis.

6.1. Enumeration of virus-specific T cells within synovial fluid and peripheral blood using HLA-peptide tetrameric complexes

HLA-peptide tetrameric complexes were used to analyse the frequency of $CD8^+$ T cells specific for two EBV lytic protein epitopes, the HLA-A2 restricted epitope (GLCTLVAML) from BMLF1 [Steven *et al*, 1997; Scotet *et al*, 1996] and the HLA-B8 restricted epitope (RAKFKQLL) from BZLF1 [Bogedain *et al*, 1995; Steven *et al*, 1997] and for an EBV latent protein epitope, the HLA-B8 restricted epitope (FLRGRAYGL) from EBNA3A [Burrows *et al*, 1992]. For comparison, the frequency of $CD8^+$ T cells specific for an immunodominant HLA-A2 restricted epitope (GILGFVFTL) from influenza A matrix protein was also analysed [Morrison *et al*, 1992].

Table 6.1. Details of patients

Patients	HLA-A	HLA-B	Clinical details
RhA1	2, 29	44, 49	rheumatoid arthritis
RhA2	2, 29	39, 44	rheumatoid arthritis
RhA3	2, 3	7, 62	rheumatoid arthritis
RhA4	2, 31	7, 8	rheumatoid arthritis
RhA5	1, 2	8, 62	rheumatoid arthritis
RhA6	2	-[a]	rheumatoid arthritis
RhA7	1, 29	8, 44	rheumatoid arthritis
RhA8	2, 30	44, 52	rheumatoid arthritis
RhA9	2, 3	7, 35	rheumatoid arthritis
RhA10	29, 32	40, 44	acute rheumatoid arthritis
NR1	1, 2	27, 49	reactive arthritis
NR2	2, 36	8, 60	psoriatic arthritis
NR3	1, 2	7, 62	osteoarthritis
NR4	1, 31	40, 52	osteoarthritis

[a] *the HLA-B type of patient RhA6 was not determined.*

Details of the patients studied are given in Table 6.1. Eleven patients suffering from inflammatory arthritis were HLA-A2$^+$. In patient RhA6, 9.5% CD8$^+$ T cells within synovial fluid were specific for the GLCTLVAML epitope, whereas only 0.5% CD8$^+$ T cells within peripheral blood were specific for same (Figure 6.1.*a*). Likewise in patient RhA5, T cells specific for the GLCTLVAML epitope were clearly enriched within synovial fluid (4.5% CD8$^+$ T cells) when compared to peripheral blood (1.0%) (Figure 6.1.*b*). In four of the six other HLA-A2$^+$ individuals with rheumatoid arthritis (RhA), we also found higher frequencies of T cells specific for GLCTLVAML in synovial fluid than in peripheral blood (RhA2, RhA3, RhA8, RhA9) (summarised in Table 6.2.). This finding was not restricted to patients with RhA; enrichment of GLCTLVAML-specific T

cells was also detected in synovial fluid taken from patients with psoriatic arthritis (NR2) and osteoarthritis (NR3) (Table 6.2.).

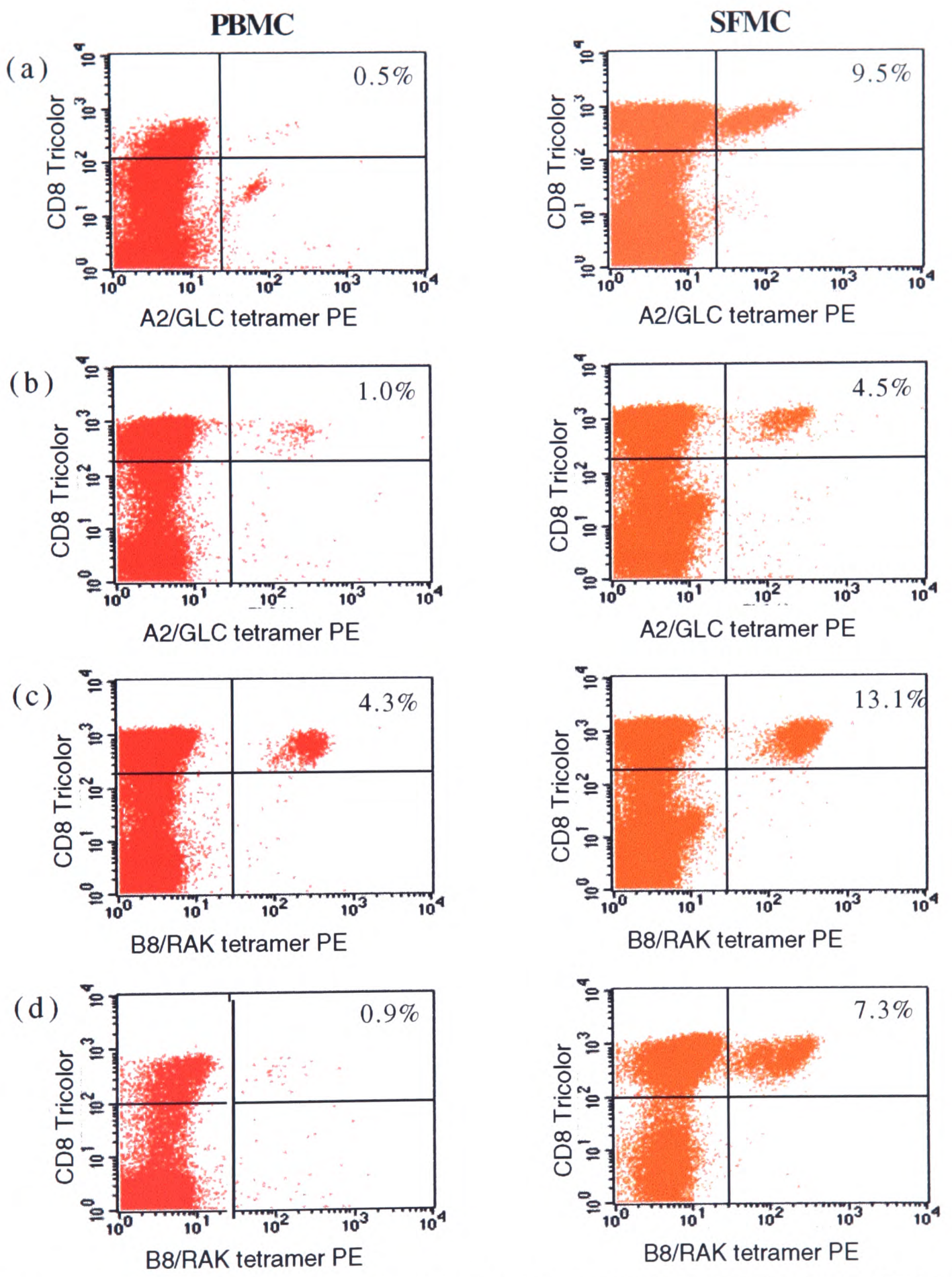


Figure 6.1. Staining of paired samples of peripheral blood and synovial fluid with HLA-peptide tetrameric complexes. Paired samples of PBMC (*left column*) and SFMC (*right column*) from donors were stained with Tricolor-conjugated anti-CD8 and (*a-b*) PE-conjugated A2/GLC tetrameric complex, or (*c-d*) PE-conjugated B8/RAK tetrameric complex. Samples were taken from donors (*a*)RhA6, (*b & c*) RhA 5, and (*d*) RhA7. The frequency of $CD8^+$ T cells that stain with the relevant tetrameric complex is expressed as the percentage of total $CD8^+$ cells.

This cohort included four HLA-B8$^+$ patients, three of whom suffered from RhA (RhA4, 5 and 7) and one from PsA (NR2). In patient RhA5, 13.1% CD8$^+$ T cells within synovial fluid reacted with the B8/RAKtetrameric complex compared with 4.3% within peripheral blood (Fig 6.1.*c*). Similarly in patient RhA7, we found 7.3% CD8$^+$ T cells were specific for the RAKFKQLL epitope in synovial fluid compared with 0.9% CD8$^+$ T cells within the periphery (Fig 6.1.*d*). In the other two individuals, the frequencies of T cells specific for the RAKFKQLL epitope were also higher in synovial fluid when compared with peripheral blood, as summarised in Table 6.2.

Table 6.2. Frequency of CD8$^+$ T cells which stain with the A2/GLC, A2/FluM, B8/RAK and B8/FLR tetrameric complexes in peripheral blood and synovial fluid

Patient	A2/GLC		A2/FluM		B8/RAK		B8/FLR	
	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC
RhA1	undet.[a]	undet.	1.6[b]	2.6	N/A[c]		N/A	
RhA2	0.1	2.0	-[d]	-	N/A		N/A	
RhA3	undet.	0.5	-	-	N/A		N/A	
RhA4	undet.	undet.	-	-	0.8	6.8	undet.	undet.
RhA5	1.0	4.5	undet.	undet.	4.3	13.1	undet.	0.1
RhA6	0.5	9.5	-	-	N/A		N/A	
RhA7	N/A		N/A		0.9	7.3	0.5	0.4
RhA8	0.1	1.1 (L)[e] 1.3 (R)	0.2	0.2	N/A		N/A	
RhA9	0.6	4.2	undet.	undet.	N/A		N/A	
NR1	0.7	0.2	0.4	0.4	N/A		N/A	
NR2	0.4	1.0	-	-	2.5	7.7	0.1	0.1
NR3	0.1	2.3	0.3	0.3	N/A		N/A	

[a]undet., undetectable; [b]expressed as % of CD8$^+$ tetramer-reactive T cells; [c]not applicable, ie samples from donors without the relevant HLA type; [d]denotes not done; [e](L) denotes left knee & (R) denotes right knee.

T cells specific for the HLA-B8 restricted epitope (FLRGRAYGL) from the EBV latent protein EBNA3A were either undetectable or only present at very low frequencies in three of the four HLA-$B8^+$ patients studied (Table 6.2.). This is consistent with the results presented in Chapter 5, showing that the frequency of T cells specific for the FLRGRAYGL epitope is usually lower than the frequency of T cells specific for the RAKFKQLL epitope and is often below the threshold for detection by HLA-peptide tetrameric complexes. In the fourth donor (RhA7) the frequency of FLRGRAYGL-specific T cells was 0.5% $CD8^+$ T cells in peripheral blood and 0.4% $CD8^+$ T cells in synovial fluid. Thus there was no evidence, using this technique, of enrichment of T cells specific for this EBV latent epitope in synovial fluid.

A tetramer of HLA-A2 complexed to a peptide (GILGFVFTL) derived from the influenza A matrix protein was synthesised to identify influenza-specific T cells in six HLA-$A2^+$ donors. Again, in keeping with previous reports [Dunbar *et al*, 1998], T cells specific for this epitope were present at very low or undetectable frequencies in the majority of patients. In only one donor, RhA1, was any enrichment of influenza-specific T cells detected in synovial fluid (Table 6.2.).

In general the frequencies of EBV and influenza reactive T cells within peripheral blood of the patients studied were similar to those previously reported in healthy individuals [Chapter 5; Dunbar *et al*, 1998], despite the use of immunosuppressive drugs.

6.2. Quantitation of EBV-specific T cells within synovial fluid and peripheral blood using an ELISpot assay for IFNγ secretion

ELISpot assays for IFNγ secretion were used to confirm the synovial enrichment of T cells specific for the two epitopes from the EBV lytic cycle proteins and to further investigate frequencies of T cells specific for the FLRGRAYGL epitope from the EBV latent protein and for the GILGFVFTL epitope from the influenza A matrix protein.

Estimates of the frequency of virus-specific T cells obtained with the ELISpot assay were a mean of 19.9 and 20.1 fold lower than those obtained by tetramer staining in peripheral blood and synovial fluid respectively. In the preceding chapter I showed that estimates of the frequency of EBV-specific T cells obtained with the ELISpot assay in healthy control individuals were a mean of 4.4 fold lower than those obtained by staining with tetrameric complexes [Chapter 5]. Thus the ability of the EBV-specific T cells to secrete IFNγ appeared to be impaired in this patient group. Furthermore, the correlation between the estimates of frequency obtained with ELISpot analysis and tetramer staining was poor, suggesting inter-individual and inter-site differences in the functional capacity of the antigen-specific cells. However the overall mean functional capacity of the T cells was similar in synovial fluid and peripheral blood.

The frequency of T cells specific for the GLCTLVAML epitope and the RAKFKQLL epitope from EBV lytic proteins, as estimated by the ELISpot assay, was higher in synovial fluid than in peripheral blood in all the donors studied (summarised in Table 6.3.). Some enrichment of T cells specific for the EBV latent FLRGRAYGL epitope and the influenza GILGFVFTL epitope was also detected, using this method (Table 6.3.). However, the numbers of T cells specific for these two epitopes was always relatively low, even in synovial fluid.

Table 6.3. Frequency of IFNγ-secreting antigen-specific T cells assayed by ELISpot

		GLCTLVAML BMRF1	GILGFVFTL Flu Matrix	RAKFKQLL BZLF1	FLRGRAYGL EBNA3A
RhA1	PBMC	undet.[a]	-[c]	N/A[d]	N/A
	SFMC	490[b]	-	N/A	N/A
RhA5	PBMC	520	-	1,390	20
	SFMC	1,395	-	8,440	40
RhA6	PBMC	5	-	N/A	N/A
	SFMC	410	-	N/A	N/A
RhA7	PBMC	N/A	N/A	110	70
	SFMC	N/A	N/A	3,635	250
RhA8	PBMC	10	20	N/A	N/A
	SFMC	365	235	N/A	N/A
RhA9	PBMC	0	20	N/A	N/A
	SFMC	1,290	390	N/A	N/A

[a]undet., undetectable; [b]expressed as the number of responding cells per 10^6 PBMC or SFMC; [c]denotes not done; [d]not applicable, ie. samples from donors without the relevant HLA type.

6.3. The phenotype of EBV-specific T lymphocytes within peripheral blood and synovial fluid

The expression of activation and differentiation cell surface markers by EBV-specific PBMC and SFMC was analysed in donor RhA7 (Figure 6.2.). CD69, CD38 and HLA-DR, which are all upregulated by activated $CD8^+$ T cells [ref xxx?], were found to be expressed at higher levels on RAKFKQLL-specific T cells within synovial fluid compared with those found in peripheral blood (Figure 6.2.*a* and data not shown). L-selectin (CD62L) is normally downregulated after antigen stimulation, with expression often being regained in the stable memory state [Spertini *et al*, 1991]. This molecule was

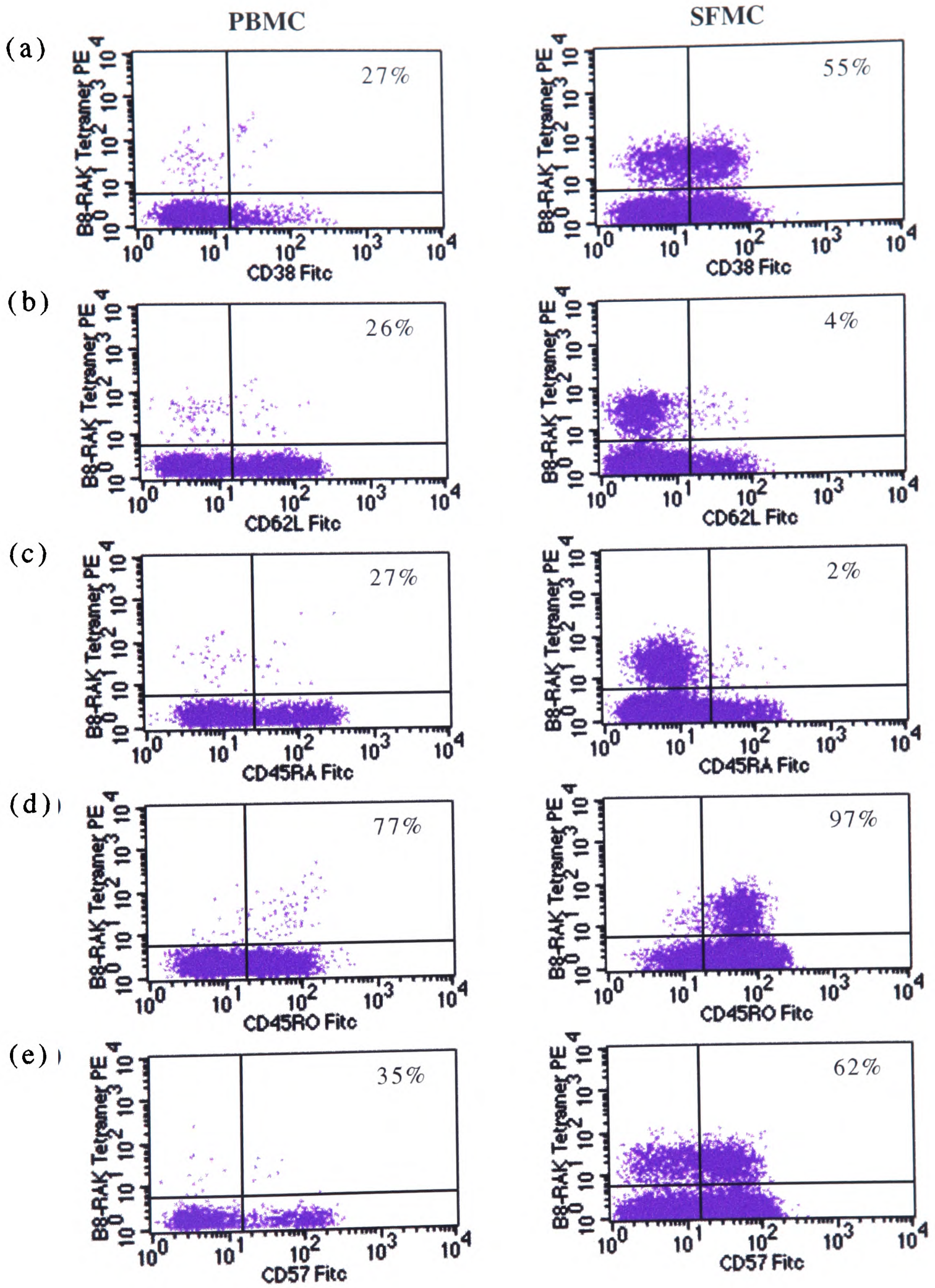


Figure 6.2. The phenotype of RAKFKQLL-specific T cells in peripheral blood and synovial fluid from patient RhA7. Samples of PBMC (*left column*) and SFMC (*right column*) were stained with PE-conjugated B8/RAK tetrameric complex, Tricolor-conjugated anti-CD8 and FITC-conjugated anti- (*a*) CD38, (*b*) CD62L, (*c*) CD45RA, (*d*) CD45RO, and (*e*) CD57. The frequency of cells staining with a particular phenotypic marker is expressed as the percentage of the tetramer-reactive cells.

expressed on only 4% of the RAKFKQLL-specific synovial fluid lymphocytes, compared to 26% of the cells within peripheral blood (Figure 6.2.*b*). CD45RA was not expressed at high levels by significant numbers of RAKFKQLL-specific cells within synovial fluid but was expressed by 27% of those within peripheral blood (Figure 6.2.*c*). The vast majority of RAKFKQLL-specific cells within synovial fluid were CD45RO bright, whilst 77% of these antigen-specific cells within the periphery expressed CD45RO [Akbar *et al*, 1988] (Figure 6.2.*d*). CD28 is a costimulatory molecule which binds B7; downregulation of CD28 expression is associated with a diminished proliferative capacity and thought to reflect a state of late/terminal differentiation [Azuma *et al*, 1993]. This molecule was expressed on 54% of RAKFKQLL-specific T cells within peripheral blood and on 28% of T cells in synovial fluid (data not shown). Expression of CD57, a glycoprotein of unknown function, is believed to indicate $CD8^+$ T cells in a late differentiation compartment [D'Angeac *et al*, 1994]. This molecule was expressed on 35% of RAKFKQLL-specific T cells within peripheral blood and on 62% of these cells within synovial fluid (Figure 6.2.*e*). Analysis of GLCTLVAML-specific T cells within peripheral blood and synovial fluid from two further donors, with rheumatoid arthritis and osteoarthritis respectively, revealed the same pattern, with EBV-specific T cell within the synovial fluid showing an increase in markers of activation and late differentiation as compared with those in peripheral blood (data not shown).

I went on to study the TCR repertoire of EBV-specific T cells within paired samples of peripheral blood and synovial fluid from five of the patients. In patient RhA5, T cells expressing BV14 accounted for 40% of the RAKFKQLL-specific T cells within peripheral blood. Within synovial fluid BV14 was also the TCR Vβ chain most commonly used by RAKFKQLL-specific T cells (Figure 6.3.*a*). In patient RhA7, the

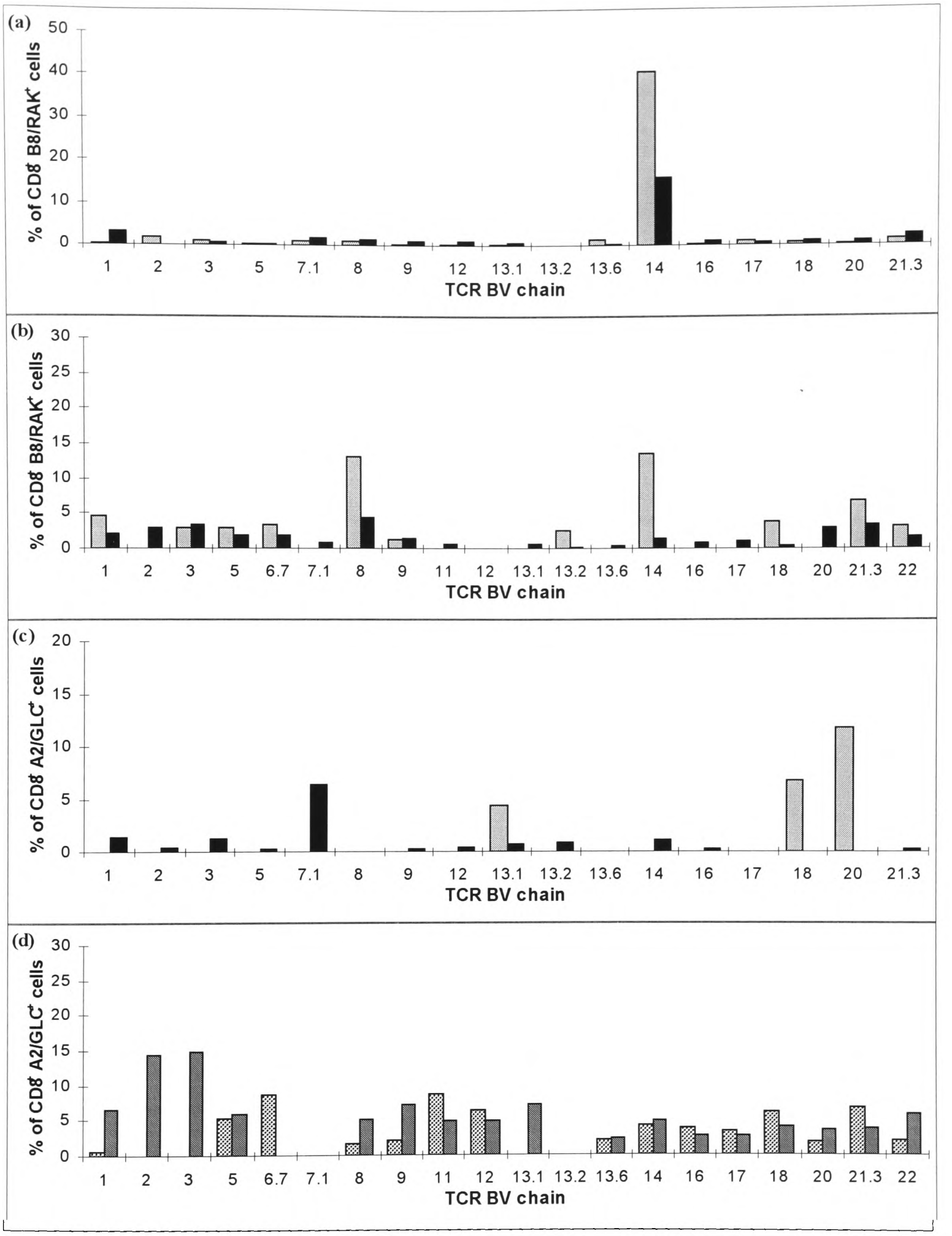


Figure 6.3. The TCR repertoire of EBV-specific T cells in samples of peripheral blood and synovial fluid. Samples from donors (*a*) RhA5, (*b*) RhA7, (*c*) RhA6 and (*d*) RhA8 were stained with (*a-b*) PE-conjugated B8/RAK or (*c-d*) PE-conjugated A2/GLC tetramers, in addition Tricolor-conjugated anti-CD8 and a panel of mAb specific for TCR BV chains. The % frequency of $CD8^+$ tetramer-reactive T cells expressing each TCR BV chain is shown for PBMC (*light grey bars*), SFMC (*black bars*); and only in (*d*) SFMC, left knee (*stippled bars*), SFMC, right knee (*dark grey bars*).

TCR repertoire of the synovial fluid RAKFKQLL-specific T cells was polyclonal with no obviously dominant TCR use. Within peripheral blood however, preferential use of BV8 and BV14 by RAKFKQLL-specific T cells was noted (Figure 6.3.*b*). In patient RhA6 peripheral blood GLCTLVAML-specific T cells expressing BV13S1, BV18 and BV20 were most abundant, whilst the most common TCR Vβ chain used by GLCTLVAML-specific T cells in synovial fluid was BV7S1 (Figure 6.3.*c*). It was possible to compare the TCR repertoire of GLCTLVAML-specific synovial fluid lymphocytes taken from both the left and right knee joints in patient RhA8. The repertoires were generally similar however certain TCR BV chains were only detected in one joint and not the other (Figure 6.3.*d*). These differences in the phenotype and TCR use of EBV-specific T cells within synovial fluid versus peripheral blood could reflect preferential recruitment of subpopulations of cells from the periphery and/or local stimulation and proliferation of the T cells within the joint.

6.4. Expression of chemokine receptors and integrins by $CD8^+$ T cells within peripheral blood and synovial fluid

Chemokines and integrins play important roles in T cell trafficking [Lazarovits & Karsh, 1993; Sallusto *et al*, 1998; Qin *et al*, 1998; Bonecchi *et al*, 1998; Yokota *et al*, 1995; Issekutz, 1992]. Expression of chemokine receptors CCR2, CCR3, CCR5, CXCR3 and CXCR4 [Sallusto *et al*, 1998; Qin *et al*, 1998, Bonecchi *et al*, 1998], and integrins $\alpha_L\beta_2$ (LFA1) [Yokota *et al*, 1995; Dustin & Springer, 1988], $\alpha_4\beta_1$ (VLA4) [Issekutz, 1992], $\alpha_4\beta_7$ [Lazarovits & Karsh, 1993] on $CD8^+$ T cells was investigated within paired samples of peripheral blood and synovial fluid from five donors. Virtually all $CD8^+$ T cells within synovial fluid expressed LFA1 in the five donors studied. However, LFA1 was also

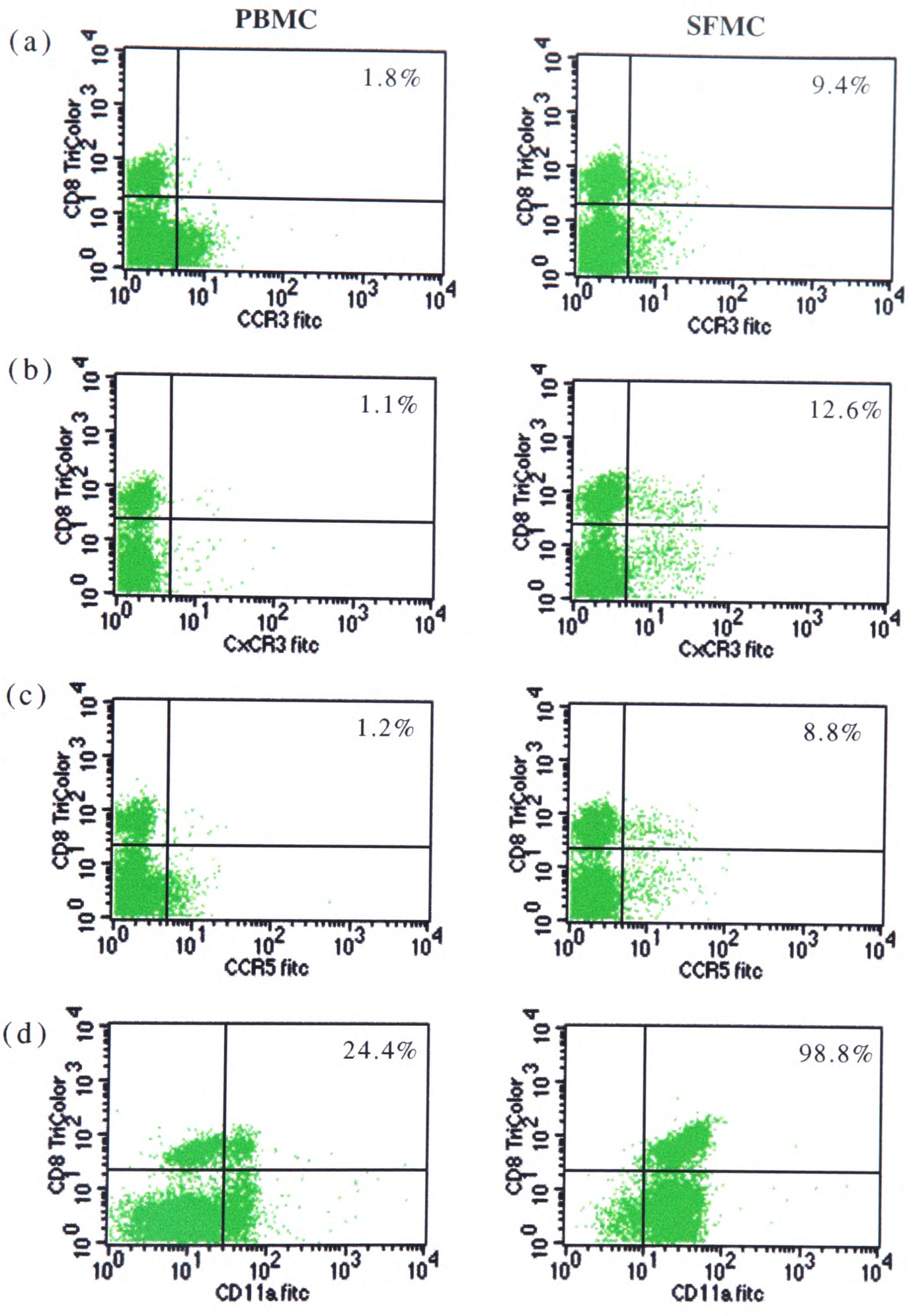


Figure 6.4. Cell surface expression of chemokine receptors and LFA1 integrin on $CD8^+$ T cells in peripheral blood and synovial fluid in patient RhA10. PBMC (*left panels*) and SFMC (*right panels*) were stained with Tricolor-conjugated anti-CD8 and anti- (*a*) CCR3, (*b*) CXCR3, (*c*) CCR5, and (*d*) LFA1. The % of $CD8^+$ T cells stained with each mAb is shown.

expressed on the majority of $CD8^+$ T cells within peripheral blood in three of the five donors (Fig 6.4.*a*; data not shown). Results from a donor RhA10 revealed greater than two-fold enrichment for $CD8^+$ T cells expressing CCR3, CXCR3 and CCR5 (Fig 6.4.*b-d*). $CD8^+$ T cells expressing CXCR3 were also enriched greater than two-fold within synovial fluid from one other individual (data not shown). There were no clear differences in levels of expression of CCR2, CXCR4, VLA4 or $\alpha_4\beta_7$ between peripheral blood and synovial fluid $CD8^+$ T cells in any of the donors studied. In three donors the expression of the integrins and chemokine receptors on total $CD8^+$ T cells was compared to that on RAKFKQLL-specific T cells; the pattern of expression on the antigen-specific cells closely reflected that found on the overall $CD8^+$ T cell population. These results suggest that selective recruitment of subpopulations of T cells based on the expression of those integrins or chemokine receptors studied do not alone account for the demonstrated enrichment of EBV-specific T cells within synovial fluid.

6.5. Proliferation of lymphocytes within synovial fluid

In order to find evidence of local proliferation of virus-specific T cells within synovial fluid, paired samples of peripheral blood and synovial fluid from donors RhA7, RhA8 and RhA9 were stained with Ki67, an antibody specific for a cell proliferation-associated nuclear protein [Schluter *et al*, 1993], to identify any proliferating T cells. Subpopulations of $CD8^+$ T cells within the synovial fluid from all these donors showed evidence of cell cycling. In donor RhA7, 17% of RAKFKQLL-specific T cells within synovial fluid were in cell cycle compared with 0% in peripheral blood (Figure 6.5.*a*). There was no evidence of proliferation of the FLRGRAYGL-specific T cells in either synovial fluid or peripheral blood from this donor (Figure 6.5.*b*). In donors RhA8 and 9, 11% and 0.7% respectively,

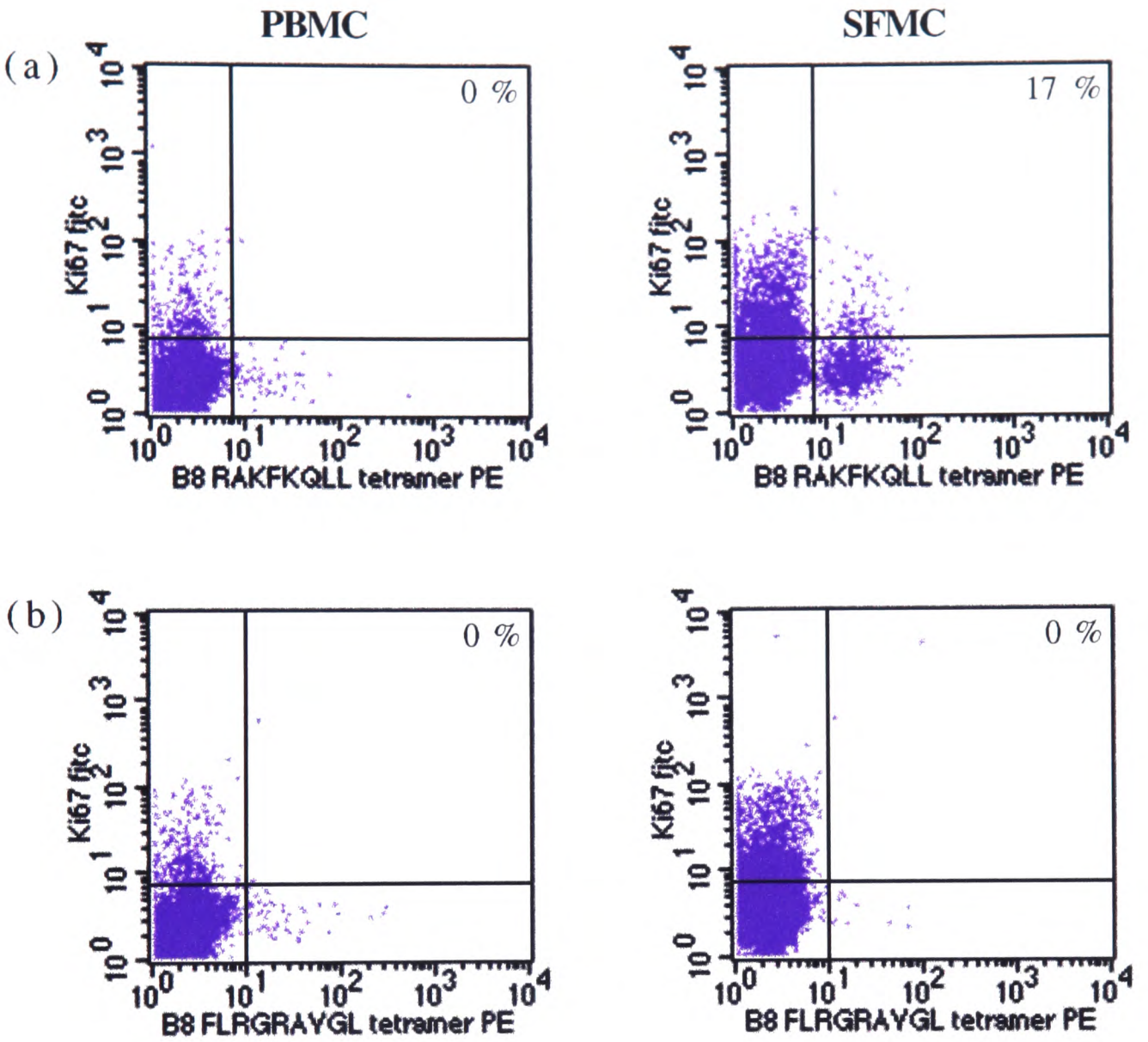


Figure 6.3. Co-staining of cells from patient RhA7 with HLA/peptide tetrameric complexes and the Ki67 mAb. PBMC (*left column*) and SFMC (*right column*) were stained with FITC-conjugated Ki67 and (a) PE-conjugated B8/RAK, or (b) PE-conjugated B8/FLR tetrameric complexes. The % of tetramer-reactive cells which stained with Ki67 is shown.

of GLCTLVAML-specific T cells within synovial fluid were in cycle compared with 0% in peripheral blood in both donors. Cycling of the GILGFVFTL-specific T cells in donor RhA8 was not detectable.

Discussion

This study shows that virus-specific $CD8^+$ T cells are enriched within synovial fluid from individuals with inflammatory arthritis. In particular, $CD8^+$ T cells specific for two epitopes from EBV lytic cycle antigens (GLCTLVAML from BMLF1 and RAKFKQLL from BZLF1) may be present at very high frequencies within the joints of EBV seropositive patients. In donor RhA5, staining with tetrameric HLA-peptide complexes showed that T cells specific for these two epitopes accounted for 17.6% of all $CD8^+$ T cells within synovial fluid (over 10^6 cells in a single joint aspirate). The findings described were not specific for rheumatoid arthritis and similar results were obtained in a patient with psoriatic arthritis (NR2) and in a patient with recurrent knee effusions secondary to osteoarthritis (NR3). Enrichment of T cells specific for an epitope (FLRGRAYGL) from the EBV latent protein EBNA3A (by ELISpot analysis) and of T cells specific for an epitope (GILGFVFTL) from the influenza A matrix protein was also detected, although the frequencies were relatively low in these cases.

Additional data concerning frequencies of cytomegalovirus (CMV)-specific T cells in a separate group of rheumatoid arthritis patients was also provided by Marc Bonneville [personal communication]. In particular, in one patient they found that up to 13% of synovial fluid T lymphocytes reacted with a HLA-A2 tetrameric complex refolded with the CMV tegument protein $pp65_{395\text{-}403}$ NLVPMVATV epitope, demonstrating that $CD8^+$ T cells specific for other herpesvirus are also present within synovial fluid. Furthermore

the synovial lymphocytes were able to secrete TNF upon stimulation with the same minimal CTL epitope.

Enrichment of virus-specific $CD8^+$ T cells within synovial fluid could reflect preferential migration of these T cells from peripheral blood into the joint. T cell migration to sites of inflammation is a carefully controlled process in which adhesion molecules and chemokines play important roles [Sallusto *et al*, 1998; Qin *et al*, 1998; Cush *et al*, 1992; Kohem *et al*, 1996; Borthwick *et al*, 1997]. In the patients studied, LFA1 was expressed on variable numbers of $CD8^+$ T cells within peripheral blood but was always found on more than 94% of $CD8^+$ T cells within synovial fluid, suggesting that the interaction between LFA1 and ICAM plays a role in recruiting or retaining $CD8^+$ T cells within synovial fluid. However, expression of LFA1 on EBV-specific T cells within peripheral blood reflected that of the overall $CD8^+$ population and therefore selective recruitment of $LFA1^+$ T cells would not result in enrichment of EBV-specific T cells within the joint. Furthermore there was no evidence to support the idea that recruitment of T cells expressing other integrins or particular chemokine receptors accounted for the degree of enrichment observed. It is possible that selective migration of subgroups of T cells on the basis of expression of other molecules may be occurring and may help to explain the high frequency of virus-specific T cells within the synovial fluid of patients with inflammatory arthritis.

The results suggest that proliferation of T cells specific for the RAKFKQLL and GLCTLVAML epitopes within the joint at least partially accounts for the high frequency of these T cells within synovial fluid. The differences in the TCR repertoire of antigen-specific T cells within peripheral blood and synovial fluid would be consistent with this as would the increase in markers of activation and late differentiation seen on the antigen-specific T cells within the joints. It is possible that the stimulus to proliferation might be

the relevant EBV antigen itself. Both B cells and T cells are recruited into inflamed joints and in EBV seropositive individuals a subpopulation of these B cells would be latently infected with EBV. One might therefore expect to find the virus within inflamed joints. Whilst some early studies have found no evidence of EBV infections within joints [Brousset *et al*, 1993], other reports describe detection of EBV DNA within the joints of patients with rheumatoid arthritis [Zhang *et al*, 1993; Mousavi-Jazi *et al*, 1998] and one study describes the use of *in situ* hybridisation to detect EBV-encoded small RNA1 and LMP1 transcripts in synovial lining cells from rheumatoid arthritis patients [Takei *et al*, 1997]. Furthermore Koide *et al* [1997] have derived a fibroblastoid cell line expressing EBV proteins from the synovium of a patient with rheumatoid arthritis. Very recently Edinger *et al* [1999] have reported detection of EBV DNA within synovia from 10/11 patients with RhA. This study also provides evidence of transcription of EBV EBER1 and BZLF1 in samples of synovia from patients with RhA and OA. Thus expression of BZLF1 within the joint may be stimulating T cells specific for the HLA-B8 restricted RAKFKQLL epitope from BZLF1. Transcription of BMLF1 has however not been detected within synovia, suggesting that alternative mechanisms may be responsible for driving the proliferation of $CD8^+$ T cells specific for the HLA-A2 restricted epitope (GLCTLVAML) from BMLF1. One possible alternative is that antigen-presenting cells such as dendritic cells may take up EBV antigens and subsequently be recruited to joints where they present epitopes from the EBV antigens by 'cross-presentation' [Albert *et al*, 1998]. A second possibility is that the virus-specific T cells are being stimulated by cross-reactive self-antigens expressed within the joint. Further studies are required to distinguish between these possibilities. Whatever the stimulus to proliferation, the secretion of pro-inflammatory cytokines such as IFNγ and TNF by these virus-specific T cells is likely to contribute to synoviocyte activation and ongoing joint destruction.

It will be important to analyse synovial T cell responses to additional epitopes from EBV and also to other viruses, particularly those which infect lymphocytes and can gain access to inflamed joints. There is evidence to suggest that besides EBV and CMV [Mousavi-Jazi *et al*, 1998; Einsele *et al*, 1992], parvovirus [Saal *et al*, 1992] may be present within synovial tissue. Stimulation of virus-specific T cells within synovial fluid, by virus within the joint, by dendritic cells recruited to the joint and presenting viral epitopes or by cross-reactive self epitopes may be a general phenomenon. These localised T cell responses could be an important factor determining the severity and chronicity of inflammatory arthritis.

CHAPTER 7

Concluding Discussion

Epstein-Barr virus is a genetically stable herpesvirus which persists for life as an asymptomatic infection in the majority of the human population. Primary EBV infection is associated with high levels of virus replication in the oropharynx and with virus-driven proliferation of latently-infected B cells throughout the lymphoid system [Rickinson & Kieff, 1996]. This elicits a vigorous cytotoxic T cell response from the host which eventually controls the infection and continues to provide long-term immune surveillance throughout the persistent phase of infection [Rickinson, 1986; Rickinson & Moss, 1997]. This is most convincingly demonstrated in the frequent incidence of lymphoproliferative disease in T cell-immunocompromised patients [Craig *et al*, 1993; Yao *et al*, 1996]. The B cell immortalising capabilities of this virus are associated with at least three human malignancies, Burkitt's lymphoma, nasopharyngeal carcinoma and malignant lymphomas as seen in immunosuppressed individuals [Epstein & Achong, 1986]. However the development of these tumours is a very rare event, despite the potent growth-transforming potential of the virus and its high spread in the human population [Masucci & Ernberg, 1994].

Until recently it was not possible to directly identify T cells according to their antigen specificity, due intrinsically to the low affinity and fast off-rates of their interactions with peptide-MHC complexes [Matsui *et al*, 1991, Davis *et al*, 1998]. Antigen-specific T cells could only be detected indirectly (and inefficiently), using functional assays such as

LDA. The results from these assays led researchers to believe that only about 1% of the activated $CD8^+$ lymphocytes that arise during the acute phase of a viral infection are antigen-specific [Doherty *et al*, 1992; Ahmed & Gray, 1996]. Fluorochrome-labelled tetrameric complexes of class I MHC-peptide molecules have now been developed by Altman *et al* [1996], which bind to their relevant cognate TCR with greater avidity and thus enable their detection by FACS analysis. The use of tetramers has enabled us to directly visualise populations of antigen-specific T cells in *ex vivo* samples, regardless of their function. Consequently, several studies have since revealed that the majority of expanded, activated $CD8^+$ lymphocytes do not result from bystander activation, as previously believed, but rather direct stimulation by antigen. The use of MHC-peptide tetrameric complexes has introduced new possibilities for assessing the nature of primary and memory responses to virus infection.

7.1. The high frequencies of antigen-specific T cells in primary EBV infection

As described in Chapter 4, staining of PBMC from individuals with acute infectious mononucleosis using MHC-peptide tetrameric complexes revealed clear populations of $CD8^+$ T cells recognising EBV lytic and latent protein CTL epitopes. A very high frequency of EBV-specific T cells recognised the two EBV lytic cycle peptides in this study (up to 6.6% of $CD8^+$ T cells for A2/GLCTLVAML and up to 44% for B8/RAKFKQLL), whilst the frequency of T cells recognising the latent cycle peptide, B8/FLR was relatively low (up to 2.2% of $CD8^+$ T cells).

Similarly high frequencies of antigen-specific cells have been demonstrated recently in some other systems. By tracking the proliferation of adoptively-transferred transgenic GP_{33-41}-specific $CD8^+$ T cells in B6 mice infected with LCMV, Butz and Bevan [1998]

showed that at least half of the activated cells were antigen-specific at the peak of primary infection. Furthermore, about 10% of spleen cells secreted IFNγ in response to each of the two dominant D^b-restricted peptide epitopes, GP_{33-41} and $NP_{396-404}$, in ELISpot assays. Another study showed that at the peak of the LCMV primary response, 40% of $CD8^+$ T cells reacted with a D^b/GP_{33-41} MHC-peptide tetrameric complex [Gallimore *et al*, 1998a]. By using tetramer binding and assays for IFNγ-production at single-cell level, a third group calculated that 50-70% of activated $CD8^+$ T cells were LCMV-specific at peak of primary response in BALB/c and C57B/6 mice [Murali-Krishna *et al*, 1998]. Here, T cells specific for an $L^d/NP_{118-126}$ tetramer accounted for 56% of $CD8^+$ spleen cells taken from BALB/c mice at peak of primary LCMV infection. Likewise primary influenza pneumonia in mice generated fairly high frequencies (>15%) of $CD8^+$ T cells within bronchial alveolar lavage that were recognised by a $D^b/NP_{366-374}$ tetramer [Flynn *et al*, 1998]. In contrast, when mice were primed intravenously with *Listeria monocytogenes*, less than 2% in total of $CD8^+$ splenocytes were specific for the four major epitopes, as assessed by staining with K^d tetramers, with the immunodominant response to the LLO_{91-99} epitope constituting about 1.4% [Busch *et al*, 1998]. Together these results indicate that high frequencies of antigen-specific T cells appear to be a common phenomenon in virus infections, but this may not necessarily be the case with other pathogens.

A common theme that emerges throughout these different studies is that dominant CTL responses tend to be highly focused towards one or a few epitopes, whilst other epitopes induce only weak responses. In this respect, the unexpectedly high frequencies of $CD8^+$ T lymphocytes specific for two recently characterised EBV lytic cycle epitopes are notable. The B8-restricted BZLF1 lytic cycle peptide, RAKFKQLL, induced the largest responses in the IM patients studied here and appears to be the dominant response in all HLA-$B8^+$

IM patients studied to date [Dr M Callan, unpublished observations]. Although previously shown to be a dominant latent epitope, the HLA-B8 restricted EBNA3A FLRGRAYGL epitope consistently elicited weaker responses. The relatively high frequencies of A2/GLCTLVAML-reactive cells were also surprising since this allele does not present any dominant reactivities within EBV latent proteins.

CTL responses to EBV latent proteins are well-characterised and a great number of individual epitopes have been defined. The vast majority of these epitopes map to the EBNA3A, 3B and 3C proteins and only a small number of epitopes, if any, derive from the remaining five latent proteins [Murray *et al*, 1992; Khanna *et al*, 1992; Steven *et al*, 1996; reviewed in Rickinson & Moss, 1997]. Such marked immunodominance is evident even during acute primary infection, and is not seen in other human viral systems; in influenza and HIV1 infection, for instance, memory CTL responses tend to be diversified across a wider range of the available target proteins [Gotch *et al*, 1987; Parker & Gould, 1996; Nixon & McMichael 1991]. This biased distribution of epitopes does not appear to be simply due to differences in the levels of expression of different latent proteins, but have been suggested to be due to differences in the levels of peptide representation at the cell surface, as discussed later.

Immunodominance was not greatly apparent at the level of the antigen receptors expressed by the T cells responding to the B8/RAKFKQLL epitope in IM59, although restricted TCR usage has been well documented for other EBV epitopes [Callan *et al*, 1998] and in other acute infections [MacDonald *et al*, 1993; Pantaleo *et al*, 1994]. More recently Ahmed and co-workers have used both immunoscope analysis and co-staining with tetrameric complexes and TCRBV-specific antibodies to show that the $CD8^+$ T cell response to the dominant LCMV epitope in BALB/c mice, $NP_{118\text{-}126}$, is restricted to TCRs expressing BV10, BV8S1 and BV8S2 [Sourdive *et al*, 1998].

The factors that influence immunodominance of particular epitopes or T cell receptors during an immune response may reflect limitations of the antigen presentation pathway, particularly factors which affect antigen density on the surface of antigen presenting cells, or the characteristics and diversity of the naive T cell repertoire. A major determinant of T cell recognition is the binding affinity of a potential epitope for class I MHC molecules, and the stability of the resultant complex. In this context, Sette and colleagues have shown a positive correlation between peptide affinity and immunogenicity; the findings indicate that the majority of peptides recognised by $CD8^+$ T cells bind to their respective class I molecules with a minimum threshold affinity (Kd < 500 nM) [Sette *et al*, 1994a and 1994b]. The efficiency with which peptides may be generated by antigen processing machinery [Villanueva *et al*, 1994; Vijh *et al*, 1998] and also the stability of peptide-MHC complexes contribute to the relative abundance of a particular epitope on the cell suface of APCs [Busch & Pamer, 1998]. In support of this, the fine specificity of proteasome degradation [Niedermann *et al*, 1995; Ossendorp *et al*, 1996], TAP binding [Neisig *et al*, 1995; van Endert *et al*, 1995], and molecular chaperone proteins [Suto & Srivastava, 1995; Nieland *et al*, 1996] have been shown capable of exerting potential effects on peptide generation. One study on LCMV infection of C57BL/6 mice has found that the magnitude of the CTL response generated does indeed depend on the abundance of each epitope on infected cells [Gallimore *et al*, 1998b]. On the other hand, Pamer *et al* [1997] have reported that the immunodominant $CD8^+$ response in *L. monocytogenes* infection is directed against $LLO_{91\text{-}99}$ which is the least abundant epitope presented on the cell surface. This apparent contradiction was resolved by the finding that $K^d/LLO_{91\text{-}99}$ complexes are relatively stable compared to the most abundant, but subdominant epitope, $p60_{449\text{-}457}$ [Busch & Pamer, 1998].

Qualitative differences in responding $CD8^+$ T cell clones can also, presumably, affect selection to dominance. Some T cells may interact more favourably with antigen, thereby increasing the speed at which they proliferate upon activation [Yewdell & Bennink, 1999]. T cells recognising subdominant epitopes may be further disadvantaged due to rapid clearance of virus infected cells by T cells recognising the dominant epitope, such that subdominant epitopes are expressed at suboptimal levels for CTL activation. This is the seen in LCMV infection of H-2^d mice, where it appears that rapid clearance of virus by the dominant L^d-restricted $NP_{118\text{-}126}$ $CD8^+$ T cell responses reduces the antigen load to a point below the threshold for stimulation of $GP_{283\text{-}291}$-specific responses. Alternatively the magnitude of the T cell response may simply reflect the number of precursors available at the time of infection [Murali-Krishna, 1998]. The importance of these mechanisms *in vivo* have not been fully explored.

Preferential expansion and domination of a particular response may be dependent on the nature of the resting T cell repertoire. Each individual T cell repertoire is shaped by positive and negative selection and tolerance to self antigens. It is possible to imagine that the TCR repertoire may be intrinsically biased towards certain immunodominant determinants, due to expression of distinct self antigens or TCR genes [Daly *et al*, 1995]. This has been demonstrated in H-2^d mice infected with mutant LCMV lacking the dominant $NP_{118\text{-}126}$ epitope, or in transgenic mice expressing NP in the thymus infected with wildtype virus. Both experiments result in the subdominant $GP_{283\text{-}291}$ epitope becoming dominant [Weidt *et al*, 1998; von Herrath *et al*, 1994]. The potential of negative selection for influencing immunodominance can also be seen in EBV infection. In most HLA-B8 individuals, $CD8^+$ T cells responding to the dominant B8-restricted $EBNA3A_{325\text{-}333}$ epitope express a highly conserved TCR, which are alloreactive to HLA-B*4402. However, individuals who are both $B8^+$ and $B*4402^+$ still respond to

EBNA3A$_{325-333}$, although the response is less vigorous and is mediated by alternative non-alloreactive CD8$^+$ T cells [Burrows *et al*, 1994 & 1995].

The hierarchy of immunodominance in CTL responses to EBV latent protein epitopes has been well-documented in Caucasian donors to EBV type 1 virus isolates. However, less is known about immunodominant responses to type 2 isolates, where sequence variations in EBNA3A, 3B and 3C have been shown to result in loss of recognition of many of the type 1 immunodominant epitopes [Moss *et al*, 1988; Kerr *et al*, 1996]. Furthermore CTL responses to lytic cycle epitopes have only recently been identified and the immunodominance of these antigens is still poorly defined. Thus far the apparent dominance of lytic protein reactivities over latent ones has been striking. The magnitude of responses to the BZLF1 RAKFKQLL and BMLF1 GLCTLVAML epitopes suggests that direct CTL control over active virus replicative lesions is of crucial importance in containment of primary infection as well as in long term maintainence of the asymptomatic phase. Expression of the BZLF1 gene alone in latently infected lymphocytes is sufficient to disrupt latency and trigger the full viral lytic cycle [Grogan *et al*, 1987]. This protein contains two regions that are homologous to c-*fos* and mediates the switch from latency to virus replication by activating the transcription of other immediate early and early genes [Flemington & Speck, 1990; Lieberman, 1986]. These include other transactivators (such as BMLF1) as well as genes encoding proteins involved in viral DNA replication [Kieff, 1996]. T cell recognition of early lytic genes is probably desirable for limiting the spread of virus at an early stage in the life cycle before production of virus particles. In addition, lytic genes encode a number of proteins with immunomodulatory functions; BHRF1 encodes a viral homologue of bcl-2 [Pearson *et al*, 1987], whilst BCRF1 encodes a viral homologue of IL-10 [Hsu *et al*, 1990]. These proteins may function in various ways to suppress T cell responses or enhance the

colonisation of mature B lymphocytes [Stuart *et al*, 1995; Suzuki *et al*, 1995; Spriggs, 1996]. Finally, it is also likely that some early proteins in EBV are involved in evasion strategies to avoid detection by CTL, as has been shown in other herpesviruses such as CMV and HSV [Spriggs, 1996].

In this cohort of IM patients (Chapter 4), the phenotype of the tetramer reactive cells was generally homogenous and indicative of recent activation. The majority of EBV-specific $CD8^+$ T cells identified in the different patients expressed high levels of CD38, HLA-DR and CD45RO, and low levels of CD62L and CD45RA. In addition, a greater proportion of HLA-B8 restricted RAKFKQLL-specific $CD8^+$ T cells appeared to have lost CD28 expression and gained CD57 expression, compared to HLA-B8 FLRGRAYGL-specific cells, correlating to their being more highly expanded. Although the results of this study clearly demonstrate high frequencies of activated EBV-specific T cells, their functional capacity was not addressed. It has been shown that the vast majority of activated, expanded $CD8^+$ T cells that arise during the course of a primary virus infection are destined to die apoptosis [Ahmed & Gray, 1996; Zinkernagel *et al*, 1996]. Therefore it is not clear that all these expanded cells retain the ability to kill target cells and if so, by what mechanisms of cytotoxicity. Analysis of FasL and perforin expression on tetramer-reactive cells might yield interesting information about their functional capacity, as would the analysis of cytokine secretion profiles. There is evidence that $CD8^+$ T cells may be subdivided into either Tc1 or Tc2 according to their secretion of either Th1-like or Th-2-like cytokine profiles [Sad *et al*, 1995]. The opportunity to analyse patterns of cytokine secretion in tetramer-positive cell populations may provide further evidence and characterisation of these subgroups.

7.2. Downregulation of the immune response following primary infection

It has often been shown that primary virus infections induce initial activation and expansion of antigen-specific effector T cell populations followed by contraction and establishment of a T cell memory compartment [Gessner *et al*, 1989; Uehara *et al*, 1992; Doherty *et al*, 1992; Murali-Krishna *et al*, 1998]. Accordingly, analysis of peripheral blood samples taken six to 37 months following recovery from acute IM showed significant culling of the earlier dramatic expansions of $CD8^+$ T cells; nevertheless populations of EBV-specific T cells were still easily detectable by tetramer staining. Additionally, the hierarchy of immunodominance displayed during primary infection was maintained in postconvalescent samples. Interestingly, the relative frequency of $CD8^+$ T cells recognising the EBNA FLRGRAYGL epitope, which had originally shown only modest expansion, was closely maintained between acute disease and the early memory phase. This would seem to support a model in which clones that undergo large expansions (eg. BZLF1 RAKFKQLL-specific $CD8^+$ T cells) are more drastically culled than small ones, such as the EBNA3A FLRGRAYGL-specific cells. In contrast, the study by Murali-Krishna *et al* [1998] showed that the number of $NP_{118\text{-}126}$- and $GP_{283\text{-}291}$-specific $CD8^+$ T cells present in the memory pool of LCMV-immune BALB/c mice was determined exclusively by the original burst size. Thus, 5% of the activated cells recognising each epitope survived to enter the memory pool, regardless of the fact that $CD8^+$ T cells recognising $NP_{118\text{-}126}$ were 20 times more abundant than those recognising $GP_{283\text{-}291}$ during the primary response. However an earlier study of $CD8^+$ T cell responses to EBV latent proteins also demonstrated that the differences in relative frequencies of CTL to immunodominant versus subdominant epitopes were less marked in memory than during the primary response [Steven *et al*, 1996].

The rapid contraction of the T cell response following primary infection is due to homeostatic controls that serve to maintain the size and diversity of the T cell pool. Activation-induced cell death (AICD), may be mediated by active mechanisms involving the interaction of FasL and other death cytokines with members of the TNF death receptor family, which includes Fas [Nagata & Goldstein, 1995]. Evidence for the importance of the Fas-FasL system in the regulation of T cell survival following antigen stimulation is seen in *lpr* (Fas^-) and *gld* ($FasL^-$) mutant mice, where accumulation of activated lymphocytes enhances the incidence of autoimmune disease. Fas is constitutively expressed on mature T cells; however expression is up-regulated upon antigen stimulation [Lenardo *et al*, 1999]. T cell activation by antigen also induces FasL expression on cytotoxic T cells, making them highly susceptible to Fas-mediated apoptosis [Zheng *et al*, 1998]. Since FasL can be cleaved from the cell surface by metalloproteinases, it is likely that T lymphocytes have the ability for self-killing [Brunner *et al*, 1995].

Death of activated $CD8^+$ T lymphocytes may also be mediated by withdrawal of IL-2 or lymphokines [Lenardo, 1991]. Activation of T cells in response to antigen induces production of IL-2 and its high affinity receptor, leading to T cell proliferation and further IL-2 production [Waldmann, 1991]. Antigen clearance at the end of an immune response results in down regulation of IL-2 production, causing rapid apoptosis of cycling T cells [Duke & Cohen, 1986; Lenardo, 1999].

It is not clear which factors determine the survival of a subset of T cells and their differentiation into memory cells. Cells expressing bcl-2 or other members of the bcl-2 family are able to avoid apoptosis and this may be a possible mechanism for enabling some T cells to survive into memory [Chao *et al*, 1995]. Additional members of the TNF and TNF receptor families have recently been discovered, including a soluble TNF-like

receptor that appears to be expressed by activated T lymphocytes. It has been proposed that binding of FasL and other related death cytokines to this soluble "decoy" receptor may be a novel mechanism by which cells may avoid undergoing apoptosis [Pan *et al*, 1997]. Analysis of the expression of death-inducing or death-rescuing molecules, or experiments with blocking antibodies may help to clarify this issue.

7.3. Memory responses to persistent EBV infection

The second group of patients studied here (Chapter 5) were EBV seropositive but had either no history of clinical disease (and probably underwent asymptomatic seroconversion during childhood) or had suffered acute IM over a decade ago. Therefore it was surprising that in these individuals, significant frequencies of EBV-specific T cells were still clearly detectable in peripheral blood, by staining with peptide-MHC tetrameric complexes. In addition, the frequencies of $CD8^+$ T cells recognising two immunodominant lytic protein epitopes (A2/GLCTLVAML and B8/RAKFKQLL) were fairly high (up to 5.5% of total $CD8^+$ T lymphocytes in one donor). The frequency of $CD8^+$ T cells recognising the A11-restricted immunodominant $EBNA3B_{416\text{-}424}$ epitope, though lower than the former, were also substantial, representing up to 3.8% of total $CD8^+$ T cells in one donor. Contrary to what was seen during primary infection, the memory pool appeared to be functionally heterogenous, as evidenced by differential expression of phenotypic markers on the EBV-specific cells, as well as variations in the responses to functional assays. Studies with mono-specific TCR-transgenic CD4 and CD8 cells have previously provided evidence for the existence of different functional subsets: effector cells, which are capable of rapid cytotoxic activity and memory cells, which require antigen stimulation for activation [Zinkernagel *et al*, 1996]. However in

humans it has generally been difficult to distinguish these two subsets on the basis of expression of cell surface markers alone. Furthermore at least two recent studies have highlighted that some of the antigen-specific T cells detectable using tetramers may be have diminished functional capacity. Gallimore *et al* [1998a] demonstrated that chronic infection of mice with a high dose of a rapidly replicating strain of LCMV resulted in premature induction of the $CD8^+$ effector population which exhibited reduced cytotoxicity and cytokine production. Similarly, Zajac *et al* [1998] showed that persistent LCMV infection in mice produced high levels of activated $CD8^+$ T cells that were reactive with D^b/GP_{33-41} tetrameric complexes, and appeared to be activated ($CD69^{hi}$, $CD44^{hi}$, $CD62^{lo}$), but did not elicit any antiviral effector functions. This effect was exacerbated in CD4 knockout mice, implying a requirement for CD4 help in maintaining effective CD8-mediated control of persistent infections. The dependence of $CD8^+$ cytotoxic responses on $CD4^+$ T cell help has been shown in murine models of chronic viral infections [Battegay *et al*, 1994; Cardin *et al*, 1996] while in humans, the declining levels of $CD4^+$ T cells are correlated with the loss of HIV-specific $CD8^+$ T cell responses during progression to AIDS [Rosenberg *et al*, 1997]. In light of these findings it is important that future experiments establish whether the large populations of EBV-specific tetramer-reactive cells demonstrated here are wholly functional. Chronic infection with a persistent, systemic virus such as EBV may lead to conditions that favour the induction of $CD8^+$ T cell unresponsiveness, as T cells become increasingly sensitive to the regulatory mechanisms that down-regulate excessive and potentially self-reactive activated T lymphocytes.

7.4. Final Considerations

The detection of potent CTL responses to lytic cycle antigens in memory strongly suggests that EBV replicative lesions continue to arise throughout life and are subject to direct CTL control *in vivo*. Thus the fall in oropharyngeal virus shedding that occurs with primary infection in IM is very probably brought about by the emerging lytic antigen-specific CTL response. Likewise chronic EBV replication in epithelial cells of the tongue, giving rise to the characteristic lesions of oral hairy leukoplakia, are often observed in immunocompromised AIDS patients. CTL control over latent B lymphocyte infection is also important for maintaining the balance between host and pathogen in the prevention of immunoblastic lymphomas and other malignancies.

The ability of EBV-associated malignancies to flourish despite such potent and comprehensive CTL responses is intriguing. Part of this success is due to the fact that malignant cells in Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease only express a small range of latent proteins, and more importantly, these proteins are not the major targets of cell-mediated EBV-specific responses. The EBNA1 protein is expressed in all EBV-associated malignancies and is required for maintenance of the episomal viral genome in latently infected cells [Yates *et al*, 1984]. However this essential protein is protected from the antigen processing pathway, by means of an internal glycine-alanine repeat sequence [Levitskaya *et al*, 1995; Shapiro *et al*, 1998]. Another protein, LMP1, is also expressed in nasopharyngeal carcinoma and Hodgkin's disease but again, is rarely recognised by CTL. Furthermore LMP1 has been shown to induce upregulation of bcl-2, which enhances the survival of latently infected B lymphocytes and presumably contributes to the resistance of malignant cells to immune clearance [Masucci & Ernberg, 1994]. As a consequence, a number of efforts to develop effective vaccines have concentrated on inducing effective responses to LMP2, which is

also expressed in nasopharyngeal carcinoma and Hodgkin's disease, and is the only protein that elicits reasonable levels of CTL recognition [Rickinson & Moss, 1997]. Furthermore, in comparison to the EBNA3 proteins, LMP2 sequences are relatively conserved between both virus types [Lee *et al*, 1993]. Interestingly, nasopharyngeal carcinoma occurs widely in Southeast Asia, where epitope-loss mutant virus isolates have been found. Specific mutations involving anchor residues of immunodominant A11-restricted epitopes have been shown to abrogate binding to class I MHC and CTL recognition [de Campos-Lima, 1993 & 1994]. It is tempting to speculate that loss of these reactivities may contribute to the high incidence of nasopharyngeal carcinoma that is observed there.

Persistent viruses that elicit prolonged and vigorous immune responses have often been implicated in the pathogenesis of autoimmune disease, and EBV is no exception. High frequencies of antigen-specific circulating $CD8^+$ T lymphocytes that accompany persistent infection with EBV can significantly influence the balance of reactivities within the circulating $CD8^+$ T cell pool. This highlights the immunopathologies that might arise if the virus-induced response were to include reactivities either potentially cross-reactive with self antigens or cross-reactive with an allo-HLA antigen present on grafted tissue. Evidence that EBV plays a direct role in the pathogenesis of rheumatoid arthritis remains controversial. Nevertheless their elevated presence and ability to secrete pro-inflammatory cytokines within affected joints may have important implications in long term maintenance of chronic joint disease. The results in Chapter 6 indicate that at least some EBV-specific T cells are activated to proliferate within arthritic joints. This may be due to the presence of EBV-infected B lymphocytes that are also recruited into the synovium in the course of inflammation, and which may present viral antigens to $CD8^+$ T lymphocytes. Alternatively cross-presentation by dendritic cells or stimulation by

cross-reactive self-antigens may be responsible for T cell activation. The involvement of such mechanisms are not mutually exclusive. T lymphocytes specific for other persistent viruses have also been found in the joints of patients with arthritis. Future experiments may uncover general mechanisms by which such T cells contribute to the disease process.

Epstein-Barr virus remains a paradigm for the study of immune responses to virus infection in humans, since it is so widespread and causes a clearly defined primary illness. Its successful strategies for survival within the host emphasise the evolution of a finely-tuned balance between virus and host. Our understanding of its adaptation to life within the immune system is essential for design of prophylactic vaccines for IM and therapeutic vaccines for associated malignancies. It is hoped that novel techniques for detecting $CD8^+$ T cells will not only provide the tools to further characterise immune responses to this virus, but also facilitate monitoring of responses to various treatment modalities.

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APPENDIX A. AMINO ACIDS AND GENETIC CODONS

Abbreviations for amino acids:

A - Alanine (Ala)
C - Cysteine (Cys)
D - Aspartic acid (Asp)
E - Glutamate (Glu)
F - Phenylalanine (Phe)
G - Glycine (Gly)
H - Histidine (His)
I - Isoleucine (Ile)
K - Lysine (Lys)
L - Leucine (Leu)
M - Methionine (Met)
N - Asparagine (Asn)
P - Proline (Pro)
Q - Glutamine (Gln)
R - Arginine (Arg)
S - Serine (Ser)
T - Threonine (Thr)
V - Valine (Val)
W - Tryptophan (Trp)
Y - Tyrosine (Tyr)

The genetic code (nuclear genes):

1st position of codon	*2nd position of codon*								*3rd position of codon*
	U		**C**		**A**		**G**		
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	**U**
	UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys	**C**
	UUA	Leu	UCA	Ser	UAA	Stop	UGA	Stop	**A**
	UUG	Leu	UCG	Ser	UAG	Stop	UGG	Trp	**G**
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	**U**
	CUC	Leu	CCC	Pro	CAC	His	CGC	Arg	**C**
	CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	**A**
	CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg	**G**
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	**U**
	AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser	**C**
	AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	**A**
	AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg	**G**
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	**U**
	GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly	**C**
	GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly	**A**
	GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly	**G**

APPENDIX B. COMMON EBV-ENCODED CTL EPITOPES

EBV antigen		Epitope sequence	MHC restriction
EBNA 3A	158-166	QAKWRLQTL	HLA-B8
	325-333	FLRGRAYGL	HLA-B8
	379-387	RPPIFIRRL	HLA-B7
EBNA3B	399-408	AVFDRKSDAK	HLA-A11
	416-424	IVTDFSVIK	HLA-A11
EBNA3C	881-889	QPRAPIRPI	HLA-B7
LMP2	340-350	SSCSSCPLSKI	HLA-A2
BZLF1	190-197	RAKFKQLL	HLA-B8
BMLF1	280-288	GLCTLVAML	HLA-A2

APPENDIX C. SEQUENCES OF T CELL RECEPTOR CDR3 REGIONS

Sequences of CDR3 coding joints are aligned in three sections, V - N - J. The N region incorporates nontemplated nucleotides as well as the encoded D segments, which are often difficult to distinguish due to their small size and the variability of reading frame. P nucleotides are underlined where they occur.

I. Sequence of $CD4^+$ BV6S3 T cell receptors

V region *N(D) region* *J region*

1. AGC AGC --------- CCA CCA GGT AGC C ---------- CC TAC GAG CAG TAC TTC **BJ2S7**
   S S P P G S P Y E Q Y F
2. AGC AGC ---------- CCT ACT CC ---------- C AAT GAG CAG TTC TTC **BJ2S1**
   S S P T P N E Q F F
3. AGC AGC T ---------- CT AGT GG ---------- T ACG CAG TAT TTT **BJ2S3**
   S S S S G T Q Y F
4. AGC AGC TTA GC ------- C CGG ACC CCT AAG -------- AAT GAG CAG TTC TTC **BJ2S1**
   S S L A R T P K N E Q F F
5. TGT GC ------- T TCG TCC AGT TCA TT ------- C TAC AAT GAG CAG TTC TTC **BJ2S1**
   C A S S S S F Y N E Q F F
6. AGC AGC ----- CAC CCG GGC GGA GGG CCG CGG G ------ AT GAG CAG TTC TTC **BJ2S1**
   S S H P G G G P R D E Q F F
7. AGC AG ------- A TCA CTC CTG CCC TGG G ------- AA AAC ATT CAG TAC TTC **BJ2S4**
   S S S L L P W E N I Q Y F
8. AGC AGC --------- CCA CCA GGT AGC C ---------- CC TAC GAG CAG TAC TTC **BJ2S7**
   S S P P G S P Y E Q Y F
9. AGC AGC T ---------- CT AGT GG ---------- T ACG CAG TAT TTT **BJ2S3**
   S S S S G T Q Y F
10. AGC AGC TTA GC ------- C CGG GAC C ------- CC TAC AAT GAG CAG TTC TTC **BJ2S1**
    S S L A R D P Y N E Q F F
11. AGC AG ------- A TCA CTC CTG CCC TGG G ------- AA AAC ATT CAG TAC TTC **BJ2S4**
    S S S L L P W E N I Q Y F
12. TGT GCC A --------- CT TCC CGG GC --------- G AAC ACT GAA GCT TTC TTT **BJ1S1**
    C A T S R A N T E A F F
13. AGC AGC TTA ---------- AAG AAG ---------- AAT TCA CCC CTC CAC TTT **BJ1S6**
    S S L K K N S P L H F
14. AGC AGC TTA G ------- GT CCG GGC GGG G ------- AC ACT GAA GCT TTC TTT **BJ1S1**
    S S L G P G G D T E A F F
15. AGC AGC T ---------- CG AAC TGG CAT ---------- GAG CTG TTT TTT **BJ2S2**
    S S S N W H E L F F
16. AGC AGC TTA GC - G CTG TCT AGC GGG AGC ATA A - CC TAC GAG CAG TAC TTC **BJ2S7**
    S S L A L S S G S I T Y E Q Y F
17. AGC AGC ---------- CCT AAG CAG ACA GGA GGG ---------- GAG CAG TAC TTC **BJ2S7**
    S S P K Q T G G E Q Y F
18. AGC AGC ---------- GCT CGA GGT GGA ---------- TAC GAG CAG TAC TTC **BJ2S7**
    S S A R G G Y E Q Y F
19. AGC AGC ---------- GCT GAC AG ---------- C TCC TAC GAG CAG TAC TTC **BJ2S7**
    S S A D S S Y E Q Y F

20. AGC AGC TTA ------- ACG GAC AGG GG -------- G AAC ACT GAA GCT TTC TTT **BJ1S1**
S S L T D R G N T E A F F

21. AGC AGC TTA GC ------- C AGC GGG AGT ATC CG ------- T GAG CAG TTC TTC **BJ2S1**
S S L A S G S I R E Q F F

22. AGC AGC TTA GC ---------- G A ---------- GC GGG GAG CTG TTT TTT **BJ2S2**
S S L A S G E L F F

23. AGC AGC TT ---------- C AAA C ---------- AC TAT GGC TAC ACC TTC **BJ1S2**
S S F K H Y G Y T F

24. AGC AGC --------- CCG GAC AGG GGA GG ---------- C TAT GGC TAC ACC TTC **BJ1S2**
S S P D R G G Y G Y T F

25. AGC AGC TTA GC --------- T GGA TCC GC --------- C GGG GAG CTG TTT TTT **BJ2S2**
S S L A G S A G E L F F

26. AGC AGC TTA ----- TAT CAG GGC CGG GGG AC ------ C ACT GAA GCT TTC TTT **BJ1S1**
S S L Y Q G R G T T E A F F

27. AGC AGC T ---------- AT CGG CGG GTG G ---------- AC ATT CAG TAC TTC **BJ2S4**
S S Y R R V D I Q Y F

28. AGC AGC TT ---------- G GGC AC ---------- C AAC GAG CAG TTC TTC **BJ2S1**
S S L G T N E Q F F

29. AGC AGC TT --------- T GCG GAG GGA ---------- TCC TAC GAG CAG TAC TTC **BJ2S7**
S S F A E G S Y E Q Y F

30. AGC AGC TTA --------- ATT TGG CAG CTG G ---------- AC GAG CAG TAC TTC **BJ2S7**
S S L I W Q L D E Q Y F

31. AGC AGC TTA G --------- GT GAA AGG A --------- CC TAC GAG CAG TAC TTC **BJ2S7**
S S L G E R T Y E Q Y F

32. AGC AGC TTA GC --------- G GAC AGG G --------- AT CAG CCC CAG CAT TTT **BJ1S5**
S S L A D R D Q P Q H F

33. AGC AG ---------- T CTA ATC CGG GAC GGC ---------- GAA GCT TTC TTT **BJ1S1**
S S L I R D G E A F F

34. AGC AGC TT ------- G ACT GGG GGG AGC CCC TC ------- C GAG CAG TAC TTC **BJ2S7**
S S L T G G S P S E Q Y F

35. AGC AGC -------- CCT AAG AGA GTG GTG GGT -------- AAT GAG CAG TTC TTC **BJ2S1**
S S P K R V V G N E Q F F

II. Sequence of $CD8^+$ BV6S3 T cell receptors

V region *N(D) region* *J region*

1. AGC AGC TTA -------- CAC CAG GGG CCA -------- TCC TAC GAG CAG TAC TTC **BJ2S7**
S S L H Q G P S Y E Q Y F

2. AGC AGC T ---------- GG GGA CAG G ---------- CC TAC GAG CAG TAC TTC **BJ2S7**
S S W G Q A Y E Q Y F

3. AGC A --- CG CCG AGG ACG GAT AGA TCC TTT C --- CC TAC GAG CAG TAC TTC **BJ2S7**
S T P R T D R S F P Y E Q Y F

4. AGC AGC ----- CCT AGT GTG GGA CTA GCG G ------ AC AAT GAG CAG TTC TTC **BJ2S1**
S S P S V G L A D N E Q F F

5. AGC AG ------- A TCA CTC CTG CCC CGG G ------- AA AAC ATT CAG TAC TTC **BJ2S4**
S S S L L P R E N I Q Y F

6. AGC AGC TTA ------- CTA GCG GGA GGG C -------- CA GAT ACG CAG TAT TTT **BJ2S3**
S S L L A G G P D T Q Y F

7. AGC AGC ---------- CCG GGA CTC AGG GGG ---------- GAG CAG TAC TTC **BJ2S7**
S S P G L R G E Q Y F

8. AGC AGC TT ------- C GCT AGC GGT C ------- GC ACA GAT ACG CAG TAT TTT **BJ2S3**
   S S F A S G R T D T Q Y F

9. TGT GC ------- T TCG TCC AGT TCA TT ------- C TAC AAT GAG CAG TTC TTC **BJ2S1**
   C A S S S S F Y N E Q F F

10. AGC AGC TT ---------- T AAC TCC GGC TAC ---------- GGC TAC ACC TTC **BJ1S2**
    S S F N S G Y G Y T F

11. AGC AG ---------- T CCT ACT CC ---------- C AAT GAG CAG TTC TTC **BJ2S1**
    S S P T P N E Q F F

12. AGC ---- T CAT GAG AGG CTA AGC GCG AGA TC ----- C TAC GAG CAG TAC TTC **BJ2S7**
    S H E R L S A R S Y E Q Y F

13. AGC AGC ---------- CCG TAC AG --------- T AGC AAT CAG CCC CAG CAT TTT **BJ1S5**
    S S P Y S S N Q P Q H F

14. AGC AGC TT ---------- CGG ACA GGA ---------- AGC GGG GAG CTG TTT TTT **BJ2S2**
    S S F R T G T G E L F F

15. AGC AGC T --- CC TGG ACT AGC GGG GGG GCG GG --- A GAG ACC CAG TAC TTC **BJ2S5**
    S S W T S G G A G E T Q Y F

16. AGC AGC TT ---------- T GGA CAG GGG ACA ---------- GAG CAG TAC TTC **BJ2S7**
    S S F G Q G T E Q Y F

17. AGC AGC -- ATA ACC AGC CCC TTA AGG ACT -- AGC ACA GAT ACG CAG TAT TTT **BJ2S3**
    S S I T S P L R T S T D T Q Y F

18. AGC AG -------- C CAT AGC GGG GGT CAA ------- CAA GAG ACC CAG TAC TTC **BJ2S5**
    S S H S G G Q Q E T Q Y F

19. AGC AGC TTA G ------- GG CTA GCC AGA CA ------- A GAT ACG CAG TAT TTT **BJ2S3**
    S S L G L A R Q D T Q Y F

20. AGC AGC TT ------- G GGA TGG ACA TGG G ------- AC ACT GAA GCT TTC TTT **BJ1S1**
    S S L G W T W D T E A F F

21. AGC AGC TTA - AAG GGA CAG GGG AGG ATT TG -- G AAC ACT GAA GCG TTC TTT **BJ1S1**
    S S L K G Q G R I W N T E A F F

22. AGC AGC T ---------- CC TCC GAG ATG GGG GGA AA ---------- C TTC **BJ1S2**
    S S S S E M G G N F

23. AGC AGC T ---------- CC TAC CGA ---------- AAT CAG CCC CAG CAT TTT **BJ1S5**
    S S S Y R N Q P Q H F

24. AGC AGC ------- CTG CGC ATG GGG CGG G -------- AC ACT GAA GCT TTC TTT **BJ1S1**
    S S L R M G G D T E A F F

25. AGC AGC T -------- CG GGG GGG CGC CA ---------- A GAT ACG CAG TAT TTT **BJ2S3**
    S S S G G R Q D T Q Y F

26. AGC AGC TTA G ---------- GG GTG AGA AAT ---------- CAG TTC TTC **BJ2S1**
    S S L G V R N Q F F

27. AGC AGC TT - G AGA TGG GTA GGA GGA ATC G - CT AAC TAT GGC TAC ACC TTC **BJ1S2**
    S S L R W V G G I A N Y G Y T F

28. AGC AGC TTA GC ------- T GGT GGG GAA GGA CT ------- C GAG CAG TAC TTC **BJ2S7**
    S S L A G G E G L E Q Y F

29. AGC AGC TTA G ---------- GC CGG ---------- TCC TAC GAG CAG TAC TTC **BJ2S7**
    S S L G R S Y E Q Y F

30. AGC AGC TTA -- NNT CAN NNC GTC CAN ACT AGC ATG A - AG ACC CAG TAC TTC **BJ2S5**
    S S L X X X V x T S M K T Q Y F

31. AGC AGC T --------- AC TTA ---------- TAC GAG CAG TAC TTC **BJ2S7**
    S S Y L Y E Q Y F

32. AGC AGC TT ----- T ACT AGC GGG GGG GCC CC ----- A GAG ACC CAG TAC TTC **BJ2S5**
    S S F T S G G A P E T Q Y F

33. AGC AGC TTA GC ----- N NNN NNN NNN NNN NNN NN ----- C GAG CAG TAC TTC **BJ2S7**
    S S L A X X X X X X E Q Y F

34. AGC AGC TTA G ---------- TC TGG GG ---------- C TAT GGC TAC ACC TTC **BJ1S2**
S S L V W G Y G Y T F

35. AGC AGC T ------- CA ATA TGG CAC GTT G ------- CC TAC GAG CAG TAC TTC **BJ2S7**
S S S I W H V A Y E Q Y F

36. AGC AGC ------- GCA GAC GGA CAC TCG ACG A -------- AT GGC TAC ACC TTC **BJ1S2**
S S A D G H S T N G Y T F

37. AGC AGC TTA GC ----- N NNN NNN NGG GAA C ----- CA GAT ACG CAG TAT TTT **BJ2S3**
S S L A X X X E P D T Q Y F

III. Sequence of $CD4^+$ BV7S1 T cell receptors

V region *N(D) region* *J region*

1. AGC AGC CAA G ----- TA TTG GCG GAA AAG GG ----- C TAC GAG CAG TAC TTC **BJ2S7**
S S Q V L A E K G Y E Q Y F

2. AGC AGC CAA -------- ATA GTT AAG GCC ATC -------- GAA AAA CTG TTT TTT **BJ1S4**
S S Q I V K A I E K L F F

3. AGC A ------- AG GTC GCA GTG GA ------- T AGC AAT CAG CCC CAG CAT TTT **BJ1S5**
S K V A V D S N Q P Q H F

4. AGC AG --------- A TCG AGG ACA GGG AT --------- C ACT GAA GCT TTC TTT **BJ1S1**
S R S R T G I T E A F F

5. AGC AGC CAA G ---------- TT TTA GG ---------- T ACG CAG TAT TTT **BJ2S3**
S S Q V L G T Q Y F

6. AGC AGC CAA GA ----- T AGA GTC CCT CGG ------ TAC AAT GAG CAG TTC TTC **BJ2S1**
S S Q D R V P R Y N E Q F F

7. AGC AGC CAA G -------- GG GCG TCG -------- CT GGA AAC ACC ATA TAT TTT **BJ1S3**
S S Q G A S G N T I Y F

8. AGC AGC CAA GA ---------- T GGG GG ---------- T GAG CAG TTC TTC **BJ2S1**
S S Q D G G E Q F F

9. AGC AGC CAA G ---------- TT TTA GG ---------- T ACG CAG TAT TTT **BJ2S3**
S S Q V L G T Q Y F

10. AGC AGC CAA G --------- GT CAT TT --------- C CAA GAG ACC CAG TAC TTC **BJ2S5**
S S Q G H F Q E T Q Y F

11. AGC AGC CAA G --------- CG AGA CTG T --------- AC ACT GAA GCT TTC TTT **BJ1S1**
S S Q A R L Y T E A F F

12. AGC AGC -------- TAC GGG ACA GGG CGA AG ------- C ACT GAA GCT TTC TTT **BJ1S1**
S S Y G T G R S T E A F F

13. AGC AGC CAA GA -------- T CCG GCC AAG GGG -------- AG CCC CAG CAT TTT **BJ1S5**
S S Q D P A K G P Q H F

14. AGC AGC ------- TAC GGG ACA GGG CGA AG -------- C ACT GAA GCT TTC TTT **BJ1S1**
S S Y G T G R S T E A F F

IV. Sequence of $CD4^+$ BV8S1 T cell receptors

V region *N(D) region* *J region*

1. AGC AGT TT --- T AGG TCA GCG AGA TA --- T AGC AAT CAG CCC CAG CAT TTT **BJ1S5**
S S F R S A R Y S N Q P Q H F

2. AGC AGT TTA ----- TGG GGG AGC GGT TC ----- C ACA GAT ACG CAG TAT TTT **BJ2S3**
S S L W G S G S T D T Q Y F

3. AGC AGT T --------- CA GTT CAA GT --------- C ACA GAT ACG CAG TAT TTT **BJ2S3**
S S S V Q V T D T Q Y F

4. AGC AGT TTA GC ----- T CGT ATG GGG CAT G ----- AT CAG CCC CAG CAT TTT **BJ1S5**
S S L A R M G H D Q P Q H F

5. AGC ---------- TCA ACT CTT ACT CCG ---------- GAG ACC CAG TAC TTC **BJ2S5**
S S T L T P E T Q Y F

6. AGC AGT ---------- GCT TCG GGA TCG ---------- GAT ACG CAG TAT TTT **BJ2S3**
S S A S G S D T Q Y F

7. AGC AGT T ------- NN NGG GGG GAA -------- GGG GCC AAC GTC CTG ACT TTC **BJ2S6**
S S X X G E G A N V L T F

8. AGC AG ---------- T AGC GAA CC ---------- C AAT GAG CAG TTC TTC **BJ2S1**
S S S E P N E Q F F

9. AGC AG ---------- C ATA CGG GG ---------- C GGG GAG CTG TTT TTT **BJ2S2**
S S I R G G E L F F

10. AGC A - TT CTC GGG TCT GCC AAC AGT CG - C TCT GGA AAC ACC ATA TAT TTT **BJ1S3**
S I L G S A N S R S G N T I Y F

11. AGC AGT T --------- AG CCT AGC ACG NGA TAC ---------- GAG CAG TTC TTC **BJ2S1**
S S P S T X Y N E Q F

12. AGC AGT TTA G ----- GT CAG CAG TA ----- T AGC AAT CAG CCC CAG CAT TTT **BJ1S5**
S S L G Q Q Y S N Q P Q H F

13. AGC AGT -------- ATA GGT GTG GAC AGG -------- TCC TAC GAG CAG TAC TTC **BJ2S7**
S S I G V D R S Y E Q Y F

14. AGC AGT T ----- CA GTG AGT ATA GAA CTC CTG AC ----- T GAG CAG TTC TTC **BJ2S1**
S S S V S I E L L T E Q F F

15. AGC AGT ----- CCA GTA GCT CTT GGA GCG GCG TAT AA ------ G CAG TAT TTT **BJ2S3**
S S P V A L G A A Y K Q Y F

16. AGC AGT ---------- CGA CAG AAC CA ---------- T GAA GCT TTC TTT **BJ1S1**
S S R Q N H E A F F

17. AGC AGT ---------- CTT CGG TGC AGG CAC TAT ---------- GAG CAG TAC TTC **BJ2S7**
S S L R C R H Y E Q Y F

18. AGC AGT T ---------- CA TTG CGG CC ---------- T GAG CAG TTC TTC **BJ2S1**
S S S L R P E Q F F

19. AGC AGT TTA G ------- GG TCC AAA AGG GAG -------- GAG ACC CAG TAC TTC **BJ2S5**
S S L G S K R E E T Q Y F

20. AGC AG ---------- A CCC CGG ACC TCG GGG CC ---------- G CAG TAC TTC **BJ2S7**
S R P R T S G P Q Y F

21. AGC AG ------- G CAG GAA GCG GGA GGC CGA -------- AAT GAG CAG TTC TTC **BJ2S1**
S S L E A G G R N E Q F F

22. AGC AGT TTA G --------- TG GGG AAC C --------- AT TCA CCC CTC CAC TTT **BJ1S6**
S S L V G N H S P L H F

23. AGC AGT TTA GC --- A ATA GGT AGC GGG GAC C --- CC TAC GAG CAG TAC TTC **BJ2S7**
S S L A I G S G D P Y E Q Y F

24. AGC AGT ---------- CCT AAA GCG C ---------- AC ACT GAA GCT TTC TTT **BJ1S1**
S S P K A H T E A F F

25. AGC AGT ---------- CGT AGC GGG AGT ---------- GAG ACC CAG TAC TTC **BJ2S5**
S S R S G S E T Q Y F

26. AGC AGT ---------- CCC TCG GGG ---------- GAG CAG TAC TTC **BJ2S7**
S S P S G E Q Y F

27. AGC AG ---------- G CTA CGG GAG CTT ---------- GAG ACC CAG TAC TTC **BJ2S5**
S S L R E L E T Q Y F

28. AGC AG -------- C AGC ATC GAC AGG A ---------- CC TAC GAG CAG TAC TTC **BJ2S7**
S S S I D R T Y E Q Y F

29. AGC AG ---------- C CTG ACA GGC ---------- AAT CAG CCC CAG CAT TTT **BJ1S5**
S S L T G N Q P Q H F

30. AGC AGT ---------- CAA GAG ACC CAG TAC TTC **BJ2S5**
S S Q E T Q Y F

31. AGC AG --------- C CTC TCG GCG GAC ACG TCC GAT ---------- TAC ACC TTC **BJ1S2**
S S L S A D T S D Y T F

32. AGC AGT TT ---------- G ACA ---------- GGA AAC ACC ATA TAT TTT **BJ1S3**
S S L T G N T I Y F

33. AGC AG ----- G CAT TAC ACC GGG ACA GGG ----- TAC AAT GAG CAG TTC TTC **BJ2S1**
S S H Y T G T G Y N E Q F F

34. AGC ---------- GTT CAG GCA GCG GG ---------- T TCA CCC CTC CAC TTT **BJ1S6**
S V Q A A G S P L H F

35. AGC AGT TTA GC ---------- G CTC TTG ---------- GAA AAA CTG TTT TTT **BJ1S4**
S S L A L L E K L F F

36. AGC AG ---------- G GGA TAC GG ---------- T GAG CAG TTC TTC **BJ2S1**
S S G Y G E Q F F

37. AGC AGT ------- ACA CAG GGG AGG ATA GG -------- C TAT GGC TAC ACC TTC **BJ1S2**
S S T Q G R I G Y G Y T F

38. AGC AGT --------- CTC TCC AGC GT ---------- C CAA GAG ACC CAG TAC TTC **BJ2S5**
S S L S S V Q E T Q Y F

39. AGC AGT TTA GC ------- G TCG AGT TCT CT ------- C TAT GGC TAC ACC TTC **BJ1S2**
S S L A S S S L Y G Y T F

40. AG ------- G CAG CAA GCA CAG GGG AGC -------- AAT TCA CCC CTC CAC TTT **BJ1S6**
S Q Q A Q G S N S P L H F

41. AGC AG --------- C TCG GGG TCC ---------- AGC ACA GAT ACG CAG TAT TTT **BJ2S3**
S S S G S S T D T Q Y F

42. AGC AGT TT --- T GTT ACT GGG ACA GGG GGT TTT ATC ---- GGC TAC ACC TTC **BJ1S2**
S S F V T G T G G F I G Y T F

43. AGC AGT ------- CCC GGC AGG GTC CTC C -------- CC TAC GAG CAG TAC TTC **BJ2S7**
S S P G R V L P Y E Q Y F

44. AGC AG ---------- C CCG GGA CAG GGG GAA ---------- GGC TAC ACC TTC **BJ1S2**
S S P G Q G E G Y T F

45. AGC AGT T --------- CC CTC CGG CGG GC --------- C GGG GAG CTG TTT TTT **BJ2S2**
S S S L R R A G E L F F

46. AGC AG ----- A AAC AGG GTA TCG GCT ------ AGC ACA GAT ACG CAG TAT TTT **BJ2S3**
S S N R V S A S T D T Q Y F

47. AGC AGT TT --------- C TGG GGG TGG AAA ---------- ACT GAA GCT TTC TTT **BJ1S1**
S S F W G W K T E A F F

48. AGC AGT --------- ACG CGG TCA GGG CTT AT ---------- T GGC TAC ACC TTC **BJ1S2**
S S T R S G L I G Y T F

49. AGC ---------- GGT ATT GAC AGG GT ---------- A AAC ATT CAG TAC TTC **BJ2S4**
S G I D R V N I Q Y F

50. AGC AGT TT --------- C CAG GGG TGG AC --------- C GGG GAG CTG TTT TTT **BJ2S2**
S S L Q G W T G E L F F

V. Sequence of $CD8^+$ BV8S1 T cell receptors

V region *N(D) region* *J region*

1. AGC AGT TTA G --------- GT TGC ACC ACG AGG ---------- GGC TAC ACC TTC **BJ1S2**
S S L G C T T R G Y T F

2. AGC AGT ---------- CAG CAG GAT ---------- GAG ACC CAG TAC TTC **BJ2S5**
   S S L Q Q D E T Q Y F

3. AGC AGT TTA G ---------- GT GCA CGA CGA ---------- GGC TAC ACC TTC **BJ1S2**
   S S L G A R R G Y T F

4. AGC AG ---------- A GAC GGT CT ---------- C TCC TAC GAG CAG TAC TTC **BJ2S7**
   S S D G L S Y E Q Y F

5. AGC AGT ---------- ATG TTG CC ---------- C AAT CAG CCC CAG CAT TTT **BJ1S5**
   S S M L P N Q P Q H F

6. AGC AGT T ---------- AC AGC AAT N ---------- AG ACC CAG TAC TTC **BJ2S5**
   S S Y S N X T Q Y F

7. AGC AGT TT ------- T ATC GAG ACA -------- AGC ACA GAT ACG CAG TAT TTT **BJ2S3**
   S S F I E T S T D T Q Y F

8. AGC ---------- GCA GGG GGA GG ---------- C TCC TAC GAG CAG TAC TTC **BJ2S7**
   S A G G G S Y E Q Y F

9. AGC ---------- GCA GGG GGA GG ---------- C TCC TAC GAG CAG TAC TTC **BJ2S5**
   S A G G G Q E T Q Y F

10. AGC AG ---------- A TAC GAC AGG GTG AT ---------- A GCT TTC TTT **BJ1S1**
    S S Y D R V I A F F

11. AGC AGT TTA G ----- GG CTA GCG GG ----- C TCC TAC AAT GAG CAG TTC TTC **BJ2S1**
    S S L G L A G S Y N E Q F F

12. AGC AGT T ---------- AC AGG GAT TG ---------- G ACC CAG TAC TTC **BJ2S5**
    S S Y R D W T Q Y F

13. AGC AGT TT ------- T AGG CTC AGG AGA -------- AAC TAT GGC TAC ACC TTC **BJ1S2**
    S S F R L R R N Y G Y T F

14. AGC A ------- TC CAC GGA CCA TTA TA ------- C ACA GAT ACG CAG TAT TTT **BJ2S3**
    S I H G P L Y T D T Q Y F

15. AGC AGT TTA ------ TTA TGG AGT CTG ------ AGC ACA GAT ACG CAG TAT TTT **BJ2S3**
    S S L L W S L S T D T Q Y F

16. AGC AGT TTA GC ---------- C AGG GAT N ---------- AG ACC CAG TAC TTC **BJ2S5**
    S S L A R D X T Q Y F

17. AGC AGT TT ---------- G AGT GGA AAT ---------- AAT GAG CAG TTC TTC **BJ2S1**
    S S L S G N N E Q F F

18. AGC AG ---------- C ATC A ---------- AG ACC CAG TAC TTC **BJ2S5**
    S S I K T Q Y F

19. AGC AGT TTA GC ----- A ATT AGC TAC CAA ------ TAC AAT GAG CAG TTC TTC **BJ2S1**
    S S L A I S Y Q Y N E Q F F

20. AGC AGT TTA G ---------- AC TTG ---------- CAA GAG ACC CAG TAC TTC **BJ2S5**
    S S L D L Q E T Q Y F

21. AGC AGT TTA GC ---------- G ACT A ---------- AA GAG ACC CAG TAC TTC **BJ2S5**
    S S L A T K E T Q Y F

22. AGC AG ---------- C TAT TGG ACG CG ---------- C AAT GAG CAG TTC TTC **BJ2S1**
    S S Y W T R N E Q F F

23. AGC AGT TTA ----- CAG TTC CGC GGG GAT GAA GC ------ T ACG CAG TAT TTT **BJ2S3**
    S S L Q F R G D E A T Q Y F

24. AGC AGT T ---------- CG ACA GGG GGC TG ---------- T ACG CAG TAT TTT **BJ2S3**
    S S S T G G C T Q Y F

25. AGC AG --------- G TGG GGG GGC AGG GG --------- C ACT GAA GCT TTC TTT **BJ1S1**
    S S W G G R G T E A F F

26. AGC AGT ---------- CCC TCA GGG ---------- AGC AAT CAG CCC CAG CAT TTT **BJ1S5**
    S S P S G S N Q P Q H F

27. AGC AGT TTA ---------- ATC GGC TT ---------- T GAA GCT TTC TTT **BJ1S1**
    S S L I G F E A F F

28. AGC AGT ---------- CTG CAG GGG TTT A ---------- AC GAG CAG TAC TTC **BJ2S7**
S S L Q G F N E Q Y F

29. AGC AGT T ------- CG ACA GGG GGC TG ------- G AAC ACT GAA GCT TTC TTT **BJ1S1**
S S X T G G W N T E A F F

30. AGC AG ------- C CGG ACT AGC GAG GGA CTA GG ------- T GAG CAG TTC TTC **BJ2S1**
S S R T S E G L G E Q F F

31. AGC AGT TTA GC ------- G TAT AGG ATC -------- AAC ACT GAA GCT TTC TTT **BJ1S1**
S S L A Y R I N T E A F F

32. AGC AGT T --------- CA GAG GGT C --------- TG AAC ACT GAA GCT TTC TTT **BJ1S1**
S S X E G L N T E A F F

33. AGC AGT TT ---------- G AGT ---------- TAC GAG CAG TAC TTC **BJ2S7**
S S L S Y E Q Y F

34. AGC AGT TTA ---------- NNN NGT GGA ACT ATT A --------- GC TAC ACC TTC **BJ1S2**
S S L X X G T I S Y T F

VI. Sequence of $CD4^+$ BV9S1 T cell receptors

V region *N(D) region* *J region*

1. AGC AGC CAA G ----- TC GCG TTT GGC GCT ------ CAA GAG ACC CAG TAC TTC **BJ2S5**
S S Q V A F G A Q E T Q Y F

2. AGC AGC CAA GA ------- G GGT GAT AG ------- C TCC TAC GAG CAG TAC TTC **BJ2S7**
S S Q E G D S S Y E Q Y F

3. AGC AGC C --------- GA ACA GGG G --------- AC CGA GAG ACC CAG TAC TTC **BJ2S5**
S S R T G D R E T Q Y F

4. AGC AGC CAA GA --------- A ACG AGG GGA TCC ---------- GGC TAC ACC TTC **BJ1S2**
S S Q E T R G S G Y T F

5. AGC AGC C ----- CG GGA TAT CCC CTA AG ----- C TCC TAC GAG CAG TAC TTC **BJ2S7**
S S L G Y P L S S Y E Q Y F

6. AGC AGC CAA G ---------- CG TCG AGG ---------- GGA AAA CTG TTT TTT **BJ1S4**
S S Q A S R G K L F F

7. AGC AGC CAA GA ------- C GT ------- C TCT GGG GCC AAC GTC CTG ACT TTC **BJ2S6**
S S Q D V S G A N V L T F

8. AGC AGC CAA GA --- A AGG GGT AGC GGA GTC CC --- C GGG GAG CTG TTT TTT **BJ2S2**
S S Q E R G S G V P G E L F F

9. AGC AGC C --- CC CTC GAT AGC GGG AGT CT --- C TAC AAT GAG CAG TTC TTC **BJ2S1**
S S P L D S G S L Y N E Q F F

10. AGC AGC CA ---------- C CAG GGG A ---------- CC TAC GAG CAG TAC TTC **BJ2S7**
S S H Q G T Y E Q Y F

11. AGC AGC CAA G --------- GG GGA CTC CTT GGG ---------- GAG CAG TAC TTC **BJ2S7**
S S Q G G L L G E Q Y F

12. AGC AGC C --------- CG GAC TCC TTC C --------- AC AAT GAG CAG TTC TTC **BJ2S1**
S S P D S F H N E Q F F

13. AGC AGC CAA G ------- CG GCT AGC GGA GG ------- C TAC GAG CAG TAC TTC **BJ2S7**
S S Q A A S G G Y E Q Y F

14. TGT GCC A ------- TT GGG GAC ACC -------- ACT AAT GAA AAA CTG TTT TTT **BJ1S4**
C A I G D T T N E K L F F

15. AGC AGC C ---------- CA GGG GT ---------- C TAC GAG CAG TAC TTC **BJ2S7**
S S P G V Y E Q Y F

16. AGC A -------- CC CTG AGG ACT AGC GGG GGT GGG ------- GAG CAG TAC TTC **BJ2S7**
S T L R T S G G G E Q Y F

17. AGC AGC CA ---------- G GA ---------- A GAT ACG CAG TAT TTT **BJ2S3**
S S Q E D T Q Y F

18. AGC AG ---------- G GGA CAG CCT C ---------- AT GGC TAC ACC TTC **BJ1S2**
S S G Q P H G Y T F

19. AGC AGC ---------- TTC CGG GGT GG ---------- C GAG CAG TAC TTC **BJ2S7**
S S F R G G E Q Y F

20. AGC AGC CA ---------- C CGG CGG ---------- AAT CAG CCC CAG CAT TTT **BJ1S5**
S S H R R N Q P Q H F

21. AGC AGC CAA GA --- T TTC ACG GGG CA --- T AGC AAT CAG CCC CAG CAT TTT **BJ1S5**
S S Q D F T G H S N Q P Q H F

22. AGC A ------- CC CTG AGG ACT AGC GGG GGT GGG -------- GAG CAG TAC TTC **BJ2S7**
S T L R T S G G G E Q Y F

23. AGC AGC C --------- CG GAC TCC TTC C --------- AC AAT GAG CAG TTC TTC **BJ2S1**
S S P D S F H N E Q F F

24. AGC AGC CAA GA ------- C GT ------- C TCT GGG GCC AAC GTC CTG ACT TTC **BJ2S6**
S S Q D V S G A N V L T F

25. AGC AGC CAA GA --------- A ACG AGG GGG ATC ---------- GGC TAC ACC TTC **BJ1S2**
S S Q E T R G I G Y T F

26. AGC AGC CA ---------- C CAG GGG A ---------- CC TAC GAG CAG TAC TTC **BJ2S7**
S S H Q G T Y E Q Y F

VII. Sequence of $CD8^+$ BV9S1 T cell receptors

V region *N(D) region* *J region*

1. AGC AGC C --------- GC ACA AGG TCA T --------- AC TAT GGC TAC ACC TTC **BJ1S2**
S S R T R S Y Y G Y T F

2. AGC AGC C --- CG AGA CTC GGA CTA GCG AGG G --- AC AAT GAG CAG TTC TTC **BJ2S1**
S S P R L G L A R D N E Q F F

3. AGC AGC CAA GA ------- T TGG AGG GCC TCG G ------- CT GAA GCT TTC TTT **BJ1S1**
S S Q D W R A S A E A F F

4. AGC AGC CAA G ------- GA GCG GGG GGA G ------- CA GAT ACG CAG TAT TTT **BJ2S3**
S S Q G A G G A D T Q Y F

5. AGC AGC CAA -- AGG GGG GGG CGG ---------- CCA AGA GAC CCA GTA CTT **BJ2S5**
S S Q R G G R Q E T Q Y F

6. AGC AGC CAA --------- ACA GGG GCC G ---------- AT CAG CCC CAG CAT TTT **BJ1S5**
S S Q T G A D Q P Q H F

7. AGC AGC C --------- CT TTG GAT TGG ---------- CAA GAG ACC CAG TAC TTC **BJ2S5**
S S P L D W Q E T Q Y F

8. AGC AGC ---------- GTA GAC AC ---------- C AAT CAG CCC CAG CAT TTT **BJ1S5**
S S V D T N Q P Q H F

9. AGC AGC CAA GA --- T GGG AGG GAC TT --- G AAC AGC GGG GAG CTG TTT TTT **BJ2S2**
S S Q D G R D L N T G E L F F

10. AGC AGC C ---------- TC CGC TTT CAG ---------- GAG CAG TTC TTC **BJ2S1**
S S L R F Q E Q F F

11. AGC AGC CAA G ------- CC TTG ACT AGC GGA GG ------- G ACC CAG TAC TTC **BJ2S5**
S S Q A L T S G G T Q Y F

12. AGC AGC C ------- CT ACT AGC GGG AGT ATC T ------- AT GAG CAG TTC TTC **BJ2S1**
S S P T S G S I Y E Q F F

13. AGC AGC C ---------- CA ACC TCG GGG ---------- GAT ACG CAG TAT TTT **BJ2S3**
S S P T S G D T Q Y F

14. AGC AGC CAA G --------- TC TCT AAG GTG G --------- AT GAG CAG TTC TTC **BJ2S1**
S S Q V S K V D E Q F F

15. AGC AGC C --- CG ATA CCC GGC CAC CCC CAG GGG TCG GG --- A GCT TTC TTT **BJ1S1**
S S P I P G H P Q G S G A F F

16. AGC AGC CA ---- C TTG TCC GGC GGG AGG --- AGC ACA GAT ACG CAG TAT TTT **BJ2S3**
S S H L S G G R S T D T Q Y F

17. AGC AGC CAA GA - T TGG AGC GGG GGT AGG A ---------- TC **BJ2S1**
S S Q D W S G G R I

18. AGC AGC CAA GA --- T AGG TGG GGA AGC GGG G --- AC ACT GAA GCT TTC TTT **BJ1S1**
S S Q D R W G S G D T E A F F

19. AGC AGC CA ------- G GGA GGG GCT G ------- GC AAT CAG CCC CAG CAT TTT **BJ1S5**
S S Q G G A G N Q P Q H F

20. AGC -- CTT TTC CAA CCG GAC AGG GGC TCG C - AC AGC GGG GAG CTG TTT TTT **BJ2S2**
S L F Q P D R G S H T G E L F F

21. TGT GCC A --------- CC CAG GAA GG --------- C TAC AAT GAG CAG TTC TTC **BJ2S1**
S S T Q E G Y N E Q F F

22. AGC AGC CAA GA --- T GGG GCA GGG GGC GTC AGG ---- ACT GAA GCT TTC TTT **BJ1S1**
S S Q D G A G G V R T E A F F

23. AGC AG ---------- G CTA GCG GGG GGG CGG GAT --------- GAG CAG TAC TTC **BJ2S7**
S R L A G G R D E Q Y F

24. AGC AGC C ---------- CA CCG ACA AGT --------- CAA GAG ACC CAG TAC TTC **BJ2S5**
S S P P T S Q E T Q Y F

25. AGC AGC CA ---------- C AGG GAA AC ---------- C TAT GGC TAC ACC TTC **BJ1S2**
S S H R E T Y G Y T F

26. AGC AGC C --- CA ACC AAG GGG GGC TGG AAT GC --- A GAG ACC CAG TAC TTC **BJ2S5**
S S P T K G G W N A E T Q Y F

27. AGC AGC -------- GAC AAG AGG ACG GCT CCG -------- TAT GGC TAC ACC TTC **BJ1S2**
S S D K R T A P Y G Y T F

28. AGC AGC CAA GA - A AGT CAA GGA CAA CCA TCC CGG T - AT ACG CAG TAT TTT **BJ2S3**
S S Q E S Q G Q P S R Y T Q Y F

29. AGC AGC CAA ---------- TAC AGG GG ---------- C TAC GAG CAG TAC TTC **BJ2S7**
S S Q Y R G Y E Q Y F

30. AGC AGC ---------- NNN NNN NNN GCG GGT NT --------- T GAG CAG TTC TTC **BJ2S1**
S S X X X A G X E Q F F

31. AGC AGC CAA GA --- T CGG GAG GCG CGA TC --- C CAA GAG ACC CAG TAC TTC **BJ2S5**
S S Q D R E A R S Q E T Q Y F

VIII. Sequence of $CD4^+$ BV12S2 T cell receptors

V region *N(D) region* *J region*

1. ATC AGT GA --------- A CAG GCC ---------- AGC ACA GAT ACG CAG TAT TTT **BJ2S3**
I S E Q A S T D T Q Y F

2. ATC AGT GA --------- G GGA CAG GGC TC --------- C ACT GAA GCT TTC TTT **BJ1S1**
I S E G Q G S T E A F F

3. ATC AGT G ------- TT GGA CTA GCG GGA ACG -------- AAT GAG CAG TTC TTC **BJ2S1**
I S V G L A G T N E Q F F

4. ATC AG ---------- C CTG GAA GGC AGG ---------- ACT GAA GCT TTC TTT **BJ1S1**
I S L E G R T E A F F

5. ATC AGT GAG TC ----- G GGG GAG GGC CGT ------ CAA GAG ACC CAG TAC TTC **BJ2S5**
I S E S G E G R Q E T Q Y F

6. ATC A - CC AAA CAG GGG TTA CGA CGG AG - C TCC TAC AAT GAG CAG TTC TTC **BJ2S1**
   I S K Q G L R R S S Y N E Q F F

7. ATC AGT GAG TC --- A GGG ACA GGG GGT AA --- C TAC AAT GAG CAG TTC TTC **BJ2S1**
   I S E S G T G G N Y N E Q F F

8. ATC AGT GA ---------- A GGT GT ---------- C TCC TAC GAG CAG TAC TTC **BJ2S7**
   I S E G V S Y E Q Y F

9. ATC AGT GAG TC --- G GGG AAA CAG AGC C --- CC TAC AAT GAG CAG TTC TTC **BJ2S1**
   I S E S G K Q S P Y N E Q F F

10. ATC AGT G --------- TT GGT AGC GGA GT --------- C AAT GAG CAG TTC TTC **BJ2S1**
    I S V G S G V N E Q F F

11. ATC AGT GA ----- C GGT GGG GGG GAG GGA ------ TAC AAT GAG CAG TTC TTC **BJ2S1**
    I S D G G G E G Y N E Q F F

12. ATC AGT GA ------- C TTG GCT AGC CAT -------- AAC ACT GAA GCT TTC TTT **BJ1S1**
    I S D L A S H N T E A F F

13. ATC A ------- CC GAG GGA CAG GGG AAA GCG -------- AAT GAG CAG TTC TTC **BJ2S1**
    I S E G Q G K A N E Q F F

14. ATC AGT GA --------- T TTG ACA GCG AC --------- C GGG GAG CTG TTT TTT **BJ2S2**
    I S D L T A T G E L F F

15. ATC AG ------- C CAA GTC GCA GGT TCT -------- AAT CAG CCC CAG CAT TTT **BJ1S5**
    I S Q V A G S N Q P Q H F

16. ATC AGT GAG T ---------- NN NNN NNN N ---------- AT GAG CAG TTC TTC **BJ2S1**
    I S E X X X E Q F F

17. ATC AGT G --------- TT CCG GGA CAG GAG ---------- TAT GGC TAC ACC TTC **BJ1S2**
    I S V P G Q E Y G Y T F

18. ATC AGT GA --- A TTT AAG TCT AAA AAC AGG GCT C --- AC GAG CAG TAC TTC **BJ2S7**
    I S E F K S K N R A H E Q Y F

19. ATC AGT GA ------- C TAC GGC CCG GGT TAT G ------- AG CCC CAG CAT TTT **BJ1S5**
    I S D Y G P G Y E P Q H F

20. TGT GCC ----- GTG GAT CAA TTG ACA GCG AC ------ C GGG GAG CTG TTT TTT **BJ2S2**
    C A V D Q L T A T G E L F F

21. ATC AGT GAG TC - T CGG ACA CAA CTC CTT TGG C - CT GGA AAC ACC ATA TAT TTT **BJ1S3**
    I S E S R T Q L L W P G N T I Y F

22. ATC AG ---------- G GGA GGG GCC GCC G ---------- AC GAG CAG TAC TTC **BJ2S7**
    I R G G A A D E Q Y F

23. ATC ----- TCG GAA GGG ATA CT ----- C TCT GGG GCC AAC GTC CTG ACT TTC **BJ2S6**
    I S E G I L S G A N V L T F

24. ATC AGT GAG TC --------- G GTA GCG GGA GG --------- T GAG CAG TTC TTC **BJ2S1**
    I S E S V A G G E Q F F

25. ATC AGT GA -------- T GCG GGA GGG C ---------- CC TAC GAG CAG TAC TTC **BJ2S7**
    I S D A G G P Y E Q Y F

26. ATC AGT GAG TC --------- A GCG GGG AGG C --------- AC GAG CAG TAC TTC **BJ2S7**
    I S E S A G R H E Q Y F

27. ATC AGT ---------- CAG CAG G ---------- GC AAT CAG CCC CAG CAT TTT **BJ1S5**
    I S Q Q G N Q P Q H F

28. ATC AGT GAG --------- GTA GTC GCC ANN CG ---------- T GAG CAG TTC TTC **BJ2S1**
    I S E V V A X R E Q F F

29. ATC AGT GAG - CTA GGA CTA GCG GAG GCG CAG GAG TAC GAG CAG - TAC GAG CAG TAC TTC **BJ2S7**
    I S E L G L A E A Q E Y E Q Y E Q Y F

30. ATC ---------- GGG ACA GGG ATC GG ---------- C GAG CAG TAC TTC **BJ2S7**
    I G T G I G E Q Y F