

Defining Novel Targets for Immunotherapy of Human Cancer

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Dedicated to the memory of my dearly beloved mum

Adélia Nunes

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Summary

Cancer cells frequently exhibit defects in apoptosis, contributing to increased survival and resistance to chemotherapy. This can result from decreased expression of pro-apoptotic proteins such as Bax, through abnormal proteasomal degradation. The central hypothesis of this project is that this abnormal proteasomal degradation in cancer cells will result in the generation of peptides that bind to MHC class I molecules, and displayed at the cell surface for CD8⁺ T cell recognition. If this is correct, then T cells directed against Bax peptides should be able to recognize and kill human cancer cells.

To test this hypothesis, candidate HLA-A2 binding peptide epitopes were identified from Bax and T cell immunogenicity tested using IFN γ ELISpot assays. Positive T cell responses were detected against Bax peptides in 10/16 healthy donors. The specificity of a CD8⁺ T cell clone (KSIVB17) derived from one donor, was mapped to two similar peptide epitopes, Bax₁₃₆₋₁₄₄ (IMGWTLDFL) and Bax₁₃₅₋₁₄₄ (TIMGWTLDFL). In addition to recognition of peptide pulsed target cells, this clone was also able to recognise primary tumour cells from CLL (chronic lymphocytic leukaemia) patients, and kill cervical carcinoma and osteosarcoma cell lines. T cell reactivity correlated with abnormal proteasomal degradation of Bax in cancer cells.

Attempts to generate Bax-specific T cells in CLL patients demonstrated that naïve and memory T cell responses were compromised compared to healthy donors. This compromised T cell immunity appeared to result from increased expression of immunosuppressive molecules, increased frequency of Treg cells and a skewed memory T cell phenotype in CLL patients.

Overall, this research supports the novel concept that proteins such as Bax, which are abnormally degraded in cancer cells, can be targets for CD8⁺ T cells. However use of Bax in immunotherapy will require strategies to overcome suppression of T cell responses in CLL and other cancers.

Abbreviations

aa	Amino acid
ACT	Adoptive cell transfer
ADCC	Antibody dependent cellular cytotoxicity
AML	Acute myelogenous leukaemia
AP	Alkaline phosphatase
Apaf-1	Apoptosis-activating factor-1
APC	Antigen presenting cell
β 2m	β 2-microglobulin
B-CLL	B-cell chronic lymphocytic leukaemia (also known as CLL)
BH	Bcl-2 homology
BiTE	Bispecific T cell engagers
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDR	Complementarity determining region
CEA	Carcino-embryonic antigen
CIN	Cervical intraepithelial neoplasia
CLL	Chronic lymphocytic leukaemia (B-CLL)
CM	Central memory (T_{CM})
CMV	Cytomegalovirus
Cr	Chromium-51
C:T	Cold target: hot target
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DD	Death domain
DISC	Death inducing signal complex
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetra acetic acid
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunoabsorbent assay
ELISpot	Enzyme-linked immunospot assay
EM	Effector memory (T_{EM})
EMRA	Effector (T_{EMRA})
ER	Endoplasmic reticulum
E:T	Effector:Target
FACS	Fluorescence-activated cell sorter
FADD	Fas-associated death domain protein
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HCV	Hepatitis C virus
HD	Healthy donor
HPLC	High-performance liquid chromatography
HPV	Human Papillomavirus
HSP	Heat shock protein
hTERT	Human telomerase reverse transcriptase

ICAM	Intercellular adhesion molecule
ICOS	Inducible costimulator
ICS	Intracellular cytokine staining assay
IDO	indoleamine 2,3-dioxygenase and galectin-1
IL	Interleukin
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IAPs	Inhibitor of apoptosis proteins
ITAM	Immunoreceptor tyrosine-based activation motif
LAMP	Lysosomal-associated membrane protein
LFA	Lymphocyte function associated antigen
MAGE	Melanoma antigen genes
MESNA	Sodium 2-mercaptoethanesulfonate
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MICA/B	MHC-class I polypeptide related sequence A/B
MIIC	MHC class II compartment
NK	Natural killer cell
NKG2D	Natural-killer group 2, member D
NKT	Natural killer T cell
PAF	Paraformaldehyde
PAMPs	Pathogen-associated molecular patterns
PAP	Prostatic acid phosphatase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCa	Prostate cancer
PCR	Polymerase Chain Reaction
PD-1	Programmed death-1
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PMA	Phorbol myristate acetate
PPP	Positive peptide pool
PRR	Pattern recognition receptors
PSA	Prostate-specific antigen
PSCA	Prostate stem cell antigen
PSMA	Prostate specific membrane antigen
PTH-rp	Parathyroid hormone-related protein
PVDF	Polyvinylidene difluoride
RAE1	Retinoic acid early transcript 1
RAG	Recombinase activating genes
RECIST	Response Evaluation Criteria In Solid Tumours
RNA	Ribonucleic acid
SCT	Stem cell transplantation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFC	Spot forming cell
SMACs	Supramolecular activation clusters
STEAP	Six-transmembrane epithelial antigen of the prostate
TAA	Tumour-associated antigen
TAP	Transporter associated with antigen processing
TCR	T-cell Receptor
TGFβ	Transforming growth factor-β

Th	T-helper
TILs	Tumour infiltrating lymphocytes
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNFR-associated death domain
TRAIL	TNF related apoptosis inducing ligand
TSA	Tumour-specific antigen
Treg	T regulatory cell
VEGF	Vascular endothelial growth factor
WT1	Wilm's Tumour 1
ZAP-70	ζ -associated protein 70

Contents

	Page
Chapter 1 Introduction	1
1.1 Cancer	1
1.2 The immune system	1
1.2.1 Innate immunity	2
1.2.2 Adaptive immunity	4
1.2.2.1 Humoral immunity	4
1.2.2.2 Cell-mediated immunity	5
<i>APCs in priming T cell responses</i>	5
<i>CD4+ T cells and their effector subsets</i>	7
<i>CD8+ T cells and their cytotoxicity</i>	9
<i>T regulatory cells</i>	12
<i>Memory T cells</i>	13
1.3 MHC and antigen presentation	17
1.3.1 MHC class I structure and function	17
1.3.1.1 Structure	17
1.3.1.2 Antigen processing and presentation	18
<i>Degradation of cytosolic proteins</i>	18
<i>Peptide translocation into the ER</i>	20
<i>Assembly of MHC class I complex</i>	20
<i>Cross-priming</i>	21
1.3.2 MHC class II structure and function	24
1.3.2.1 Structure	24
1.3.2.2 Antigen processing and presentation	24
<i>Uptake of extracellular proteins and processing</i>	24
<i>Synthesis and trafficking of the MHC class II molecule</i>	25
1.3.3 The HLA genes	25
1.4 The T cell receptor (TCR)	26
1.4.1 TCR structure	26
1.4.2 T cell activation	27
1.5 Tumour Immunity	30
1.5.1 Tumour antigens	31

1.5.2 Immunity to tumours	35
1.5.3 Tumour escape	38
1.6 Tumour immunotherapy	40
1.6.1 Monoclonal antibodies	41
1.6.2 Cytokines	43
1.6.3 Prophylactic and Therapeutic Vaccines	44
1.6.4 Adoptive cell transfer	49
1.7 Prostate cancer (PCa)	54
1.8 B-cell chronic lymphocytic leukaemia (B-CLL/CLL)	56
1.9 Apoptosis	59
1.9.1 Pathways	60
1.9.1.1 Extrinsic pathway	61
1.9.1.2 Intrinsic pathway	63
1.9.2 Pro-apoptotic Bax protein and its dysregulation in cancer	64
1.10 Aims of project	67
Chapter 2 Material & Methods	69
2.1 Tissue culture basics	69
2.1.1 Tissue culture media and buffers	69
2.1.2 Tissue culture plastics	70
2.1.3 Cell viability counting	70
2.1.4 Routine maintenance of cell lines	70
2.2 Peptide Antigens	72
2.2.1 Positive peptide pool (PPP)	72
2.2.2 MART-1/Melan-A peptide	73
2.2.3 Bax peptide pools	74
2.3 Peptide binding assay	76
2.4 Blood origin and preparation	76
2.4.1 Blood donors	76
2.4.2 Isolation of peripheral blood mononuclear cells (PBMC)	77
2.5 Primary PBMC /T cell cultures	77
2.5.1 Short-term <i>in vitro</i> culture of PBMC/T cells with peptides	77
2.5.2 Cryopreservation of PBMC	78
2.5.3 Restimulation of PBMC/T cell cultures	78

2.5.4	Enrichment of CD8+ T cells from PBMC using specific microbeads	78
2.6	Detection of peptide-specific T cells	79
2.6.1	IFN γ ELISpot assay	79
2.6.2	IFN γ -Intracellular Cytokine Staining (ICS) analysis	82
2.6.3	CD107 assay	82
2.6.4	CD137 assay	83
2.7	Expansion of T cells using an allogenic feeder system	83
2.8	Enrichment of peptide specific T cells using CD107 or CD137 assay	84
2.9	Enrichment of IFN γ -secreting T cells using magnetic beads	84
2.10	Generating peptide-specific T cell lines	86
2.10.1	Culture of enriched peptide-specific T cell population	86
2.10.2	Generation of T cell lines from peptide specific T cells under limiting dilution conditions	86
2.10.3	Selection of peptide-specific T-cell lines from cloning plates	87
2.11	Chromium-51 (^{51}Cr) release cytotoxicity assays	87
2.11.1	Cold-target competition assay	89
2.11.2	Antibody-blocking assay	89
2.12	IFN γ treatment of target cells	90
2.13	Treatment of cells with proteasome inhibitors	90
2.14	Flow cytometric analysis and monoclonal antibodies	90
2.14.1	Monoclonal antibodies	91
2.14.2	Single colour immunofluorescence staining	93
2.14.3	Multicolour immunofluorescence staining	93
2.14.4	Human Foxp3 staining	94
2.15	Western blotting	95
2.15.1	Buffers	95
2.15.2	Generation of total cell lysates	95
2.15.3	SDS-PAGE	96
2.15.4	Transfer onto PVDF membranes	96
2.15.5	Immunodetection of protein on PVDF blots	97
2.16	Statistical analysis	97

Chapter 3 Results	98
Generation of CD8+ T cell Responses against Bax derived peptides in healthy donors	
3.1 Defining CD8+ T cell responses against Bax pool 1-15	100
3.1.1 Peptide binding to HLA-A2 on the T2 cell line	100
3.1.2 Donor responses	102
3.2 Generation of a Bax-specific polyclonal T cell line using CD107 assay	106
3.2.1 Mapping epitopes from Bax pool 1-15 responses using the JSB line	108
3.2.2 Loss of Bax peptide specificity through repeated expansions of the JSB line	110
3.3 Defining CD8+ T cell responses against Bax pool 601-23	112
3.3.1 Peptide binding to HLA-A2 on the T2 cell line	114
3.3.2 Donor responses	116
3.4 Testing different protocols for the detection of peptide specific T cells	118
3.5 Generation of T cell lines against Bax pool 601-23 using IFN γ secretion assay	121
3.5.1 Enrichment of IFN γ -secreting cells from donor 11 using magnetic-based cell sorting	121
3.5.2 Generation of KSI line through the antigen-independent expansion of IFN γ -enriched T cells	124
3.5.3 Generation of Bax-specific KSI 10B7 T cells using the limiting dilution protocol	124
3.6. Generation of T cell lines from JSB cells using IFN γ secretion assay	126
3.6.1 Enrichment of IFN γ -secreting cells from JSB cells using magnetic-based cell sorting	126
3.6.2 Generation of JSBI line through the antigen-independent expansion	128
3.6.3 Generation of Bax-specific JSBI 10B6 T cells using the limiting dilution protocol	128
3.7 Discussion	130

Chapter 4 Results	138
Characterisation of CTL generated against Bax derived peptides	
4.1 Characterisation of Bax-specific T cells derived from the JSB line	138
4.1.1 Epitope recognition by JSBI line using an ELISpot assay	138
4.1.2 Epitope recognition by JSBI 10E6 using an ELISpot assay	139
4.1.3 Analysis of the TCR V β chains used by Bax-specific polyclonal T cell lines	139
4.1.4 JSBI 10E6 is able to lyse peptide-pulsed targets	142
4.2 Characterisation of Bax-specific T cell lines derived from donor 11 using Bax pool 601-23	144
4.2.1 Mapping epitopes from Bax peptide pool 601-23 responses using KSI line	144
4.2.2 Mapping epitopes from Bax peptide pool 601-23 responses using KSI 10B7	146
4.2.3 Analysis of the TCR V β chains used by Bax-specific polyclonal T cell lines	148
4.2.4 KSI line and KSI 10B7 are able to lyse target cells pulsed with Bax pool 601-23	150
4.3 Generation of TCR V β 5.1+ and V β 17+ T cells from KSI 10B7	153
4.4 Avidity of KSIVB17 CTL for Bax p610 and p613	156
4.5 Recognition of tumour cells by KSIVB17 CTL	158
4.5.1 Pca cell lines	158
4.5.2 Lymphoid malignancies	160
4.5.3 HPV-transformed cervical cancer cell lines	162
4.5.4 Osteosarcoma cell lines	164
4.5.5 Other cancer cell types	164
4.5.6 Negative controls	167
4.5.7 Summary of targets	167
4.6 Specificity of KSIVB17 clone	170
4.7 The effect of proteasome inhibition on MHC class I presentation of Bax peptides to KSIVB17 clone	176
4.8 Discussion	178

Chapter 5 Results	185
Analysis of Bax expression in cancer cells	
5.1 Bax expression in human PCa cell lines	186
5.2 Bax expression in CLL patients	186
5.3 Bax expression in cervical cancer cell lines	189
5.4 Bax expression in osteosarcoma cell lines	191
5.5 Bax expression in normal (non-cancerous) cells	191
5.6 Discussion	194
 Chapter 6 Results	 198
Generation of T cell Responses in CLL patients	
6.1 T cell responses against PPP	199
6.2 T cell responses against MART-1	202
6.3 T cell responses against Bax peptides`	205
6.4 Discussion	206
 Chapter 7 Results	 211
Immunophenotyping of CLL patients	
7.1 Frequencies of peripheral blood lymphocyte populations	212
7.2 Expression of immunosuppressing molecules CD200, CD200R and PD-1	215
7.3. CD3+ T cells	221
7.3.1 CD8+ T cells	223
7.3.2 CD4+ T cells	228
7.4 Discussion	234
 Chapter 8 Final Discussion	 241
 Bibliography	 251

List of Figures

	Page
Chapter 1	
Figure 1.1 Differentiation of T helper cells	8
Figure 1.2 Effector functions of CTLs	11
Figure 1.3 Differentiation from naïve to memory T cells	15
Figure 1.4 Structure of MHC class I and MHC class II molecules	22
Figure 1.5 MHC class I and II pathways	23
Figure 1.6 Structure of the TCR	28
Figure 1.7 Extrinsic and Intrinsic apoptotic pathways	62
Figure 1.8 Cancer cells and dysregulation of anti-apoptotic and pro-apoptotic proteins	66
Figure 1.9 Schematic overview of the main hypothesis of the project: Degradation of Bax protein in healthy & cancer cells	68
Chapter 2	
Figure 2.1 Detection of peptide specific T cells by IFN γ ELISpot assay	81
Chapter 3	
Figure 3.1 General plan for the generation of Bax specific T cells	99
Figure 3.2 Binding of candidate peptides from Bax (pool 1-15) to HLA-A2	101
Figure 3.3 Bax T cell responses from healthy donors after 2 to 4 weeks of stimulation (Bax pool 1-15)	103
Figure 3.4 Bax T cell response from donor 1 after 5 weeks of stimulation	105
Figure 3.5 The approach used to derive a Bax peptide specific T cell line (JSB line) by surface expression of CD107a	107
Figure 3.6 Mapping the Bax response from the JSB line to split pools and individual peptides	109
Figure 3.7 Demonstration of Bax specificity loss of the JSB line after repeated expansions using CD107 assay	111
Figure 3.8 Binding of candidate peptides from Bax (pool 601-23) to HLA-A2	115
Figure 3.9 Bax T cell responses from healthy donors after 2 to 4 weeks of stimulation (Bax pool 601-23)	117
Figure 3.10 Comparison of methods for detection of MART-1 specific T cells	120
Figure 3.11 Overview of the process by which Bax-specific T cell lines were generated from donor 11	122
Figure 3.12 Enrichment of IFN γ -secreting Bax-specific T cells from donor 11 using a magnetic bead cell sorting system	123
Figure 3.13 T cell lines (100 in total) generated using limiting dilution culture conditions were	125

	screened for recognition of Bax pool by ELISpot assay	
Figure 3.14	Enrichment of IFN γ -secreting Bax-specific T cells from JSB line using a magnetic bead cell sorting system	127
Figure 3.15	T cell lines (10 in total) generated using limiting dilution culture conditions were screened for recognition of Bax peptides by ELISpot assay	129
Figure 3.16	Summary of the <i>in vitro</i> approach used to generate Bax-specific T cell lines	137

Chapter 4

Figure 4.1	Epitope recognition by JSBI line using an ELISpot assay	140
Figure 4.2	Epitope recognition by JSBI 10E6 using an ELISpot assay	140
Figure 4.3	Staining of Bax-specific T cell lines (from donor 1) for TCR V β expression	141
Figure 4.4	Ability of JSBI 10E6 to lyse target pulsed with Bax peptides	143
Figure 4.5	Mapping the Bax 601-23 response from KSI line to individual peptides	145
Figure 4.6	Mapping the Bax 601-23 response from KSI 10B7 to split pools and individual peptides	147
Figure 4.7	Staining of Bax-specific T cell lines (from donor 11) for TCR V β expression	149
Figure 4.8	Ability of the KSI line to lyse target pulsed with Bax pool 601-23	151
Figure 4.9	Ability of KSI 10B7 to lyse target pulsed with Bax pool 601-23	151
Figure 4.10	Generation of the TCRV β 5.1+ and TCR V β 17+ T cells from KSI 10B7	154
Figure 4.11	KSI V β 17+ cells are able to lyse target pulsed with Bax p610 or p613	155
Figure 4.12	Peptide dose-response for KSIVB17 recognition of Bax p613 and Bax p610 – pulsed target cells	157
Figure 4.13	KSIVB17 clone does not lyse HLA-A2+ PCa cell lines LNCaP and CA-HPV10	159
Figure 4.14	KSIVB17 clone recognises HLA-A2+ CLL cells using ELISpot assay	161
Figure 4.15	KSIVB17 clone does not lyse HLA-A2+ lymphoid malignant cell lines	161
Figure 4.16	KSIVB17 CTL recognition of HPV-transformed cervical cancer cell lines	163
Figure 4.17	KSIVB17 CTL recognition of HLA-A2+ osteosarcoma cell lines	165
Figure 4.18	KSIVB17 clone lyses a hepatocellular cell line but not a breast cancer or melanoma cell lines	166
Figure 4.19	KSIVB17 clone does not lyse healthy PHA blasts	168
Figure 4.20	KSIVB17 clone does not lyse healthy skin fibroblasts and K562 cell line	168
Figure 4.21	Antigen specificity of KSIVB17 clone against TK143	171
Figure 4.22	Antigen specificity of KSIVB17 clone against MS751	172
Figure 4.23	Anti-HLA-A2 antibody inhibition of TK143 and Bax-pulsed T2 lysis by KSIVB17 clone	174

Figure 4.24	Anti-HLA-A2 antibody inhibition of MS751 lysis by KSIVB17 clone	175
Figure 4.25	Bortezomib inhibits presentation of endogenous Bax epitopes in tumour cell lines	177
Figure 4.26	Presentation of endogenous Bax epitopes in MS751 is blocked with various concentrations of the proteasome inhibitor Bortezomib	177

Chapter 5

Figure 5.1	Expression of Bax in PCa cell lines in the presence or absence of the proteasome inhibitor, clasto-lactacystin	187
Figure 5.2	Representative immunoblots showing expression of Bax in CLL cells in the presence or absence of the proteasome inhibitor, clasto-lactacystin	188
Figure 5.3	Expression of Bax in cervical cancer cell lines in the presence or absence of the proteasome inhibitor, bortezomib	190
Figure 5.4	Expression of Bax in osteosarcoma cell lines in the presence or absence of the proteasome inhibitor, bortezomib	192
Figure 5.5	Expression of Bax in normal cells in the presence or absence of the proteasome inhibitor, clasto-lactacystin	193

Chapter 6

Figure 6.1	Memory T cell responses against recall antigens in CLL patients	200
Figure 6.2	Memory T cell responses against recall antigens in CLL patients and healthy donors	201
Figure 6.3	Naive T cell responses against Mart-1 peptide in CLL patients (A) and healthy donors (B)	203
Figure 6.4	MART-1 T cell responses after 2-3 weeks of stimulation in CLL patients (A) and healthy donors (B).	204

Chapter 7

Figure 7.1	Frequency of the peripheral blood lymphocyte populations in CLL patients (n=51) and healthy donors (n=10)	213
Figure 7.2	Frequency of Tregs in CLL patients (n =43) and healthy donors (n =10)	214
Figure 7.3	Representative results of a CLL patient (A) and healthy donor (B)	216
Figure 7.4	CD200 expression in CLL patients (n =50) and healthy donors (n =8)	218
Figure 7.5	CD200R expression in CLL patients (n=43) and healthy donors (n=8-9)	219
Figure 7.6	PD-1 expression in CLL patients (n=42) and healthy donors (n=10)	220
Figure 7.7	Representative results of T cell analysis	222
Figure 7.8	Expression of CD28, CD45RO and CD57 in CD8+ T cells of CLL patients (n=49) and healthy donors (n=10)	224

Figure 7.9	CD8+ T cell subsets of CLL patients (n=34) and healthy donors (n=8)	226
Figure 7.10	Percentage of subsets that are CD28- and CD57+ in total CD8+ T cells of CLL patients (n=34) and healthy donors (n=8)	227
Figure 7.11	Expression of CD28, CD45RO and CD57 in CD4+ T cells of CLL patients (n=49) and healthy donors (n=10)	229
Figure 7.12	CD4+ T cell subsets of CLL patients (n=42) and healthy donors (n=8)	231
Figure 7.13	Percentage of subsets that are CD28- and CD57+ in total CD4+ T cells of CLL patients (n=42) and healthy donors (n=8)	232

List of Tables

Table 1.1	Types of tumour antigens	34
Table 2.1	Description of established cell lines used	71
Table 2.2	Positive Peptide Pool (PPP)	73
Table 2.3	Bax peptide pool 1-15	74
Table 2.4	Bax peptide pool 601-23	75
Table 2.5	Monoclonal antibodies	91
Table 2.6	Panels of monoclonal antibodies for immunophenotyping	92
Table 3.1	Bax pool 1-15 (A) and Bax pool 601-23 (B)	113
Table 4.1	Summary of Bax-specific T cell lines generated in this study	152
Table 4.2	Summary of target cells tested in ⁵¹ Cr release assays and their respective lysis	169
Table 6.1	Summary of T cell responses against Bax in CLL patients	206
Table 7.1	Summary of the immunophenotyping in CLL patients	233

List of suppliers

Applied Biosystems	Applied Biosystems, Warrington, UK
ATCC	LGC Promochem, Teddington Middlesex UK
BD	Becton Dickinson UK Ltd., Oxford, UK
Becton Dickinson	Becton Dickinson (UK) Ltd, Oxford
Bio-Rad	Bio-Rad, Watford, UK
Biostat	Biostat Ltd, Stockport, UK
Cadama Medical	Cadama Medical, Stourbridge, UK
Calbiochem	Calbiochem Ltd, Nottingham, UK
Caltag	now Invitrogen
Dako	Dako Ltd, Ely, Cambridge, UK
Ebiosciences	by Insight Biotechnology Ltd, Middlesex, UK
ECACC	European Collection of Cell Cultures, Salisbury, UK
Falcon	Fisher Scientific, Loughborough, UK
Fisher	Fisher Scientific, Loughborough, UK
GE-Healthcare Life sciences	GE-Healthcare Life sciences Buckinghamshire, UK
Greiner	Greiner Bio-one Ltd., Stonehouse, UK
Heatsystems-Ultrasonics	Heatsystems-Ultrasonics Inc, Beds, UK
Invitrogen	Invitrogen Ltd., Paisley, UK
Leica MZ6	Leica, Milton Keynes, UK
Mabtech	Mabtech, Nacka Strand, Sweden
Merck Laboratory Supplies	Merck Ltd, Dorset, UK
Millipore	Millipore (U.K.) Ltd., Watford, UK
Miltenyi	Miltenyi Biotec Ltd., Bisley, Surrey UK
Mimotopes	Mimotopes Ltd., Wirral, UK
Nunc	Fisher Scientific, Loughborough, UK
Perkin Elmer	PerkinElmer, Massachusetts, USA
ProImmune	ProImmune Limited, Oxford, UK
Proleukin	Chiron Corporation, Emeryville, CA., U.S.
R&D Systems	R & D Systems Europe Ltd, Oxfordshire, UK
Serotec	Serotec Ltd., Kidlington, Oxford, UK
Severn Biotech	Severn Biotech, Kidderminster, UK
Sigma, UK	Sigma, Poole, Dorset, UK
Wallac	now Perkin Elmer
Welsh Blood Transfusion Service	WBTS, Pontyclun, Port Talbot, UK
Xograph Imaging Systems	Xograph Imaging Systems Ltd, Gloucestershire, UK

Chapter 1

Introduction

1.1 Cancer

Cancer is a group of more than 200 diseases characterized by the uncontrolled growth of abnormal cells that have the ability to spread locally by invasion and systematically to other organs by metastasis. Most cancers acquire crucial capabilities during their development. These physiologic changes include insensitivity to growth-inhibitory signals, self-sufficiency in growth signals, limitless replicative potential, sustained angiogenesis, evasion of programmed cell death (apoptosis), and tissue invasion and metastasis. Each of these alterations in cell physiology helps to breach the anticancer defence mechanism present in normal cells and tissues (Hanahan & Weinberg, 2000).

It is imperative to understand and control cancer, because to date cancer is one of the leading causes of death worldwide. More than a quarter of a million new cases of cancer are diagnosed each year in the UK (Cancer Research UK). There is great interest in developing new and effective strategies to complement conventional methods of cancer treatment, such as surgery, radiation, and chemotherapy. One such area is the use of the immune system to mediate tumour protection and regression.

1.2 The immune system

The immune system provides protection against infectious pathogens. Host defence is composed by the early responses of innate immunity and the later responses of adaptive immunity. Innate immunity mediates a rapid induced, non-specific response against infections, which does not result in immune memory. Adaptive immunity arises from a breach in the innate immune response resulting in the generation of antigen specific effector cells,

and memory cells that will limit or prevent subsequent infection with the same organism (Abbas *et al.*, 2007).

1.2.1 Innate immunity

The innate immune system detects a pathogen and acts as the front line of defence. This system is made up of several distinct components, including epithelia, cells present in the circulation and tissues, cytokines and plasma proteins, which includes proteins of the complement system. A major component of the innate system is the physical barrier provided by epithelia (skin, gastrointestinal tract, and respiratory tract), which can prevent the entrance of pathogens and therefore an infection from becoming established. Where epithelial defences have been breached, a number of other innate system components come into play to control or destroy pathogen (Janeway *et al.*, 2008).

The components of innate system recognise structures called pathogen-associated molecular patterns (PAMPs) that are characteristic of microbes. The receptors that bind to these conserved structures are called pattern recognition receptors (PRRs) and are expressed on the cell surface or secreted. PRRs can recognise various bacterial components, which include receptors for lipids (Toll-like receptor 4 (TLR4)), bacterial carbohydrates (mannose and glucan receptors), and other pathogen-derived components (scavenger receptor, and TLRs) The main functions of these molecules are activation of pro-inflammatory signalling pathways, opsonisation of the microorganism, activation of the lectin pathway of the complement, phagocytosis, and induction of apoptosis (Janeway & Medzhitov, 2002).

The principal effector cells of the innate immune responses are phagocytes (neutrophils, monocytes/macrophages), natural killer (NK) cells, and dendritic cells (DCs). Phagocytes, including neutrophils and macrophages, are recruited to sites of infection, where they identify, ingest (phagocytose) and destroy microbes. Monocytes ingest microbes in the circulation and in tissues, where these cells mature and become macrophages. Neutrophils

ingest microbes in the blood, and can also migrate and enter tissues at sites of infections. Once at the site of infection, neutrophils and macrophages ingest the microbes by phagocytosis for intracellular destruction, secrete cytokines that contribute to an inflammatory response, and promote tissue remodeling at the sites of infection (Janeway *et al.*, 2008).

NK cells are lymphocytes that are mainly important in the clearance of virally infected and malignantly transformed cells. The activation of these cells depends on the balance between activating and inhibitory signal provided by cell surface receptors. NK cells preferentially kill target cells that express few or no major histocompatibility complex (MHC) class I molecules. NK cell lytic activity can be induced by the down regulation of MHC class I expression by virally infected cells and cancer cells (Karre, 2002). This down regulation is an immune evasion strategy acquired by a number of viruses and cancer cells in order to avoid recognition by cytotoxic T lymphocytes (CTLs).

NK cells kill by either engaging Fas or releasing perforin and granzyme, which are associated with cytotoxicity. The activated NK cells will mediate their activities through the release of cytokines, such as interferon- γ (IFN γ), which is involved in activating macrophages and neutrophils, and upregulation of HLA class I and class II expression on antigen presenting cells (APCs) (Boehm *et al.*, 1997). NKT cells are innate-like lymphocytes that are characterised by the co-expression of semi-variant $\alpha\beta$ T cell receptor (TCR) together with NK cell associated receptors. The TCRs of NKT cells recognise CD1d molecules displaying glycolipid or non-peptidic ligands (Janeway *et al.*, 2008).

Another type of innate-like lymphocytes are the $\gamma\delta$ T cells. In humans, $\gamma\delta$ T cells make up 50% of intraepithelial lymphocytes and represent 1-5 % of lymphocytes that circulate in the blood. These lymphocyte express both the TCR $\gamma\delta$, that recognises non-peptidic ligands, and the NK associated NKG2D (natural-killer group 2, member D) receptor (Carding & Egan, 2002).

Dendritic cells have phagocytic capabilities and respond to microbes by secreting cytokines. They have an important role in adaptive immune responses by capturing, processing and presenting microbial antigens to T lymphocytes (Banchereau & Steinman, 1998) (later discussed in section 1.2.2). Therefore, DCs have a critical function in linking innate and adaptive immune responses. This is particularly important when pathogenic microbes successfully invade and replicate in the cells and tissues, and protection against these pathogens requires the more specialized mechanisms of the adaptive immunity.

1.2.2 Adaptive immunity

Adaptive immunity develops more slowly, but is a more highly evolved system than the innate system, mediating a more efficient defense against infections. In this context, it provides responses to specific regions (epitopes) of a foreign molecule (antigen). These responses are accompanied by the generation of immunological memory allowing a more rapid and effective response upon subsequent exposure to the pathogen. The adaptive immune system is mediated by lymphocytes and their products, such as antibodies. There are two types of adaptive immunity, humoral immunity and cell-mediated immunity, which provide defense against extracellular microbes and intracellular microbes, respectively.

1.2.2.1 Humoral immunity

The humoral immunity is mediated by antibodies produced by B lymphocytes. This type of immune responses neutralizes and eliminates extracellular microbes and microbial toxins. B lymphocytes are activated through their B cell receptor to produce antibodies that bind to foreign antigens, which results in neutralization of viruses or bacteria or in tagging of pathogens to be destroyed by innate immune system.

1.2.2.2 Cell-mediated immunity

The cell-mediated immunity is governed by T lymphocytes. These cells express TCRs on their cell surface that recognise foreign antigens in the form of short peptides presented by MHC molecules (human leukocytes antigens (HLA) in humans, H-2 molecules in mice) expressed on the surface of APCs (Engelhard, 1994; Rudensky *et al.*, 1991). T cells can be categorised into two types based upon the cell surface expression of either CD4 or CD8. As a general principle, MHC class I molecules present antigenic peptides derived from intracellular proteins for CD8+ T cell recognition and MHC class II molecules present peptide epitopes derived from extracellular antigens for CD4+ T helper (Th) cell recognition. MHC class I is expressed in all nucleated cells, whilst MHC class II molecules are mainly found on cells of the immune system with a specialized antigen presenting function (Engelhard, 1994).

APCs in priming T cell responses

The principal APCs include DCs, macrophages and B cells (professional APCs). APCs have an important task in the generation of epitope specific T cell responses. DCs are the most effective APCs and are especially fundamental in the activation of naïve CD4+ and CD8+ T cells during a primary immune response (Guery & Adorini, 1995; Hart, 1997). B cells ingest protein antigens and display them to CD4+ Th cells during humoral immune responses. Macrophages phagocytose microbes and present antigens of these microbes to effector CD4+ T cells. All these APCs are able to activate CD4+ T cells as they express MHC class II molecules and costimulatory molecules. But, DCs are the only APCs with a unique capacity to stimulate naïve CD4+ and CD8+ T cells and initiate (prime) T cell responses (Cella *et al.*, 1997).

During an immune response, DCs must accomplish two main functions, namely antigen processing and antigen presentation to T cells. Immature DCs enter the peripheral blood system and the circulating DCs are then deposited in peripheral tissues where they are

able to constantly monitor the local environment for the presence of antigenic material through macropinocytosis of soluble proteins (Sallusto *et al.*, 1995). These DCs capture microbial protein antigens and transport the antigens to lymph nodes. After encountering microbial components, the DCs mature during migration to lymph nodes and become competent at presenting antigens and stimulating naive T cells.

The immature DCs can phagocytose microbes and apoptotic or necrotic bodies. The internalised material is processed within a phagolysosome, producing peptide fragments which bind to either HLA class I (through cross-priming) (Albert *et al.*, 2001) or HLA class II molecules which are then displayed on the surface of the DC (Banchereau & Steinman, 1998). DCs can be activated by microbes and also by CD40 ligation (Cella *et al.*, 1996) and presence of pro-inflammatory cytokines such as tumour necrosis factor α (TNF α) and interleukin-1 β (IL-1 β) secreted by infected tissue. These DCs lose their adhesion for epithelial and start to express CCR7, a chemokine receptor that is specific for chemokines produced in the T cell zones of lymph nodes.

Once activated, DCs secrete a number of cytokines such as MIP-1 α , IL-8 and RANTES that are involved in the recruitment of T cells, NK cells, phagocytes and further DCs (O'Neill *et al.*, 2004; Sallusto *et al.*, 1999b). Maturation is characterised by the up-regulation of a number of adhesion molecules (ICAM-1, ICAM-3 and LFA-3) and co-stimulatory molecules (CD80, CD86) that are respectively required for the migration of the DC and the priming of T cells (Timmerman & Levy, 1999). The maturation of the DCs is specifically directed towards efficient antigen presentation rather than phagocytosis and antigen processing.

In the lymph nodes, mature DCs present the antigen (complexed with a HLA molecule) to naïve T cells. A T cell recognises a peptide through its expression of a unique TCR. The recognition of an epitope by the TCR is not sufficient by itself to completely activate a naïve T cell. Secondary signals provided by the ligation of co-stimulatory

molecules on the DC (CD80 and CD86) with their cognate molecule (CD28) on the T cell, are required for full activation and clonal expansion of T cells (Linsley *et al.*, 1991; Sansom *et al.*, 2003).

CD4+ T cells and their effector subsets

Naïve CD4+ T cells become primed and activated through the recognition of their cognate epitope presented by MHC class II molecules expressed on mature DC. Once activated, CD4+ Th cells proliferate and differentiate into subsets of effector cells that perform different functions. The main subsets of CD4 effector T cells are Th1, Th2, and Th17. (CD4+ T cells can also be differentiated into regulatory T cells, which will be discussed separately in this section.) The first subsets to be defined were Th1 and Th2 (Mosmann & Coffman, 1989), and more recently Th17 (Harrington *et al.*, 2005). The development of these Th subsets is regulated by the stimuli that naïve CD4+ T cells receive when they encounter antigens. These three subsets produce distinct sets of cytokines and perform different functions (Zhu & Paul, 2008) (Figure 1.1). Upon triggering, CD4+ T cells can orchestrate the activation of B cells, macrophages, and CD8+ T cells, and also the activation and recruitment of neutrophils.

It has been shown that during their interaction with mature DCs, CD4+ Th cells can provide some form of stimulatory signal, enhancing the DCs capacity to effectively activate naïve CD8+ T cells via a cross-priming mechanism of antigen processing (Bennett *et al.*, 1997). This stimulation by the CD4+ Th cell is believed to be dependent on the ligation of CD40L on the activated Th cell with CD40 on the DC. This ligation has been shown to result in enhanced surface expression of co-stimulatory molecules such as CD80 and CD86, cell adhesion molecules such as ICAM-1 and also high levels of IL-12 production (Caux *et al.*, 1994; Cella *et al.*, 1996; Shinde *et al.*, 1996). In the absence of CD4+ T cell help, the interaction between mature DCs and naïve CD8+ T cells can result in T cell tolerisation.

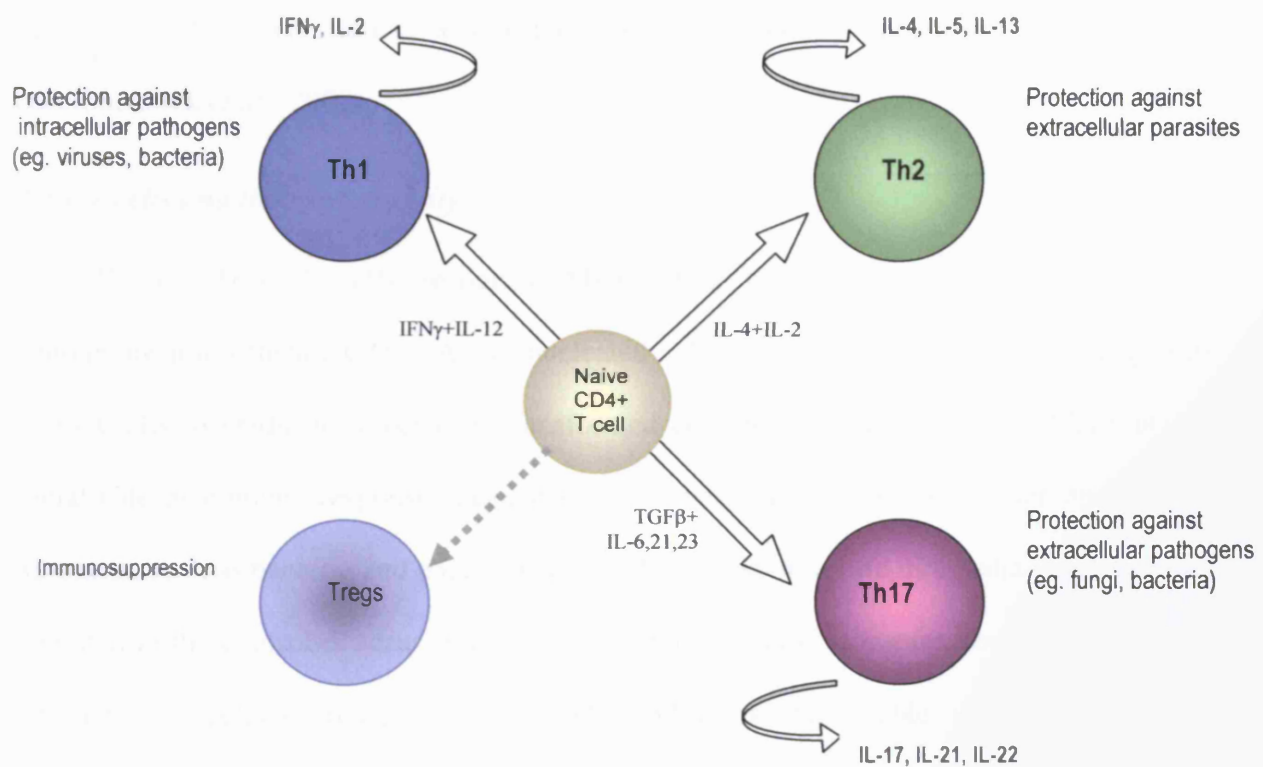


Figure 1.1 Differentiation of T helper cells (*adapted from Zhu & Paul, 2008*). The development of the CD4⁺ T cell subsets Th1, Th2, Th17 (and Tregs) is dependent on the stimuli that naïve T cells receive when they encounter antigens. These subsets produce distinct sets of cytokines and perform different functions (indicated on the diagram).

In vitro studies have revealed that this need for CD4 help can be bypassed through the addition of CD40L (Albert *et al.*, 2001). CD40/CD40L ligation is clearly an essential pathway through which CD4⁺ T cells provide cognate help. Murine studies have revealed that although CD4⁺ T cell help is not always required for a primary CD8⁺ T cell response, it is important for the generation of a robust CD8⁺ T cell memory population (Janssen *et al.*, 2003; Shedlock *et al.*, 2003)

CD8⁺ T cells and their cytotoxicity

Naïve CD8⁺ T cells recognise MHC class I-associated peptide antigens and differentiate into effector CTLs. As all nucleated cells express MHC class I molecules, this allows CTLs to eradicate infected or transformed cells in all types of tissues. CTLs play a crucial role in immune responses against many viruses, tumors cells and other intracellular pathogens, such as bacteria and parasites. CD8⁺ T cell responses are directed against peptides generated in the cytosol of virus-infected or transformed cells. However, these virus-infected or transformed cells are not professional APCs and are not be capable of activating naïve T cells. For that reason, DCs have an important role in priming naïve CD8⁺ T cells (Basta & Alatery, 2007; Heath & Carbone, 2001). DCs are able to acquire antigen from virus-infected or tumour cells, and then present peptides on their MHC class I to naïve CD8⁺ T cells. This is defined as cross-presentation and termed cross-priming when it leads to the activation of CD8⁺ T cells (Chen *et al.*, 2004; Huang *et al.*, 1994; Sigal *et al.*, 1999).

The activation of naïve CD8⁺ T cells and their differentiation may require involvement of CD4⁺ Th cells. As previously described, Th cells may contribute by secreting IL-2, which promotes CD8⁺ T cell differentiation, and/or by enhancing DC ability through CD40-CD40L interaction (Janeway *et al.*, 2008). The requirement for CD4⁺ T cell help in the generation of a primary CD8⁺ T cell response can differ depending on the infecting virus. The clearance of non-cytopathic or poorly immunogenic viruses such as hepatitis C virus (HCV)

appears to need CD4⁺ T cell help (Godkin *et al.*, 2002), whereas it is not necessary for viruses such as Influenza A (Albert *et al.*, 2001).

Subsequent to activation, CD8⁺ T cells proliferate and differentiate into effector CTLs. The activated CTLs migrate into inflamed tissue in search of virally infected or transformed cells, which are detected by the presentation of virus or tumour-derived epitopes complexed with MHC class I molecules on the cell surface. The CTLs have 3 effector functions – secretion of cytokines, induction of apoptosis via release of cytolytic granules and induction of apoptosis via engagement of the death receptors such as Fas and tumour necrosis factor receptor (TNFR) on the surface of targets (Figure 1.2). Once a target cell has been eliminated, the CTL disengages and can then continue to kill other target cells.

The principal mechanism through which a CTL kills is the release of pre-formed cytotoxic granules (Figure 1.2A). CTLs (and NK cells) have granules that contain several cytotoxic mediators, including perforin and granzyme, which contribute to target cell apoptosis. The granule membrane expresses lysosomal associated membrane glycoproteins (LAMPs), including CD107a (LAMP-1), CD107b (LAMP-2) and CD63 (LAMP-3), which become transiently exposed on the cell surface during degranulation (Betts *et al.*, 2003; Peters *et al.*, 1991). During T cell killing, cytotoxic granules are transported to the cell membrane, releasing the cytotoxic mediators into the immune synapse between the effector T cell and the target (Betts *et al.*, 2003). Both perforin and granzymes are required for efficient CTL killing of the target cells. Perforin helps by delivering the contents of the granules into the cytoplasm of target cells. Once in the cytoplasm of the target cell, granzymes trigger apoptosis by targeting the pro-apoptotic protein Bid and also caspases. It was originally thought that perforin created pores in the plasma membrane to permit the entrance of the proteolytically active granzymes (Heusel *et al.*, 1994). However, it has been shown that perforin and granzymes form multimeric complexes with the proteoglycan serglycin which acts as a scaffold (Metkar *et al.*, 2002). Although, how this is accomplished is still not well understood.

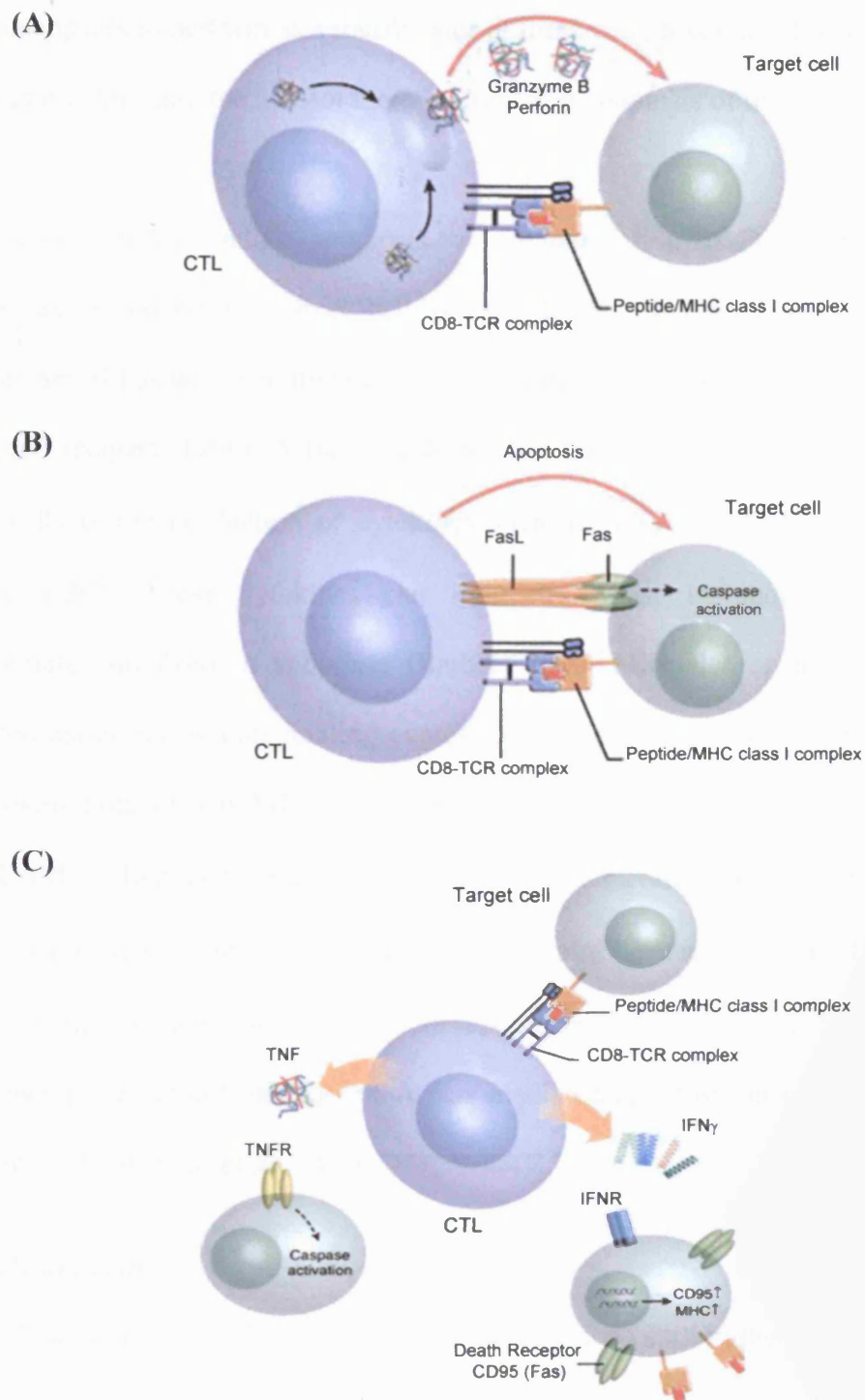


Figure 1.2 Effector functions of CTLs (*adapted from Andersen et al., 2006*). (A) Induction of apoptosis in target cells via the release of cytotoxic granules. (B) Induction of apoptosis via death receptors triggering. (C) Indirect killing by release of cytokines (TNF α and IFN γ).

Perforin appears to perform as a translocator of these complexes and direct the release of the bound granzymes into the cytosol thereby triggering apoptosis of the target cell (Metkar *et al.*, 2002).

CTL can also induce apoptosis via the interaction of Fas ligand (FasL) with Fas (CD95) expressed on the target cell (Figure 1.2B). This activates a signaling pathway downstream of Fas which culminates in the activation of caspases resulting in apoptosis of the target cell (Nagata, 1996). A further effector function of CTL when they recognise specific target cells is the production of cytokines such as IFN γ and TNF α (Jassoy *et al.*, 1993) (Figure 1.2C). These cytokines can indirectly assist in apoptosis by up-regulating intermediates involved in apoptosis (Barber, 2000; Mitsiades *et al.*, 2003), and in CTL mediated apoptosis by upregulating expression of molecules involved in antigen processing and presentation, such as MHC class I, transporter associated with antigen processing (TAP) and ICAM-1 (Epperson *et al.*, 1992). IFN γ can activate macrophages, leading to their recruitment to sites of infection. In addition, it has also been reported that TNF α and IFN γ can synergistically induce apoptosis, where apoptosis is not induced by one or the other (Sasagawa *et al.*, 2000) and to restore Fas mediated apoptosis in previously Fas apoptosis resistant cells (Spanaus *et al.*, 1998).

T regulatory cells

In addition to the CD4⁺ T cells subsets described earlier, there is another CD4⁺ T cell population to which a regulatory or suppressive function has been attributed, known as T regulatory cells (Tregs). These cells are considered to play a central role in self-tolerance (Sakaguchi, 2000). They have been categorized into two subgroups, the natural Tregs and the adaptive (induced) Tregs (Bluestone & Abbas, 2003). Natural Tregs develop in the thymus and recognise self antigen via their TCRs. These cells are characterised by the expression of CD4 and CD25 and the transcription factor Foxp3. Although all activated T cells express CD25, Tregs express the highest levels of CD25. Their principal function is to suppress the

activity (activation, proliferation and IL-2 production) of CD4⁺ and CD8⁺ T cells by cell to cell contact, and play a role in controlling autoreactive T cells (Piccirillo & Shevach, 2001; Woo *et al.*, 2002). It has also been suggested that they may also function by producing IL-10 and TGF- β cytokines that can inhibit T cell proliferation (Roncarolo *et al.*, 2006).

The adaptive Tregs are generated in the periphery under diverse tolerogenic circumstances. They are induced from naïve T cells by specific antigen mode stimulation. The adaptive Tregs include the Tr1 subset, which secrete high levels of IL-10 and the Th3 subset, which produce TGF- β (Carrier *et al.*, 2007; Roncarolo *et al.*, 2006). These cytokines suppress both naïve and memory T cell responses and down-regulate the expression of co-stimulatory molecules and inflammatory cytokines by APCs (Roncarolo *et al.*, 2006).

An increase of Tregs in the peripheral blood and the presence of Tregs at the tumour site have been reported for several types of human cancer, indicating that these cells may suppress T cell-mediated antitumour immunity (Liyanage *et al.*, 2002; Ormandy *et al.*, 2005; Piccirillo & Shevach, 2001; Wolf *et al.*, 2003; Woo *et al.*, 2002). Several groups have utilised murine models to investigate the effect of removing CD4⁺ CD25⁺ Treg cells on breaking immunotolerance to tumours (Jones *et al.*, 2002; Shimizu *et al.*, 1999). Interestingly, in the absence of these Treg cells, tumour-specific CD4⁺ CD25⁻ and CD8⁺ T cell responses develop, resulting in protection against transplanted tumours (Casares *et al.*, 2003).

Memory T cells

Following resolution of an infection the effector T cell population contracts, leaving behind some of the T cells as antigen-specific memory T cells that persist at much higher levels than the initial naïve T cell frequency. Memory T cells are maintained over long periods of time and function to permit an increasingly rapid and potent immune response with each subsequent exposure to a specific pathogen (Murali-Krishna *et al.*, 1998). The memory T cell pool consists of two predominant heterogeneous populations. Although not totally distinct, these can differ in their expression of cell surface markers such as adhesion

molecules, in the tissues in which they reside and also in their ability at exerting effector function. The two main populations are central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) (Sallusto *et al.*, 1999a).

Both T cell memory subsets can be found in the blood and spleen. T_{CM} constitutively express lymphoid homing molecules CD62L and CCR7, high levels of IL7R and reside primarily in secondary lymphoid organs, thereby patrolling against systemic antigenic challenge. Furthermore, when compared to T_{EM} cells, T_{CM} are more sensitive to antigen, exhibit an enhanced ability to proliferate and secrete IL-2 that leads to a rapid recall response (Masopust *et al.*, 2001; Sallusto *et al.*, 1999a; Wherry *et al.*, 2003). Whereas, T_{EM} do not express these lymphoid homing molecules and are located in non-lymphoid tissues, such as the gut, liver, and lungs. These cells are rapidly recruited to sites of inflammation and infection, where upon antigenic re-stimulation they exhibit immediate effector functions including the production of IFN γ and cytotoxicity, thus operating as sentinels for immediate protection against pathogen (Masopust *et al.*, 2001; Sallusto *et al.*, 1999a; Weninger *et al.*, 2001; Wherry *et al.*, 2003).

The issue of how memory T cells develop from the initial pool of activated T cells is not completely understood. It remains controversial whether the transition of activated T cells to T_{EM} cells and T_{CM} cells is linear (linear differentiation models) or they develop as separate lineages (branched differentiation model) (Baron *et al.*, 2003; Bouneaud *et al.*, 2005). Several studies support the linear differentiation pathway, in which naïve cells upon encounter with an antigen mature into T_{CM} , which differentiate to T_{EM} and finally to (terminally) highly differentiated effector cells, known as T_{EMRA} (Naïve $\rightarrow T_{CM} \rightarrow T_{EM} \rightarrow T_{EMRA}$) (Figure 1.3). Data on telomere length supports a linear pathway of differentiation. T_{EM} and T_{EMRA} exhibit a shortened telomere length in comparison to naïve and T_{CM} cells, suggesting they have undergone multiple rounds of division, and are thus further along in the linear

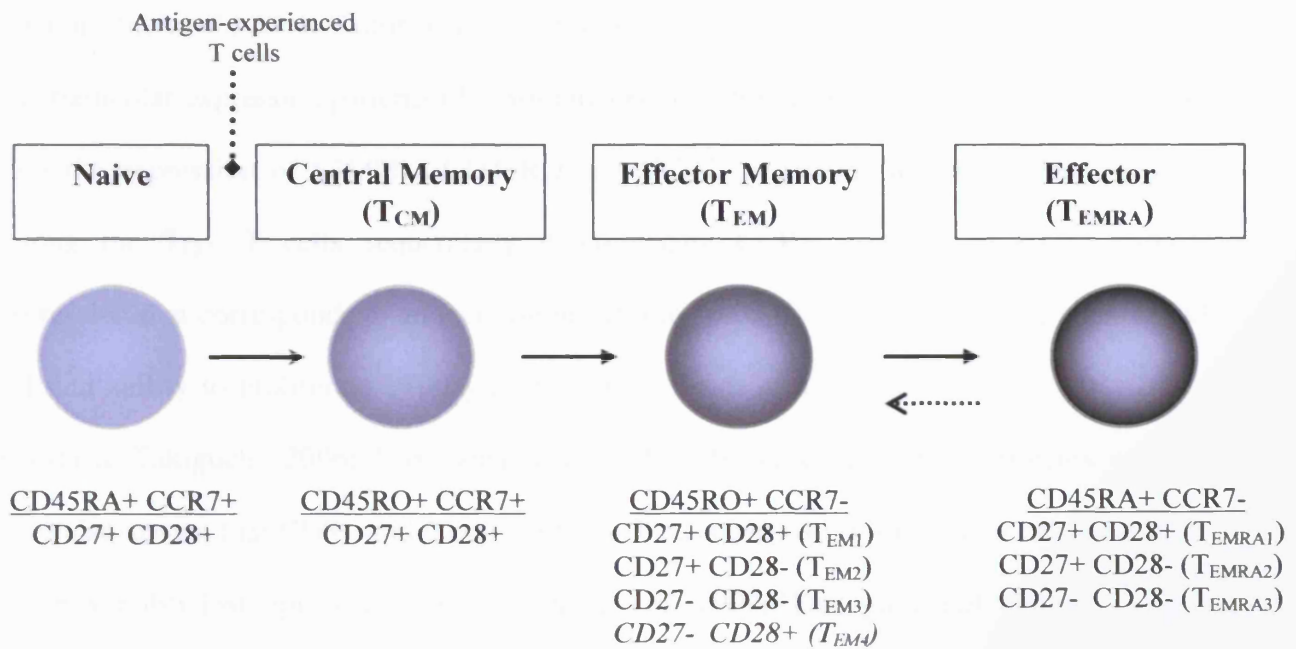


Figure 1.3 Differentiation from naïve to memory T cells. T cells can be distinguished by the expression of CD45RA, CD45RO, CCR7, CD28 and CD27. Additional heterogeneity occurs among T_{EM} (subsets T_{EM1}, T_{EM2}, T_{EM3} and T_{EM4}) and T_{EMRA} (subsets T_{EMRA1}, T_{EMRA2}, and T_{EMRA4}) by the particular expression patterns of costimulatory receptors CD28 and CD27. (based on Appay *et al.*, 2002; Okada *et al.*, 2008; Romero *et al.*, 2007; Takada & Takiguchi, 2006; and Tomiyama *et al.*, 2002)

differentiation pathway (Champagne *et al.*, 2001; Sallusto *et al.*, 2004; Sallusto *et al.*, 1999a; Tomiyama *et al.*, 2002).

Surface expression of isoforms of tyrosine phosphatase CD45 (CD45RA and CD45RO) and CCR7 have been widely used to delineate four major subsets of T cells. (Champagne *et al.*, 2001; Sallusto *et al.*, 2004; Sallusto *et al.*, 1999a; Tomiyama *et al.*, 2002). The particular expression patterns of costimulatory receptors CD28 and CD27, in conjunction with the expression of CD45RA/CD45RO and CCR7, uncovered additional heterogeneity among the T_{EM}. T cells sequentially downregulate CCR7, CD28, and CD27 surface expression that corresponds to an increase in cytokine production and cytotoxic potential and reduced ability to proliferate (Appay *et al.*, 2002; Okada *et al.*, 2008; Romero *et al.*, 2007; Takata & Takiguchi, 2006; Tomiyama *et al.*, 2002). However, recent experiments in mice have also shown that CD8⁺ and CD4⁺ memory T cells can rise from effector cells that have not irreversibly lost replicative ability (Bannard *et al.*, 2009; Harrington *et al.*, 2008).

The factors that establish T cell differentiation and generation of memory T cells are not fully understood. Memory T cells persist in an antigen-independent manner under the influence of homeostatic cytokines, particularly IL-7 and IL-15, with IL-7 enhancing survival and IL-15 inducing proliferation (Lanzavecchia & Sallusto, 2005). Signals provided from cytokine receptors can influence T cell differentiation. The presence of either IL-2 or IL-15 during activation has been shown in *in vitro* studies to influence the migratory properties of the memory T cells that are generated. IL-15 preferentially generates T_{CM} cells, whereas IL-2 promotes the formation of T_{EM} cells (Weninger *et al.*, 2001).

CD4⁺ Th cells contribute to CD8⁺ T cell function, persistence and capacity to control a secondary challenge by a pathogen (Janssen *et al.*, 2003; Sun *et al.*, 2004). The precise mechanism of CD4⁺ help remains controversial. It may take place during CD8⁺ T cell priming (Janssen *et al.*, 2003) and seems to be required for the maintenance of memory CD8⁺ T cells (Sun *et al.*, 2004). Absence of CD4⁺ T cell help during priming results in CD8⁺ T

cells that can mediate effector functions upon restimulation, but are unable to mount recall responses and undergo TRAIL (TNF related apoptosis inducing ligand)-mediated activation-induced cell death upon re-challenge. However, CTL responses against certain viruses can be primed without CD4⁺ T cell help, either to direct infection of APC or inducing inflammatory host responses (Janssen *et al.*, 2005). Furthermore, Tregs have been shown to negatively influence memory CD8⁺ T cell proliferation and responsiveness (Murakami *et al.*, 2002; Suvas *et al.*, 2003).

1.3. MHC and antigen presentation

The ability of a T cell to specifically recognise foreign antigen on the surface of either APCs or infected cell is mediated by the interaction of the TCR with MHC-peptide complexes. This refined system permits the immune system to screen both the intracellular and extracellular environment of a host for pathogens. The task of displaying cell-associated antigens for recognition of T cells is performed by MHC molecules (HLA in humans, H-2 molecules in mice). There are two main types of MHC molecules, called MHC class I molecules and MHC class II molecules.

1.3.1 MHC class I structure and function

1.3.1.1 Structure

MHC class I molecules are able to present endogenous peptides derived from an intracellular pathogen to CD8⁺ T cells. The MHC I molecule is a membrane-bound heterodimer consisting of an α chain associated non-covalently with β 2-microglobulin (β 2m). The α chain contains 3 extracellular globular domains. The polymorphic α 1 and α 2 regions form the walls of a cleft on the surface of the molecule, called the peptide binding groove (Bjorkman *et al.*, 1987). The α 3-domain is linked to the cell surface *via* a transmembrane-domain. The α 3 domain interacts with the T cell accessory molecule CD8, whilst the peptide binding groove is contacted by the antigen-specific TCR (Engelhard, 1994). The ends of the

peptide binding groove are closed, thereby restricting the length of peptide that can be accommodated. MHC class I restricted peptides tend to be between 8 and 11 amino acids in length (Rammensee, 1995) (Figure 1.4).

1.3.1.2 Antigen processing and presentation

Degradation of cytosolic proteins

MHC class I molecules present peptides that are derived from cytosolic proteins, most of which are synthesized in nucleated cells. Antigens in the cytosol may be products of viruses or other intracellular microbes. Alternatively, protein antigens that may also be produced by mutated or overexpressed genes in tumour cells. The breakdown of intracellular proteins that may take place during a viral or bacterial infection or with cancer allows the immune system to screen for infected or transformed cells.

In order to produce short peptides that will bind to MHC class I molecules the proteins are degraded in the cytoplasm by the ubiquitin-proteasome pathway. Many intracellular proteins to be degraded in the cytoplasm are first modified by ubiquitin (Michalek *et al.*, 1993). Ubiquitination may unfold the target proteins and act as recognition components for cytosolic proteasome complexes. Once a protein has been ubiquitinated, it is rapidly degraded by the proteasome (Ciechanover, 1994; Pamer & Cresswell, 1998).

Proteasomes are multicatalytic proteases that mediate the majority of cytoplasmic protein degradation and have an important role in the generation of MHC class I-associated peptides. Ubiquitinated proteins are rapidly degraded by the 26S proteasome complex, which is found in the cytoplasm of most cells (Goldberg *et al.*, 2002; Rock & Goldberg, 1999). The 26S proteasomes contain a barrel-shaped 20S catalytic core particle, which is arranged in four stacked rings. The outer two rings consist of seven homologous α -subunits which provide the proteasome structure, surrounding a central opening through which the substrates appear to enter. The two inner rings are each composed of seven homologous β -subunits which

surround a central chamber where proteolysis occurs. Three of the β -subunits display proteolytic activity (Pamer & Cresswell, 1998).

Exposure of cells to IFN γ induces the expression of three additional β -subunits, namely LMP2, LMP7 and MECL1 that are preferentially incorporated into newly assembling proteasomes in place of their homologous constitutive counterparts. This results in a change in the substrate specificity of the proteasome, so that peptides produced contain carboxy-terminal basic and hydrophobic amino acids, and this presumably enhances the production of peptides capable of associating with MHC class I molecules or changes the repertoire of peptides presented (Eleuteri *et al.*, 1997; Fruh *et al.*, 1994). Along with increase of MHC-class I expression, this is one mechanism by which IFN γ modulates antigen presentation. Proteasomes containing the IFN-induced subunits are termed immunoproteasomes, indicating their special role in antigen presentation. Immunoproteasomes are constitutively expressed by APCs (Eleuteri *et al.*, 1997; Kloetzel, 2004; Macagno *et al.*, 1999).

It is now evident that degradation of proteins by the proteasome is the key step in the generation of most antigenic peptides. Several studies have shown that proteasome inhibitors were able to not only block the degradation of most cellular proteins but also block most MHC class I antigen presentation of peptides derived from cytosolic proteins (Rock *et al.*, 1994). Initial studies used peptide aldehydes, for example, to demonstrate inhibition of antigen presentation (Harding *et al.*, 1995; Rock *et al.*, 1994). Several other structurally unrelated extremely specific proteasome inhibitors, such as lactacystin, also showed a reduction of class I antigen presentation (Cerundolo *et al.*, 1997; Craiu *et al.*, 1997).

The inhibitors appear to block the assembly of stable MHC class I molecules, resulting in an accumulation of MHC class I continuously bound with TAP in the endoplasmic reticulum (ER) due to the lack of proper antigenic peptides. Moreover, this loss of stable MHC class I complexes in proteasome-inhibitor cells can be reversed by the addition of antigenic peptides (Rock *et al.*, 1994). However, in some reports treatment with proteasome

inhibitors only partially prevented or even enabled presentation of particular epitopes (Morishima *et al.*, 2007; Schwarz *et al.*, 2000; Valmori *et al.*, 1999; Vinitsky *et al.*, 1997). One of the explanations could be that in these cases the generation of antigenic peptides or their precursors involved proteases other than proteasome (Vinitsky *et al.*, 1997). It could also be that proteasomal inhibition may be only partial and the remaining proteasomal activities created different peptides that might be better suited for MHC class I binding or that proteasomes were destroying epitopes (Schwarz *et al.*, 2000).

Proteasomes generate the correct C-terminus of antigenic peptides but it appears that some of the resultant peptides are further trimmed in the cytosol, or in the ER by aminopeptidases that generate the correct N-terminal sequences of presented epitope. Peptidases can also destroy epitopes by trimming peptides and making them too short to bind to MHC class I molecules. Some of the peptides escape destruction in the cytosol and bind to MHC class I in the ER (Rock *et al.*, 2004).

Peptide translocation into the ER

Peptides generated in the cytosol are transported into the lumen of the ER via TAP. TAP is a heterodimer that contains TAP-1 and TAP-2, encoded by genes in the MHC closely linked to the LMP2 and LMP7 genes. The TAP protein is located in the ER membrane, where it mediates the active, ATP-dependent translocation of peptides with 9-13 residues in length from the cytosol into the ER lumen (Pamer & Cresswell, 1998). On the luminal side of the ER membrane, the TAP protein acts as scaffold for the final stage of MHC class I assembly, as it is noncovalently attached to newly synthesized MHC class I molecules by a linker protein called tapasin.

Assembly of MHC class I complexes

Translocated peptides bind to newly assembled MHC class I molecules in the ER. Assembly of MHC class I molecules involves a multistep process in which binding of a peptide plays an important role. Class I α chains and β_2m are assembled in the ER, which is

mediated by the sequential interaction of class I α chains and β_2m with a set of ER-resident chaperones, namely calnexin, calreticulin and Erp57. These chaperones have a quality control function and ensure that misfolded proteins and incomplete MHC complexes do not leave the ER (Pamer & Cresswell, 1998). The MHC class I/ β_2m complex, associates with TAP along with tapasin, Erp57 and calreticulin to form a peptide loading complex (Farmery *et al.*, 2000). Tapasin forms a bridge between MHC class I molecule and TAP, and has been proposed to aid an efficient loading of the peptide into the MHC class I binding groove, so that peptides are bound with optimum affinity (Williams *et al.*, 2002). Peptide translocation by TAP eventually results in the formation of a stable MHC class I-peptide complex, which dissociates from the chaperone proteins, and is transported from the ER through the Golgi apparatus to the cell surface. MHC class I molecules with bound peptides are structurally stable and expressed on the cell surface for recognition by peptide-specific CD8⁺ T cells (Figure 1.5A).

Cross-priming

The cross-priming is a process by which APC such as DCs, can process exogenously derived antigen, and present the resulting peptides complexed with HLA class I molecules to CD8⁺ T cells. APC acquire antigen via endocytosis and phagocytosis. There are several proposed mechanisms as to how the antigen gains access to the HLA class I pathway. One of the major pathways is thought to involve the release of antigen from the phagosomes into the cytosol. The antigen is then degraded by the proteasome, and the resulting peptides are transported via the TAP complex into the ER where they bind to newly synthesised HLA class I molecules (Kovacsovics-Bankowski & Rock, 1995). The second main pathway is independent of TAP and proteasome activity. Instead the antigen is processed in the phagosome by the cysteine protease cathepsin S (Shen *et al.*, 2004). The resulting peptides bind to HLA class I molecules within the endocytic compartment, however the precise mechanism through which this occurs is not clear.

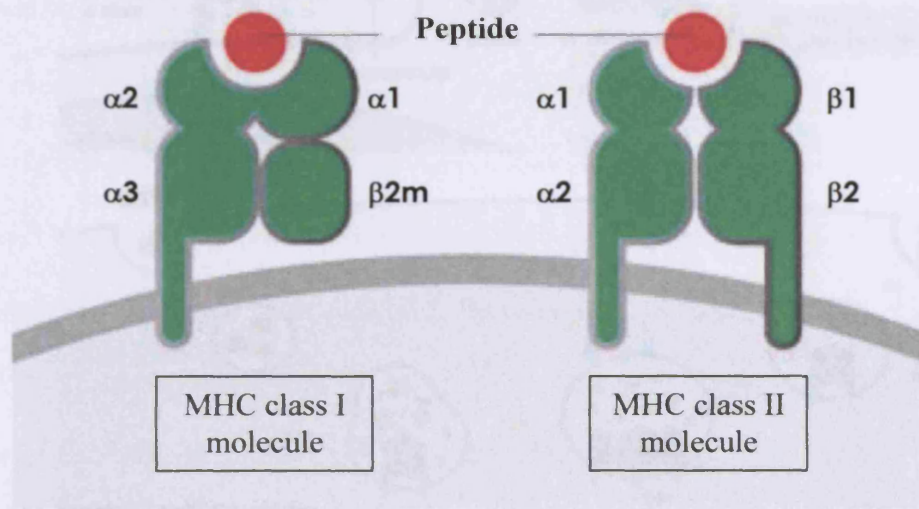


Figure 1.4 Structure of MHC class I and MHC class II molecules (adapted from DeFranco et al., 2007). The MHC class I heterodimer consists of an α chain, non-covalently associated with β 2-microglobulin (left). The α chain contains 3 extracellular globular domains (α 1, α 2 and α 3), with the polymorphic α 1 and α 2 regions forming the peptide binding groove. The α chain also contains a transmembrane domain and a cytoplasmic tail. The MHC class II heterodimer consists of an α and a β chain (right). These chains both contain two domains (α 1/ α 2 and β 1/ β 2), a transmembrane domain and a cytoplasmic tail. The α 1 and β 1 domains of the chains come together to form a polymorphic peptide binding groove.

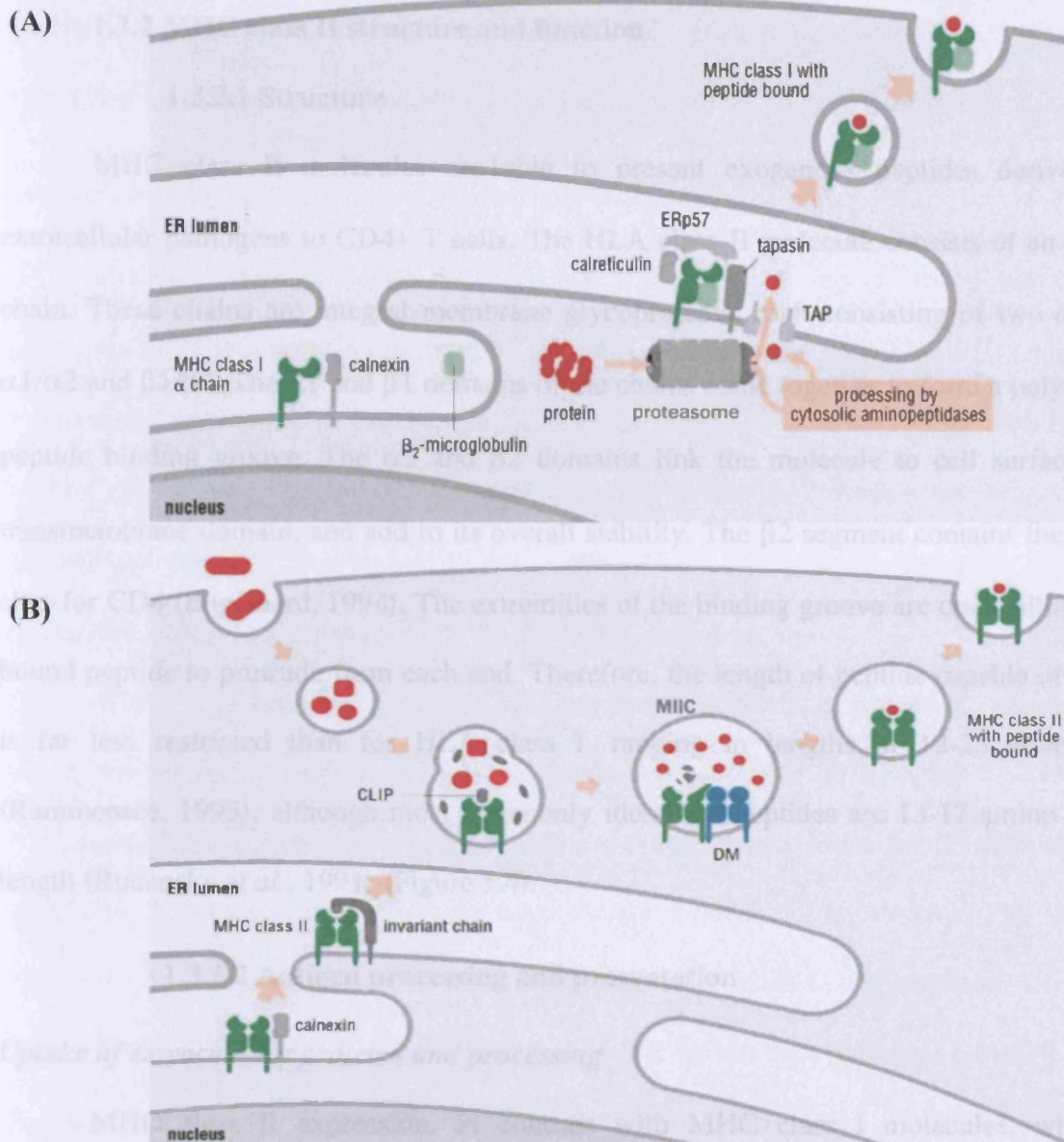


Figure 1.5 MHC class I and II pathways (adapted from DeFranco et al., 2007). (A) The MHC class I pathway presents peptides derived from endogenously generated proteins. The proteins are degraded by the proteasome (and in some cases, are further cleaved by aminopeptidases), generating short peptide fragments, which can then be transported via the TAP complex into the ER. In the ER, peptide fragments associate with the HLA class I heterodimer, assisted by several chaperone molecules. The MHC class I-peptide complex is then transported to the cell surface, where it is presented to CD8⁺ T cells. (B) The MHC class II pathway predominantly presents peptides derived from an exogenous source of protein. Protein is endocytosed by APC and enters the endocytic pathway, where it progresses through increasingly acidic endosomes, finally fusing with a lysosome. The resulting peptide fragments are introduced to the HLA class II molecules in the MHC class II compartment (MIIC). Peptide loading requires the release of the CLIP fragment from the MHC class II binding groove, which is facilitated by the HLA-DM heterodimer. The MHC class II-peptide complex is then transported to the cell surface where it is presented to CD4⁺ T cells.

1.3.2 MHC class II structure and function

1.3.2.1 Structure

MHC class II molecules are able to present exogenous peptides derived from extracellular pathogens to CD4⁺ T cells. The HLA class II molecule consists of an α and β chain. These chains are integral membrane glycoproteins, each consisting of two domains, $\alpha 1/\alpha 2$ and $\beta 1/\beta 2$. The $\alpha 1$ and $\beta 1$ domains of the chains come together to form a polymorphic peptide binding groove. The $\alpha 2$ and $\beta 2$ domains link the molecule to cell surface, via a transmembrane domain, and add to its overall stability. The $\beta 2$ segment contains the binding sites for CD4 (Engelhard, 1994). The extremities of the binding groove are open, allowing the bound peptide to protrude from each end. Therefore, the length of peptide capable of binding is far less restricted than for HLA class I, ranging in lengths of 12-25 amino acids (Rammensee, 1995), although most commonly identified peptides are 13-17 amino acids in length (Rudensky *et al.*, 1991) (Figure 1.4).

1.3.2.2 Antigen processing and presentation

Uptake of extracellular proteins and processing

MHC class II expression, in contrast with MHC class I molecules, which are expressed on all nucleated cells, is limited to specific antigen presenting immune cells, including macrophages, B cells and DCs. Extracellular proteins that are internalised by specialised APCs are processed in vesicles and displayed by MHC class II molecules. A protein antigen enters the APC by endocytosis, which includes specific receptor-mediated endocytosis and non-specific adsorptive endocytosis or phagocytosis. Endocytosed protein is then delivered into the endocytic pathway, where it progresses through endosomes/phagosomes (which become increasingly acidic), finally fusing with lysosomes. The acidic endosome environment activates a number of acid proteases, in particular cathepsins (Watts, 2001). For efficient proteolysis the antigen must be unfolded. This requires the breaking of intramolecular disulphide bonds which is catalysed by GILT (IFN γ -inducible

lysosomal thiol reductase) (Phan *et al.*, 2000). This proteolytic pathway produces peptide fragments, some of which are capable of binding to the MHC class II molecules.

Synthesis and trafficking of the MHC class II molecule

Newly synthesised MHC class II α and β chains enter the ER where they form $\alpha\beta$ heterodimers. The newly formed MHC class II heterodimer then interacts with a non-polymorphic protein called the invariant chain (Ii). Occupation of the MHC class II peptide binding groove with a region of the Ii protein known as the CLIP domain prevents binding of ER resident peptides (Romagnoli *et al.*, 1993). The $\alpha\beta$ -Ii complex are then exported to specialised endosomal compartments via the Golgi apparatus.

Within the endocytic vesicle, acid proteases, such as cathepsin S, proteolytically cleave the Ii chain, leaving the CLIP domain bound to the MHC class II heterodimer (Villadangos *et al.*, 1999). The precise vesicular compartment where peptide loading of the MHC class II molecule occurs has not been clearly defined, but is believed to be late in the endosomal pathway. It is referred to as the MIIC (MHC class II compartment) (Hiltbold & Roche, 2002). The CLIP domain is removed by the action of a molecule called HLA-DM, which is also found in the MIIC compartment (Busch *et al.*, 2005). Peptides generated by proteolysis of internalized protein antigens are then able to bind to MHC class II molecules. MHC class II molecules are stabilised by the bound peptides and these complexes are delivered to the surface of the APC for CD4⁺ T cell recognition (Figure 1.5B).

1.3.3 The HLA genes

The human HLA region is located on the chromosome 6 and extends over 4×10^6 base pairs of DNA. It can be divided into three major regions. The first contains the HLA class I - A, -B, -C, -E, -F and -G genes. The second region contains the HLA class II -DR, -DP and -DQ genes, as well as HLA-DM and the genes encoding TAP and low molecular weight proteins (immunoproteasome subunits LMP). The class III region is situated between the class I and II

regions and contains over 75 genes including those encoding several complement proteins. HLA genes can be highly polymorphic allowing the presentation of a huge range of potential peptide epitopes. There are multiple alleles for each gene, for example, HLA class I have 2678 known alleles (IMGT/HLA database, 2009). The HLA-A2 is the largest allele family at the HLA-A locus. The HLA-A*0201 is frequent in all ethnic groups and in the study population carried out by Ellis *et al.* (2000) showed that it was found in 35% of African-Americans and 50% of Caucasians. Because HLA-A2 is so widely present in the human population, peptides that bind HLA-A2 are frequently considered for peptide-based vaccines (Ellis *et al.*, 2000). In fact, several *in vitro* studies and clinical trials have used HLA-A2 to present melanoma-derived epitopes to CTLs (Clay *et al.*, 1999; Rosenberg & Dudley, 2004; Wolfel *et al.*, 1989).

1.4 The T cell receptor (TCR)

1.4.1 TCR structure

The TCR is an antigen-specific membrane-bound molecule present on the surface of T cells (Figure 1.6). Between 90 and 99% of peripheral T cells express TCR $\alpha\beta$ chains, whilst the remaining population expresses TCR $\gamma\delta$ chains. The TCR of MHC-restricted CD4⁺ and CD8⁺ T cells consists of a α chain and β chain, covalently linked by disulphide bonds. Both α chain and β chain consists of a N-terminal region (which contains a variable (V) and constant (C) domain), a transmembrane region and a cytoplasmic tail (Bentley & Mariuzza, 1996). Each TCR chain is encoded by multiple gene segments that undergo somatic rearrangements during early stages of lymphocyte maturation. The constant region is encoded by a constant gene (C), whilst the variable domain is encoded by a variable gene (V), a joining gene (J), and in the case of β chains, a diversity gene (D). The somatic rearrangement of the V-J (α) or V-D-J (β) genes occurs in the thymus. This results in the generation of large numbers of T cells expressing diverse TCRs and is referred to as combinatorial diversity.

Thymic selection narrows the peripheral TCR repertoire, eliminating cells with inept or auto-reactive receptors (Goldrath & Bevan, 1999).

The constant domain of the TCR chains continues into short hinge regions in which cysteine residues form disulfide bonds, which contributes to a link between the two chains. The variable domain of both TCR chains contain three hypervariable or complementarity-determining regions (CDR1-3) which are involved in the recognition and binding of the MHC-peptide complex. The CDR1 and CDR2 regions are responsible for contacting mostly with the MHC surface, whilst CDR3 binds mostly to the exposed peptide residues. The CDR3 regions are the most variable section of the TCR chain, and are the principal determinants of specificity (Goldrath & Bevan, 1999). The TCR has an extremely diverse variable region to allow it to specifically recognise the vast diversity of antigenic peptides. TCR diversity of an individual is estimated to be approximately 10^6 unique TCR β chain CDR3 sequences (Arstila *et al.*, 1999).

1.4.2 T cell activation

Efficient T cell signalling is a necessary event in the activation of T cells and their subsequent proliferation. T cells express the CD8 or CD4 coreceptors that bind to non-polymorphic regions of MHC class I and II molecules, respectively. These coreceptors act synergistically with TCR during antigen recognition to strengthen the adhesion between the T cells and the target and to increase the sensitivity of TCR responsiveness (Zamoyska, 1998). Upon initial T cell stimulus, a structure is formed around the region of contact between the APC and T cell, called the immunological synapse, as a consequence of a reorganisation of T cell membrane proteins, which it is thought to further facilitate TCR signalling (Grakoui *et al.*, 1999). Components required for TCR signalling congregate at the immunological synapse and form supramolecular activation clusters (SMACs). Adhesion molecules such as leukocyte function-associated antigen-1 (LFA-1) and CD2 segregate to the peripheral SMAC (p-SMAC), surrounding a central cluster (c-SMAC) (Monks *et al.*, 1998).

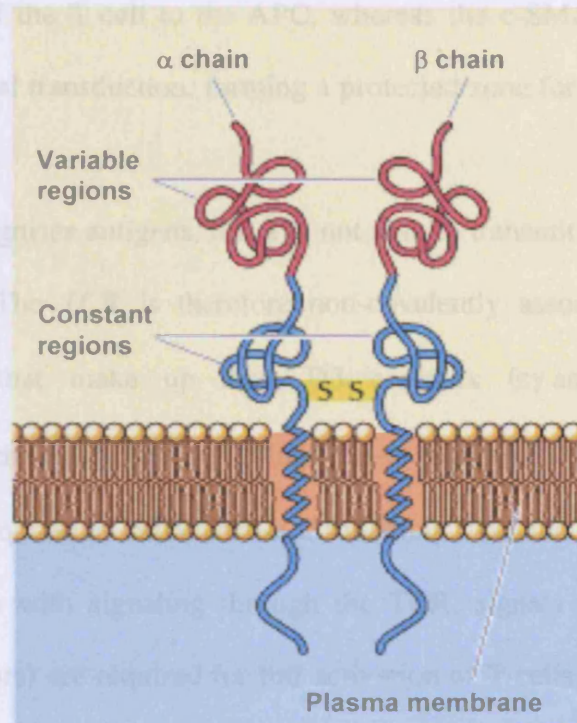


Figure 1.6 Structure of the TCR (adapted from Cooper, 2000). The schematic diagram of the $\alpha\beta$ TCR shows the domains of a typical TCR specific for a peptide-MHC complex. The TCR is a heterodimeric membrane bound molecule consisting of an α and β chain which are covalently linked by disulphide bonds. The chains consist of an N-terminal region containing a variable and constant domain, a transmembrane region and a cytoplasmic tail. The variable domains are encoded by the variable gene (V), the joining gene (J), and in the case of the β chain the diversity gene (D) (not shown). The antigen-recognition site of the TCR consists of a flattened region formed by the α and β chain.

The central SMAC (c-SMAC) includes engaged TCRs, the CD3 complex, CD28 and intracellularly the protein tyrosine kinases Lck and Fyn. The p-SMAC ensures that the APC and T cell remain together for a sufficient duration to allow signal transduction, providing an adhesive anchoring of the T cell to the APC, whereas the c-SMAC contains the necessary proteins for TCR signal transduction, forming a protected zone for TCR signalling (Monks *et al.*, 1998).

The TCR recognises antigens, but it is not able to transmit biochemical signals to the interior of the cell. The TCR is therefore non-covalently associated with a complex of polypeptide chains that make up the CD3 complex ($\epsilon\gamma$ and $\epsilon\delta$ heterodimers), and a homodimer called ζ chain (Call *et al.*, 2002). These form the TCR complex that transduce intracellular signals required to initiate effector functions when the T cell is activated.

In conjunction with signaling through the TCR, signals from accessory molecules (costimulatory receptors) are required for full activation of T cells and clonal expansion. The costimulatory molecule, CD28, is constitutively expressed by T cells and binds to the costimulatory ligands CD80 (B7.1) and CD86 (B7.2), which are expressed by activated APCs. CD28-mediated signaling is fundamental for initiating the response of naïve T cells. In the absence of CD28-B7 interactions, engagement of the TCR alone is not capable of activating T cells, and may induce a state of T cell unresponsiveness (Acuto & Michel, 2003; Lucas *et al.*, 1995). The necessity for costimulation ensures that naïve T cells are activated fully by APCs that carry harmful antigens, and not by other bystander cells that express harmless foreign substances.

In addition to CD28, other costimulatory molecules, which are expressed by T cells once activated, help to sustain or increase costimulatory signals for T cell responses. These include CD40L, CD27, ICOS (inducible costimulator) and 4-1BB (CD137) that are expressed by activated T cells and bind to counter-molecules expressed on activated APCs. These

costimulatory molecules provide additional signals that favour T cell activation and proliferation (Croft, 2003).

T cells also express inhibitory receptors that are important in regulating the immune response. Proteins homologous to CD28 are essential for limiting or terminating T cell responses. CTLA-4 is an additional receptor for B7 molecules but delivers an inhibitory signal to the activated T cells. CTLA-4 limits production of IL-2 and thereby the proliferative response of the T cell (Chen, 2004). Another member of CD28 family is the programmed death-1 (PD-1), which is expressed transiently by activated T cells and can bind to two ligands, PD-L1 (constitutively expressed by a range of cells) and PD-L2 (expressed by APCs during an inflammatory response). The binding of PD-1 on T cells to its ligands inhibits T cell activation and plays a role in maintaining peripheral tolerance (Keir *et al.*, 2008; Okazaki & Honjo, 2006).

Peripheral tolerance is induced when mature T cells recognise self-antigens or without adequate levels of costimulation that are required for T cell activation in the peripheral tissue. This leads to anergy, deletion by apoptosis (activation-induced cell death) or suppression by Tregs (see section 1.2.2.2). Anergy is the functional inactivation of T cells. T cells may engage inhibitory receptors such as CTLA-4 and PD-1 that block activation and consequently induce functional unresponsiveness. Self-antigen recognition by T cells without costimulation may also lead to an excess of intracellular proapoptotic proteins or expression of death receptors and their ligands, resulting in the apoptotic death of the cells (see section 1.9 Apoptosis) (Srinivasan & Frauwirth, 2009).

1.5 Tumour Immunity

The immune system plays an essential role in tumourigenesis, which can be associated with either the success or failure to protect against tumour development. It is involved in: early tumourigenesis, where the immune system can provide surveillance against cancer, thus preventing tumour development (e.g. NK and T cells); and also in established tumours, in part

through tolerance (e.g Tregs). In fact, one of the features that allow malignant tumours to grow is the ability of tumour cells to evade or overcome the immune system mechanisms.

Understanding the complex involvement of the immune system in tumourigenesis is crucial for the development of cancer immunotherapeutic approaches.

1.5.1 Tumour antigens

The expression of a molecule that is abnormal in appearance, concentration, or location, or is expressed at an unusual time during development in a tumour cell, may be recognised as a foreign antigen by the immune system. A wide variety of tumour antigens have been identified in humans and animal cancers. Two different approaches have been used in an attempt to identify T cell epitopes from tumour antigens. The first approach is direct and often called genetic immunology, and allowed the identification of the first tumour antigen, melanoma antigen genes-1 (MAGE-1) (van der Bruggen *et al.*, 1991). This approach typically involves tumour-reactive T cells derived from mixed lymphocyte-tumour cultures, in which T cells from a cancer patient are stimulated *in vitro* with autologous tumour cells. The tumour-reactive T cells are used to screen cDNA libraries prepared from autologous tumour cells for expression of a potential antigen. Such approach has been most successful in identifying melanoma-associated antigens. However, it has been difficult to employ this method for the identification of other cancer antigens because it is difficult to isolate and grow tumour cell lines from the majority of cancers. Another limitation is the requirement of large numbers of long-term stable T cell clones or lines for screening, which frequently cannot be achieved.

An alternative approach has been widely used to overcome these limitations, known as a reverse immunology approach. Firstly, a candidate tumour antigen is proposed based on its differential expression pattern in cancer. Potential peptide epitopes are predicted from the protein sequence using computer-based prediction of peptide:MHC binding. Several algorithms have been developed to identify candidate peptides based on their predicted binding affinities to MHC, such as BIMAS and SYFPEITHI. Synthetic peptides are then used

to prime or restimulate T cells *in vitro*, which allows for the identification of peptides that are immunogenic. This is followed by the generation of a T cell line or clone that specifically recognises the peptide, then testing of the T cells against tumour cells that naturally express the antigen of interest, in effect providing validation of the protein as a tumour antigen. This approach is not dependent on the availability of autologous tumour cells, requires isolation of fewer lymphocytes from patients when compared to the direct approach, and allows for rapid screen of a large number of candidate antigens (Viatte *et al.*, 2006). However, the algorithms are not completely accurate in predicting peptides, thus there is a risk that the predicted peptides are not actually epitopes and T cells generated against those peptides may not kill tumour cells.

Tumour antigens can be classified by various specifications. The earliest classification consists of two groups based on their patterns of expression: tumour-specific antigens (TSA), which are unique to tumour cells but not found in normal cells, and tumour-associated antigens (TAA), which can be found in normal cells but their expression in tumour cells is aberrant or dysregulated. However, a more comprehensive division of tumour antigens is based on their molecular structure and source, which includes products of mutated genes, overexpressed or abnormally expressed normal proteins, altered glycolipid and glycoprotein antigens, oncofetal antigens, tissue-specific differentiation antigens, and antigens of oncogenic viruses (Abbas *et al.*, 2007).

In many tumours, cellular oncogenes or tumour suppressor genes are frequently affected by mutations, deletion, chromosomal translocations or viral gene insertion. The products of these cellular oncogenes or tumour suppressor genes are required for malignant transformation and are synthesized in the cytoplasm of tumour cells. Therefore, this may lead to the production of altered peptides from these products by the MHC class I processing pathway. These altered genes and their peptides are not present in normal cells, thereby potential epitopes could be created for T cell recognition. Examples of oncogenes known to

be prone to having transforming mutations and used to generate potential epitopes are Ras (Linard *et al.*, 2002), p53 (Ciernik *et al.*, 1996) and Bcr-Abl (Yotnda *et al.*, 1998). Randomly mutated genes whose products are not associated in tumourigenesis may also produce tumour antigens.

In several human tumours, the antigens that elicit immune responses appear to be normal proteins that are overexpressed, or whose expression is normally limited to a particular tissue or stage of development, but is dysregulated in the tumours. Normal cellular genes can undergo amplification during malignancy, resulting in a higher level of expression by tumour cells when compared to normal cells, for example Her-2, which is normally expressed by cardiac tissue but is overexpressed in breast cancer cells (Peoples *et al.*, 1995). Overexpression of Her-2 has also been reported in melanoma, ovary, lung, pancreas and prostate cancers. Human telomerase reverse transcriptase (hTERT) (Minev *et al.*, 2000) and Wilm's Tumour 1 (WT1) (Oka *et al.*, 2004) proteins are also overexpressed proteins and are examples of targets for immunotherapy.

Tumour antigens can also be proteins that are overexpressed in cancer cells and normally found in a developing fetus, but not in adult tissue. Carcino-embryonic antigen (CEA) is an example of an oncofetal antigen. CEA is normally expressed in the liver, pancreas and intestines of a human foetus but has been shown to be increased in cancer cells found in the colon, pancreas, lung, and breast (Kawashima *et al.*, 1999). Tumour specificity can also be conferred by tumour antigens derived from altered glycolipids and glycoproteins. Alterations in protein glycosylation are frequent in cancers and are often related with progression. Elevated expression of glycolipid and some glycoprotein antigens are characteristics of many tumours, and these molecules are often structurally different from those expressed in normal cells. These tumour antigens include a variety of gangliosides, blood group antigens and mucins. For example, the glycoprotein MUC-1 has been shown to

be overexpressed and contain aberrant carbohydrate modifications in breast cancer and several other cancers (Brossart *et al.*, 1999).

Differentiation antigens are molecules present on tumour cells and their normal cell counterparts. The best studied differentiation antigens are from melanoma, for example, Melan-A/MART-1 (Coulie *et al.*, 1994), gp100 (Kawakami *et al.*, 1995) and tyrosinase (Wolfel *et al.*, 1994), which are expressed by melanoma cells as well as normal melanocytes. Other solid tumours, such as prostate cancer, also contain proteins that have been described as tissue specific differentiation antigens, which include prostate specific membrane antigen (PSMA) (Horiguchi *et al.*, 2002). Cancer/testis antigens are a type of antigens that are expressed on male germ cells and on a variety of tumours, but not in normal somatic cells. An example of which are MAGE (Chaux *et al.*, 1999) and NY-ESO-1 (Jager *et al.*, 1998).

In malignancies associated with viral infection, virally expressed proteins may also act as tumour antigens. Various human cancers have viral associations and are characterized by expression of viral gene products, for example, the Epstein-Barr virus (EBV) and human papillomavirus (HPV), which are associated with B cell lymphomas and cervical carcinoma, respectively (Khanna *et al.*, 1998; Rensing *et al.*, 1995). Because viral peptides are considered foreign and exist only in infected cells, it makes viral antigens attractive tumour antigens.

Table 1.1 Types of tumour antigens

Type of Antigen	Examples of human tumour antigens
Products of mutated genes	Ras (Linard <i>et al.</i> , 2002), p53 (Ciernik <i>et al.</i> , 1996), Bcr-Abl (Yotnda <i>et al.</i> , 1998)
Overexpressed normal proteins	Her-2 (Peoples <i>et al.</i> , 1995), hTERT (Minev <i>et al.</i> , 2000), WT1 (Oka <i>et al.</i> , 2004)
Differentiation antigens (Tissue-specific)	Melan-A (Coulie <i>et al.</i> , 1994), gp100 (Kawakami <i>et al.</i> , 1995), PSMA (Horiguchi <i>et al.</i> , 2002)
Oncofetal antigens	CEA (Kawashima <i>et al.</i> , 1999)
Cancer/Testis antigens	MAGE (Chaux <i>et al.</i> , 1999), NY-ESO-1 (Jager <i>et al.</i> , 1998)
Altered glycolipid & glycoprotein antigens	MUC-1 (Brossart <i>et al.</i> , 1999)
Antigens of oncogenic viruses	EBV (Khanna <i>et al.</i> , 1998), HPV (Rensing <i>et al.</i> , 1995)

1.5.2 Immunity to tumours

Both innate and adaptive components play an important role in tumour immunosurveillance, which involves the detection and eradication of tumour cells (Dunn *et al.*, 2004). Mice lacking expression of the recombinae activating genes (RAG) 1 and 2 have provided evidence for the role of lymphocytes in the prevention of tumour development, as they are unable to somatically re-arrange their antigen receptors and therefore cannot produce $\alpha\beta$ T cells, B cells, NKT cells or $\gamma\delta$ T cells (Shinkai *et al.*, 1992). These mice have been shown in several studies to have a higher incidence of carcinogen induced tumour formation and also spontaneous tumour development as they age (model for immunosurveillance) (Shankaran *et al.*, 2001; Smyth *et al.*, 2001).

These studies collectively suggest several mechanisms for tumour immunosurveillance that may be operating simultaneously:

- 1) Expression of tumour specific markers and pro-inflammatory danger signals: promote the immune system to act. Cells of the innate system (NK cells, NKT cells, $\gamma\delta$ T cells, macrophages and DCs) are recruited to the tumour site. The infiltrating lymphocytes such as the NK, NKT or $\gamma\delta$ T cells are stimulated to produce IFN γ (Dunn *et al.*, 2002).
- 2) Production of IFN γ : In both humans and mice with malignancies, the lack of IFN γ production leaves the host susceptible to tumour progression. Mice lacking either IFN γ , STAT-1 (which is a component of the IFN γ signalling pathway) or the IFN γ receptor have a significantly greater incidence of carcinogen induced, spontaneous and genetically driven tumour progression (Shankaran *et al.*, 2001; Street *et al.*, 2001; Street *et al.*, 2002).
- 3) Chemokines and angiogenesis: IFN γ also promotes the production of chemokines (CXCL10, CXCL9) that can inhibit angiogenesis by blocking the formation of new blood vessels within the tumour. The lack of blood supply and the effect of IFN γ accelerate tumour cell death. Tumor cell debris produced as a result of tumor death is then ingested

by DCs, followed by the migration of DCs to the draining lymph nodes (Dunn *et al.*, 2002).

- 4) Cytotoxicity induced by innate immune cells: The chemokines produced during the inflammatory responses recruit more immune cells to the site. The recruited tumour-infiltrating NK and macrophages produce IL-12 and IFN γ , which kill more cells by TRAIL, perforin and reactive oxygen and nitrogen intermediates (Dunn *et al.*, 2002).
- 5) NK cell mediated cytotoxicity: NK cells are capable of killing various types of tumour cells, particularly cells that have reduced MHC class I expression. NKG2D is a lectin-type activating receptor expressed at the surface of NK cell as well as on $\gamma\delta$ T cells. Its ligands are MICA/B (MHC-class I polypeptide related sequence A/B), and ULBPs (UL16 binding proteins) in humans, and RAE1 (retinoic acid early transcript 1) and H60 in mice. Such ligands can be expressed at the surface of infected, stressed or tumour cells (Diefenbach & Raulet, 2002). Mice depleted of both NK and NKT cells by using the anti-NK1.1 monoclonal antibody or with anti-asialo-GM1, which depletes NK but not NKT cells, were more susceptible to MCA-induced tumourigenesis than the wild-type (Smyth *et al.*, 2001) .
- 6) NKT cells: Mice that are injected with NKT cell-activating ligand (α -galactosylceramide) can block tumour metatasis, which is mediated by the production of IFN γ by NKT cells (Hayakawa *et al.*, 2003).
- 7) $\gamma\delta$ T cells: *In vivo* studies have shown that mice deficient for $\gamma\delta$ T cells (TCR $\gamma\delta^{-/-}$) have a higher incidence of carcinogen induced tumour formation (Girardi *et al.*, 2003; Girardi *et al.*, 2001). It has also been suggested that $\gamma\delta$ T cells may also be an important source of IFN γ during the initial stages of tumour surveillance (Gao *et al.*, 2003).
- 8) Induction of adaptive immunity: DCs trigger the differentiation of CD4⁺ Th1 cells in draining lymph nodes, which in turn helps the development of CD8⁺T cells. Tumour-specific CD4⁺ and CD8⁺ T cells then migrate to the tumour site, where CTLs carry out

the perforin/granzyme-mediated cytotoxicity of the antigen-bearing tumour cells that remain at the site (Dunn *et al.*, 2002). In further support of the role of $\alpha\beta$ T cells in tumour surveillance, mice lacking $\alpha\beta$ T cells (TCR $\beta^{-/-}$) are more susceptible to carcinogen induced tumour formation (Girardi *et al.*, 2003).

- 9) Induction of cytotoxic effector cells: The ability of the immune system to kill tumour cells is a crucial effector function of tumour immunosurveillance, particularly the release of perforin which is present in the cytotoxic granules from CD8⁺ T cells, NKT, NK and $\gamma\delta$ T cells. *In vivo* studies showed that mice that are deficient for perforin have an increased incidence of carcinogen induced and spontaneous tumour formation (Street *et al.*, 2001; van den Broek *et al.*, 1996).

There are several lines of evidence showing that tumour immunosurveillance exists in humans. Individuals with immunodeficiencies have a higher incidence of tumour development when compared with immunocompetent individuals. Moreover, transplant patients on immunosuppressive therapy are predisposed to developing tumours, especially virally induced malignancies, but other tumours with no known viral association can also occur, such as melanoma (Buell *et al.*, 2005; Swann & Smyth, 2007). More direct evidence for tumour surveillance in humans comes from the positive association between the presence of infiltrating lymphocytes in a tumour and increased patient survival. A correlation between positive prognosis and tumour infiltrating lymphocytes was first observed in patients with melanoma, showing that patients with high levels of CD8⁺ T cell infiltration survive longer (Clark *et al.*, 1989; Clemente *et al.*, 1996). In concurrence with this observation, correlations have also been found in patients with other cancers, such as breast, bladder, colorectal, prostate and ovarian cancers (Mercader *et al.*, 2001; Ropponen *et al.*, 1997; Tomsova *et al.*, 2008).

1.5.3 Tumour escape

It has now become apparent that interaction between the immune system and tumourigenesis is dynamic and complex, with the immune system playing a fundamental role in shaping tumour development. The tumour may escape elimination and exist in a state of equilibrium, where it is chronically maintained by the immune system in a sub-clinically observed state (Dunn *et al.*, 2002). One example of tumour equilibrium has been demonstrated in transmission of cancer from organ transplant donor to recipients. Two organ transplant recipients developed metastatic melanoma, post kidney transplant from the same donor. It was found that the kidney donor had been treated for primary melanoma sixteen years prior to donation indicating that the tumour had been maintained in a state of equilibrium by the immune system of the donor (MacKie *et al.*, 2003).

However, the genetic instability of a tumour allows it to evolve and consequently escape the attention of the immune system. The surviving variant tumour cells acquire insensitivity to immunological detection and/or elimination and begin to expand in an uncontrolled way. Many malignant tumours have mechanisms that enable them to evade or resist host immune responses:

- 1) Antigen loss: Tumour immunogenicity decreases under selective pressure of a fully functional immune system, which could allow selective growth of antigen-loss mutants. Tumour cells can lose their antigens by mutation, which could be a loss of the whole protein or changes in immunodominant T cell epitopes, and therefore affect their recognition by T cells, antigen processing or binding to the MHC (Campoli *et al.*, 2002).
- 2) Alteration of antigen processing and presentation: A mechanism by which tumour cells avoid immune detection is by altering or losing molecules involved in the antigen processing and presentation pathways, such as TAP and the immunoproteasome subunits LMP2 and LMP7 (Seliger *et al.*, 2000). Tumours are able to evade CTL mediated immune rejection by downregulation or loss of MHC class I molecules (Campoli *et al.*, 2002).

- 3) Tumour unresponsiveness to IFN γ : indirectly leads to antigen processing or presentation defects due to the lack or abnormal function of the IFN γ receptor signalling pathway, and consequently failure to upregulate their existing low levels of MHC class I (Kaplan *et al.*, 1998).
- 4) Immunosuppressive cytokines & Tregs: IL-10 and TGF- β can act in an autocrine fashion to directly facilitate tumour growth and also alter or inhibit an immune response, directly or indirectly by activating Treg cells (Chen *et al.*, 2005; Kawamura *et al.*, 2002). Tregs can suppress anti-tumour CTL responses and their presence at the site of tumour development is associated with poor survival of cancer patients (see section 1.2.2.2).
- 5) Escape apoptosis: tumour cells express molecules that are able to prevent cell death, e.g. anti-apoptotic proteins survivin and BCL-X_L (Dohi *et al.*, 2004; Hinz *et al.*, 2000).
- 6) Resistance to cytotoxic pathways: by mutations in the gene encoding Fas, or mutations in the gene encoding TRAIL (Shin *et al.*, 2001; Wohlfart *et al.*, 2004). Upregulation of FasL by the tumour cells can directly affect T cell responses, as FasL engagement of Fas on T cell can induce T cell death.
- 7) Tolerogenic environment: Tumour cells may suppress the induction of proinflammatory danger signals, which lead to impaired DC differentiation, maturation and function. This in turn renders the T cells that engage these DCs to be anergized rather than activated. The tumour environment may promote the development and recruitment of suppressive immune cells, e.g. IL-13-producing NKT cells, and Tregs (Terabe & Berzofsky, 2004). Tumour instructed immature myeloid cells can overproduce nitric oxide or arginase-1, which can inhibit T cell function. Some tumours can also overproduce inhibitors of T cells function such as IDO (indoleamine 2,3-dioxygenase and galectin-1) (Serafini *et al.*, 2006).

Taking these observations together, it is evident that both innate and adaptive immunity participate not only in the protection the host from tumour development but also in shaping or editing the immunogenicity of tumours that may ultimately appear. A term known

as cancer immunoediting has been proposed to encompass tumour surveillance (elimination), tumour equilibrium and escape (Dunn *et al.*, 2002)

1.6 Tumour immunotherapy

There are currently several conventional treatment strategies to tackle a developing tumour, including surgery, radiotherapy and chemotherapy. These treatments have increased the overall survival rate of cancer patients. For example, the use of chemotherapy following surgery reduces the risk of death from operable pancreatic cancer by around 30% (Cancer Research UK). The first step in cancer treatment is often surgery, which involves removing the primary tumour and often the surrounding tissue. Surgery is obviously not an option when the malignancy is diffuse, such as leukaemias, nor when the tumour has metastasised to multiple sites throughout the body.

Conventional radiotherapy and/or chemotherapy are normally implemented after surgery was performed or ruled out. Both therapies exploit the continuous proliferative capacity of tumour cells by targeting rapidly dividing cells, and therefore this enables the tumour cells to be targeted over resting cells that undergo normal rounds of proliferation. However, these therapies may also have harmful short and long term side effects that often affect the quality of life of the cancer patient. The side effects, such as nausea, vomiting and loss of ability to fight infection, arise because radiation and many chemotherapeutic drugs can also harm normal, rapidly dividing cells. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body. Accumulation of the chemotherapeutic drugs and their metabolites can occur in the liver and kidney, leading to hepatic and nephritic toxicity. Unfortunately, the high doses of radiation and chemotherapeutic drugs that are applied may encourage tumour cells to become resistant to these treatments (Mak & Saunders, 2006).

Because of the inefficiencies, tumour resistance, and side effects associated with conventional therapies, the treatment of cancer still remains challenging. For these reasons, novel treatments used as an alternative, or in combination with conventional therapies are

required. There is a great interest in developing approaches that harness the immune system or its components to arbitrate tumour protection and regression. The major strategies for cancer immunotherapy strive to provide anti-tumour effectors to patients, actively immunize patients against their tumours, and stimulate the patients' own anti-tumour immune responses. There are two broad classes of immunotherapies: active and passive immunotherapy. Passive immunotherapy involves adoptive transfer of *ex vivo* activated immune cells, tumour-specific antibodies or immunomodulators (cytokines), thus conferring anti-tumour immunity to a tumour-bearing host. Conversely, active immunotherapy is designed to elicit or augment an anti-tumour response within a tumour-bearing host in order to mediate tumour regression (Rescigno *et al.*, 2007).

1.6.1 Monoclonal antibodies

Monoclonal antibody therapy has proven to be one of the most successful forms of immunotherapy for a variety of haematologic malignancies and solid tumours. Several monoclonal antibodies that target tumour antigens, such as tumour growth factors, are clinically approved for tumour treatment, with less toxicity than conventional therapy. Their effects are exerted by mechanisms which include antibody dependent cellular cytotoxicity (ADCC), activation of complement, functional interference of pathways essential for tumour growth, and recruitment of other immune effector cells to mediate lysis (King *et al.*, 2008).

Successful therapy has been seen with monoclonal antibodies that bind to surface proteins that are highly expressed on hematologic malignancies, which include, for example, CD20 in non-Hodgkin's lymphoma (rituximab) (Maloney, 2005), CD52 in chronic lymphocytic leukaemia (CLL) (alemtuzumab) (Keating *et al.*, 2002), and CD33 in acute myelogenous leukaemia (AML) (gemtuzumab) (Bross *et al.*, 2001). Other monoclonal antibodies have been approved for the treatment of solid tumours, which include monoclonal antibodies directed against Her-2 (trastuzumab) for breast cancer (Hudis, 2007), vascular endothelial growth factor (VEGF) (bevacizumab) for colorectal and lung cancer (Ferrara *et*

al., 2004), and epidermal growth factor receptors (EGFR) (cetuximab) for colorectal cancer (Galizia *et al.*, 2007). Some monoclonal antibodies may also be conjugated with toxins or with radioisotopes so that the antibodies deliver these toxic agents specifically to the tumour cells. The immunoconjugate kills the tumour cells because the toxin or radioisotope is internalised following antibody binding to the tumour cell (Dougan & Dranoff, 2009).

The potential of use monoclonal antibodies with immune-modulating activity in humans is being evaluated for cancer treatment. These include antibodies that block CTLA-4, which plays an important role in regulating immune responses by reducing the proliferation of activated T cells. However, a common complication of the treatment using anti-CTLA-4 antibodies in clinical trials has been the development of autoimmune reactions (O'Day *et al.*, 2007). Another example is the negative immunoregulatory receptor PD-1, which is currently been evaluated in clinical trials as an antibody target for immunotherapy (Hirano *et al.*, 2005).

Antibodies against CD28 are also known to potentiate anti-tumour immunity. Some anti-CD28 antibodies, known as superagonists, bind to a particular epitope in CD28 and can induce T cell proliferation in absence of antigen recognition. These superagonist anti-CD28 antibodies were evaluated in clinical trials (Suntharalingam *et al.*, 2006). However, a phase I clinical trial with agonist antibodies against CD28 (TGN1412) resulted in a dramatic clinical toxicity in a cohort of normal volunteers. This led to over activation of T cells and a violent cytokine storm (substantial increases in the concentration of several inflammatory cytokines), precipitating the release of endogenous molecules affecting other cells. Each of the volunteers receiving the first dose required hospitalization for cytokine-release syndrome, including severe cases of multiorgan failure (Suntharalingam *et al.*, 2006). The outcome of this trial clearly indicates that caution must be taken with the clinical development of other immunostimulatory antibodies and very careful preclinical and clinical testing in humans is required to minimise toxicity risks.

An alternative approach to direct the cytotoxic potential of effector cells has been the use of bispecific T cell engagers (BiTE) antibodies. BiTEs are based on single-chain antibodies, recognising with one arm a cell surface protein on tumour cells (like a conventional monoclonal antibody) and with the other arm, recognising the CD3 complex associated with TCR, leading to the formation of synapse and consequently lysis of tumour cell without requirement of TCR or peptide:MHC complex. Clinical activity of a BiTE antibody known as blinatumomab, with dual specificity for CD19 and CD3 has been evaluated in non-Hodgkin's lymphoma patients. All seven patients treated with low doses of blinatumomab experienced tumour regression. A phase II trial is currently investigating the activity of this BiTE antibody in acute lymphoblastic leukaemia (Bargou *et al.*, 2008). The activity has a passive effect and will be ceased once the reagent is removed from the body and involves effector cells that have no specificity for the target.

The efficiency of monoclonal antibody application is dependent on the presence of a tumour antigen on the cell surface that can be bound successfully by an antibody, the extent to which antibodies distribute once administered, and the expression profile of the target antigen. It has a number of limitations, which include requirement of repeated antibody infusions because of their half-life and clearance from circulation; a memory response is not generated with this type of therapy; also antibodies can only recognise specific proteins presented on the surface. As intracellular proteins are processed into large numbers of peptides and expressed on the cell surface in association with MHC (see section 1.3), these peptides-MHC complexes can then be recognised by T cells, and therefore T cell based immunotherapy may target a wider range of intracellular tumour antigens (King *et al.*, 2008).

1.6.2 Cytokines

Many cytokines have the potential to induce nonspecific inflammatory responses, which by themselves may have anti-tumour reactivity (see section 1.5.2). Cytokine based tumour therapies involve the systemic transfer of purified cytokines to tumour-bearing

patients, in order to promote an anti-tumour response. IL-2 promotes the expansion of tumour-specific T cells and counteracts the effects of immunosuppressive cytokines. However, IL-2 has only been effective in approximately 10-20% of patients with melanoma and renal cell carcinoma (Atkins *et al.*, 1999; Rosenberg *et al.*, 1994). A limitation of cytokine based therapies is the toxic side effects of administering high systemic concentrations of cytokine that are necessary to induce an anti-tumour response. In order to overcome this problem, approaches have been generated to deliver cytokines to the site of tumour development in a sustained manner, instead of systemic administration (Veelken *et al.*, 1997). However, the generation of cytokine-expressing cells can be time-consuming and often delays treatment, allowing the tumour to advance.

1.6.3 Prophylactic and Therapeutic Vaccines

Vaccine administration to combat tumourigenesis presents an attractive aim of tumour immunotherapy. With respect to cancer prevention, the only prophylactic vaccines currently available are those that prevent infections by pathogens that have the potential to induce cancer development. The most common pathogen cancer vaccines are those directed against the oncogenic viruses. The ongoing vaccination program against hepatitis B virus has resulted in a reduction of hepatocellular carcinoma (Chang *et al.*, 2000). More recently, newly developed HPV vaccines promise to reduce the incidence of cancers associated with HPV infections. Indeed, these vaccines have been shown to be clinically successful for protection from infection and cervical diseases associated with HPV16 and/or HPV18 types (Garland *et al.*, 2007; Kahn & Burk, 2007). In addition, a vaccine against *Helicobacter pylori* is under development, with the purpose of reducing the incidence of gastric lymphomas and carcinomas (Wong *et al.*, 2004).

Development of prophylactic vaccines for tumour with non-pathogen aetiology would be ideal but has yet to be accomplished. Therapeutic vaccines are aimed to activate the patient's own immune system to combat already established cancer. They provide an

advantage over antibody therapy that, if successful, can induce long-lasting immunological memory that can protect against tumour recurrence. Although various attempts are being made to generate therapeutic vaccines against various cancers, encouraging results have only been seen in a small number of clinical trials, and there is currently no FDA (Food and Drug Administration) approved therapeutic vaccine. Various approaches for generating therapeutic immune responses to tumours have been endeavoured, including antigen-specific vaccines (peptide, protein, DNA), whole tumour cell vaccines, heat shock protein (HSP) vaccines, and DC vaccines (Dougan & Dranoff, 2009; Itoh *et al.*, 2009).

Peptide based vaccines involves the transfer of either peptide or the protein from which the antigenic peptides are derived. A concern when using a whole protein is the competition between the desired and already existing peptides for MHC binding. Another concern is the condition of *in vivo* processing by either the standard proteasome or immunoproteasome, which if absent may prevent the generation of the desired peptides. Vaccination using peptide or recombinant protein along with adjuvants has been conducted in several trials involving patients with metastatic melanoma, eosophogeal, colon, breast and prostate cancer (Dougan & Dranoff, 2009; Itoh *et al.*, 2009).

Most clinical research to date on humans using tumour antigens has been done with melanoma and cancer-testis antigens, as these are usually highly immunogenic and therefore easier to mount an antigen-specific response. For example, vaccination of melanoma patients with peptides derived from tyrosinase, gp100 or MelanA/MART-1 antigens have induced immune responses against these peptides, with tumour regression occurring in all three cases (Jager *et al.*, 1996). NY-ESO-1-based vaccines have also been examined in multiple trials for a variety of cancers, including melanoma, ovarian carcinoma and non-small cell lung cancer (Gnjatic *et al.*, 2006). Peptide-specific immune responses have also been observed in 92% patients with breast cancer received Her-2 vaccine plus GM-CSF (Disis *et al.*, 2002), and in

55% of prostate cancer patients vaccinated with prostate-specific antigen (PSA) (Perambakam *et al.*, 2006).

The results from clinical trials of peptide vaccines suggest that few cancer patients show improved clinical benefits that correlate with peptide-specific immune responses. Alternatives to transferring the tumour antigen alone have been considered to increase the likelihood of generating an anti-tumour response, such as the use of whole tumours, HSPs, DCs, or cytokines as adjuvants (Dougan & Dranoff, 2009; Itoh *et al.*, 2009) .

An approach to stimulate immune responses against tumours is to vaccinate patients with autologous or allogeneic tumour cells, which can be genetically modified and/or administered in the presence of adjuvant. Clinical responses have been shown in several initial clinical studies but these studies failed to show significant efficacy in the randomized phase III trials (Itoh *et al.*, 2009). For example, treatment with a combination of allogeneic melanoma cells with BCG (Canvaxin trial) showed efficacy in the early stages of clinical development (5 years overall survival: 49% treated patients vs 37% controls), but further evaluation of this approach in phase III trials showed no significant difference between patients receiving this treatment compared with the controls (Faries & Morton, 2005). Other treatments involving the immunisation of prostate cancer patients with a vaccine that consists of two prostate cancer lines genetically modified to secrete GM-CSF (GVAX) did not succeed in showing clinical benefits in the phase III and the trials were discontinued (Itoh *et al.*, 2009; Small *et al.*, 2007).

A major drawback to whole tumour cell vaccines is the weak antigen presentation and therefore poor ability to stimulate a potent immune response. Tumour cells, such as allogeneic cells, might not be an appropriate source of a therapeutic vaccine for all cancer patients. These cells may induce immune response to irrelevant antigens that can competitively suppress anti-tumour responses. Other disadvantages of this approach are the cost and time to

grow autologous tumour cells from a patient, which sometimes are impossible to isolate in sufficient quantity to provide adequate source material (Itoh *et al.*, 2009).

Tumour-derived HSPs have been evaluated as cancer vaccine adjuvants for various types of cancer. HSPs are not antigen themselves but can promote immune responses. They can act as chaperones for peptides between necrotic cells and APCs, therefore contributing to the process of T cell cross-priming. HSPs are not particularly specific for peptides and proteins they chaperone, allowing both known purified peptides or a mixture of peptides from tumour cell lysates to be combined with HSPs prior to transfer. A major disadvantage of this approach is the requirement of personalized vaccine preparation for each patient. Although there are contradictory views about the efficacy of HSP-based vaccines, a vaccine using HSP as adjuvant was recently approved in Russia for the treatment of patients with kidney cancer at intermediate risk for disease recurrence (Itoh *et al.*, 2009; Wood *et al.*, 2008).

DCs are potent inducers of adaptive immunity, driving the activation of T cells and therefore they are an attractive approach for tumour vaccination. Their *ex vivo* activation may also overcome the lack of *in vivo* inflammatory signals that can leave DCs in an immature state (see section 1.5.4). The delivery of antigen to DCs may be tumour cell lysates, antigenic peptides or a mixture of long overlapping peptides, exposure to whole recombinant proteins, transfection of antigen-encoding DNA or RNA, and fusion with irradiated tumour cells (Dougan & Dranoff, 2009; Itoh *et al.*, 2009). Although a number of DC cancer vaccine trials have shown some encouraging preliminary results, only one has thus far succeeded in showing clinical benefits in phase III trials. Results from a phase III clinical trial demonstrated for the first time that a cancer vaccine known as Sipuleucel-T/Provenge, which consisted of autologous DCs loaded *ex vivo* with PAP/GM-CSF, can significantly prolong overall survival by 4.1 months in patients with advanced prostate cancer (Higano *et al.*, 2009). It is expected that Sipuleucel-T will be approved by the FDA by 2010.

Apart from the success of this trial, DC-based vaccines for other cancers have not been successful in showing clinical benefits. This is a personalized treatment, and it is difficult and expensive to grow large number of autologous DCs from all the patients. Inconsistency in trials results may be associated with a number of variables, such as the type and quality of DCs that vary with method of generation and maturation, choice of tumour antigen target and the form it takes, choice of adjuvant and frequency of vaccination. Collectively, the results from clinical trials suggest that DCs as vehicle for therapeutic cancer vaccination could hold the promise to circumvent some of the strategies tumours use to evade the immune system, but there are still technical issues to be resolved before the development of an effective therapeutic DC-based vaccine.

Despite the encouraging initial results observed in several clinical trials using different approaches, unfortunately clinical efficacy of cancer vaccines has so far been disappointing. According to response evaluation criteria in solid tumours (RECIST), only less than 5% overall objective response rates were observed in patients with metastatic cancer who underwent different types of active immunotherapy (Itoh *et al.*, 2009; Rosenberg *et al.*, 2004). Overall, there are significant hurdles to be overcome before therapeutic cancer vaccines are to be considered effective in mediating cancer regression.

Given that established tumours clearly subvert the immune system, it is possible that single components therapies such as vaccine-only strategies are unlikely to ever be effective. Inadequate numbers or low avidity of T cells, failure of tumour to activate lymphocytes, tolerance, suppressor functions of tumour cells or the immune system itself are among the factors that prevent an effective anti-tumour T cell response and clinical responses upon active vaccination. Therefore, elimination of both tumour and lymphocyte-mediated immunosuppressive mechanisms without affecting anti-tumour effector cells seems to be a promising way to improve current therapy. Indeed, approaches that target multiple parts of

immunity and integrate elements that target tumour escape simultaneously are more likely to result in clinical success (Andersen *et al.*, 2008; Copier *et al.*, 2009).

Combination of anti-cancer vaccination with other immunotherapeutic agents, or with conventional therapy are been evaluated for treatment of cancer. Various studies are focussed on combining vaccines with cytokines such as IL-2 and GM-CSF, with immunomodulatory antibodies such as those against CTLA-4, PD-1, or specifically blocking secreted immunosuppressive molecules such as TGF- β , IL-10 as well as eliminating Treg cells may also be required (Andersen *et al.*, 2008; Copier *et al.*, 2009; Dougan & Dranoff, 2009). Further investigation of these manipulations in conjunction with vaccination may lead to a greater magnitude of immunological and clinical responses, and ultimately improve the efficacy of current immunotherapy.

1.6.4 Adoptive cell transfer

In attempts to overcome the obstacles associated with therapeutic vaccination, alternative T cell immunotherapy strategies have been considered. One strategy that carries great potential for generation of productive tumour immunity is adoptive cell transfer (ACT). ACT therapy relies on the *in vitro* expansion of T cells with appropriate specificity and avidity to mediate tumour regression upon reintroduction to a cancer patient. T cells can be obtained from various sites, including the peripheral blood, malignant effusions, lymph nodes and tumour biopsies (Rosenberg *et al.*, 2008).

Multiple clinical trials have been performed to establish the efficacy of using adoptive T cell therapy. ACT therapy has been particularly successful with EBV associated malignancies. The transfer of EBV specific allogeneic or autologous PBMC or T cell lines has shown to be efficient for the prevention and treatment of EBV-related diseases. These malignancies can develop in patients receiving immunosuppressed drugs after organ or bone marrow transplantation (Khanna *et al.*, 1999; Rooney *et al.*, 1998). The effectiveness of ACT directed against EBV has shown the feasibility of developing ACT approaches for solid

cancers, by demonstrating that administration of an anti-tumour T cell with high avidity and targeting a highly expressed antigen can indeed result in cancer regression (June, 2007).

ACT has achieved clinical responses in a small number of patients with metastatic melanoma. In one trial, CD8⁺ T cells specific for MelanA/MART-1 were purified from the PBMC of patients with metastatic melanoma, expanded *ex vivo* in the presence of IL-2 and reintroduced to patients. Eight out of twenty patients exhibited minor, mixed or stable anti-tumour responses (Yee *et al.*, 2002). Encouragingly, this resulted in T cell infiltration into both the skin and tumour tissue, but there was a selective outgrowth of tumours lacking expression of MART-1 (Yee *et al.*, 2000). Similarly, in another trial using T cells specific for MART-1, CTLs engrafted successfully, but disappointingly selected for tumours that did not express the antigen (Mackensen *et al.*, 2006). These results highlight the limitation of transferring monoclonal tumour specific CD8⁺ T cells, since tumour cells that either lack expression of the antigen or evolve under the selective pressure of the T cells to not express the antigen, are not affected and continue to develop. It is possible that the emergence of antigen escape variants can be overcome by, for example, infusing CTL clones with multiple antigenic specificities (June, 2007).

Another strategy of ACT is the use of CD8⁺ tumour infiltrating lymphocytes (TILs), which are isolated from tumour biopsy specimens. The tumour-specific T cells are selected *ex vivo* using IL-2 and various cell culture methods (June, 2007). TILs might have an advantage over CD8⁺ T cells purified from blood since the T cell population in this context may have higher frequency of tumour-reactive cells and is most likely to be polyclonal for the antigens expressed by the tumour and have already express homing receptors.

In human clinical trials, the transfer of TILs to patients with metastatic melanoma or renal cancer has proven to be unsuccessful in reaching objective clinical responses (Dreno *et al.*, 2002; Figlin *et al.*, 1999). However, in effort to improve clinical outcome of ACT therapy trials involving TILs, patients with metastatic melanoma were conditioned with chemotherapy

(cyclophosphamide and fludarabine), which have an immunosuppressive effect but no anti-melanoma activity, prior to the transfer of the expanded lymphocyte population (Dudley *et al.*, 2002; Dudley *et al.*, 2005). In a clinical trial of thirteen patients, there was an overall 50% objective response rate (Dudley *et al.*, 2002). Importantly, prolonged engraftment of TILs was observed in patients who were lymphodepleted by nonmyeloablative chemotherapy, and this was correlated with the clinical responses (Dudley *et al.*, 2005; Rosenberg & Dudley, 2004).

In two additional sequential trials of ACT with autologous TILs in patients with metastatic melanoma, host immunodepletion (consisting of chemotherapy and radiotherapy) followed by TILs transfer and IL-2 resulted in an objective response rate of 72% (Dudley *et al.*, 2008). These findings indicate that combination of chemotherapy and radiotherapy prior to cell transfer would help improve the therapeutic results of ACT.

It seems important to lymphodeplete patients, prior to adoptive transfer of cells, in order for those cells to persist *in vivo*. This conditioning regimen may augment the anti-tumour efficacy of ACT therapy by increasing levels homeostatic cytokines (such as IL-7 and IL-15), reducing competition with endogenous T cells, clearing host suppressor cells like Tregs, immunosuppressive cytokines required for tumour growth and improving the immunogenicity of the tumours, and by these means allow the transferred T cells to function in an optimal way and mediate tumour regression (Wrzesinski & Restifo, 2005) .

Current limitations that may preclude the efficacy of using TILs as a model therapy include the difficulty of obtaining cells from tumour biopsies of all patients and cancers, and also the time it takes to generate sufficient numbers to infuse back to patients and the possibility of autoimmunity and infection. Several areas need to be addressed in order to accomplish objective clinical responses during ACT therapy using CD8+ T cells. In addition to lymphodepletion prior to transfer, the ability to acquire and generate tumour reactive CD8+ T cells is crucial. The CD8+ T cells used for ACT therapy are required to be of correct specificity and contain sufficiently avid TCRs and be in optimal stage of differentiation.

Moreover, CD4+ T cell help or associated cytokines, such as IL-2, IL15, and populations of CD8+ T cells with multiple specificities may also improve the effectiveness of ACT therapies (June, 2007).

The genetic modification of T cells used in ACT approaches may improve the anti-tumour capabilities of the T cells and most importantly the clinical outcome of such therapies. It may circumvent obstacles associated with the absence of CD8+ T cells with the required TCR specificity and avidity for antigen clearance, and also the difficulty in obtaining CD8+ T cells from certain types of tumours. T cells harvested from patients can be engineered to express the α and β chains of TCRs with known avidity and antigen specificity for tumour recognition (Dougan & Dranoff, 2009). A goal of such ACT therapy is to establish a persistent memory response to prevent recurrence of tumour.

Clinical trials have been performed using adoptively transferred CD8+ T cells that were transduced with genes encoding a TCR with specificity for a MART-1 peptide. Patients were also lymphodepleted prior to ACT. The T cells persisted *in vivo*, trafficked to the site of tumour development and mediated tumour regression in 2 out of 15 patients (Morgan *et al.*, 2006). This study demonstrated that retroviral TCR gene transfer can be used to provide anti-tumour specificity, and that T cells can engraft in patients and persist at high levels. However, TCR gene therapy using retrovirus may carry potential risk of retroviral insertional mutagenesis that may lead to malignancies in some circumstances. A further potential risk of TCR gene transfer may be autoimmunity. The possibility of novel TCR combinations encompassing the introduced and endogenous α and β chains could generate mixed TCR dimers with new specificities, and consequently recognise self antigens with high affinity and be autoreactive. The development of strategies that could suppress endogenous TCR expression or ensure that the exogenous TCR chains preferentially pair could potentially eliminate these concerns. Very few engineered TCRs are available and only single epitopes can be used with this strategy, therefore limiting the possibility to recognise tumours, as they

might not express that epitope. Another limitation of this approach is that TCR recognition of an antigen is MHC restricted and thus the engineered T cells can only be used in patients with matched MHC alleles (King *et al.*, 2008).

MHC restriction can be bypassed by introducing chimeric antigen receptors, termed T-bodies, to CD8+ T cells. T-bodies consist of an external antibody-based domain, that confers specificity and the cytosolic domains containing the signal components of the TCR complex. These T-bodies can directly bind tumour antigens, leading to T cell activation, and provide a T cell with antigen specificity in a non-MHC class I restricted manner. Transfer of T cells expressing T bodies has been tested in renal cell carcinoma, neuroblastoma and ovarian carcinoma (Dougan & Dranoff, 2009). Although this approach seems safe and T-bodies are well tolerated, severe autoimmune hepatitis developed in renal cell carcinoma patients (Lamers *et al.*, 2006). This approach requires further optimisation with regards to the persistence of the transferred cells, expression of the transgene, the correct avidity of the T-bodies and the identification of suitable antigens in order to circumvent some adverse effects due to the reactivity of transduced T cells against the target antigen expressed on normal tissue (June, 2007) .

On the whole, the results of ACT therapy appear promising by showing potent anti-tumour T cell activity, particularly in melanoma patients. However, as with other immunotherapy strategies, adoptive T cell therapy alone may not be sufficient to confer clinically significant responses in most cancer patients. Combination of therapies, such as anti-cancer vaccination to increase the frequency of tumour-reactive T cells prior, or immediately after, ACT therapy may be another mechanism to boost immune and clinical responses, and thus improve overall treatment effectiveness of immunotherapy.

1.7 Prostate cancer (PCa)

PCa is the most common male cancer in the UK, with almost 35,000 newly diagnosed cases and 10000 deaths in 2005 (Cancer Research UK). Although radical prostatectomy or radiation therapy is successful for patients diagnosed with localised PCa, tumour clearance is not always achieved and 20-40% of the patients develop recurrent disease (Coen *et al.*, 2002; Roehl *et al.*, 2004).

Hormone therapy can also be used in combination with radiotherapy for localised disease, and is widely used for recurrent disease or control of metastatic disease. Hormone therapies attempt to suppress the growth of the cancer by reducing androgen levels, which result in rapid and massive apoptosis of tumour cells. This androgen deprivation increases the overall median survival time of the patients, with almost all patients with advanced PCa responding initially to this therapy, but most patients eventually develop androgen-independent PCa. Chemotherapy is usually the next treatment option for hormone-refractory disease, but it only provides short-term control of the cancer. Therefore, novel treatment strategies that prolong the hormone-sensitive stage and that are valuable for hormone-refractory disease are required.

The short-term delay in disease development during androgen-dependent state provides opportunity in these patients to study additional adjuvant treatments. Significant T cell infiltration of prostate tissues can be observed after androgen deprivation treatment (Mercader *et al.*, 2001), and reversal of tolerance in prostate specific T cells can also be shown with androgen withdrawal in mouse models (Drake *et al.*, 2005). Androgen deprivation treatment and its resulting tumour cell apoptosis provide potentially beneficial immunological consequences, and thus might have significant implications for the development of immunotherapeutic strategies to treat PCa.

Immunotherapeutic strategies involving tumour-specific CTL are an attractive option for PCa treatment. Given that the prostate is a non-essential organ, immune destruction of

normal prostate cells is likely to have fewer side effects than conventional treatments. Several candidate antigens that are typically expressed in prostate tissue have been proposed for PCa immunotherapy, including PSA (Correale *et al.*, 1997), PSMA (Horiguchi *et al.*, 2002), prostatic acid phosphatase (PAP) (Machlenkin *et al.*, 2005), prostate stem cell antigen (PSCA) (Kiessling *et al.*, 2002), and prostein (Kiessling *et al.*, 2004). Other potential tumour antigens that are overexpressed in various tumours, including PCa, have also been suggested, which comprise six-transmembrane epithelial antigen of the prostate (STEAP) (Rodeberg *et al.*, 2005), parathyroid hormone-related protein (PTH-rp) (Yao *et al.*, 2004), hTERT (Hernandez *et al.*, 2002), survivin (Schmitz *et al.*, 2000), Her-2, and EGFR (Kiessling *et al.*, 2008).

Subsequent to identification of these potential targets for tumour-reactive T cells, several clinical trials have been conducted to determine immunological and clinical responses. In clinical studies, vaccination with peptides, proteins, DNA, tumour cells, or tumour antigen-pulsed DCs indicate that these treatments were feasible and safe, but only few induced immunological and clinical responses in patients with PCa (Kiessling *et al.*, 2008). A promising approach in PCa immunotherapy is vaccination of patients with DCs. Results from phase III clinical trial, in which metastatic hormone-refractory PCa patients were vaccinated with sipuleucel-T (DCs loaded with PAP/GM-CSF) demonstrated an increase in the overall survival by 4.1 months (Higano *et al.*, 2009).

Despite the mounting evidence of the impact of immunotherapy on PCa, the clinical efficacy for most of patients with metastatic PCa is still inadequate. It seems unlikely that single immunotherapy will produce long-term remissions in most of patients with advanced PCa. Thus, further investigation of current treatment modalities, with appropriate design and end points, is required to improve the response rates and the duration of response. This may be achieved by combining immunotherapy with other standard treatments, including

radiation, hormone therapy or chemotherapy, or even combining multi immunotherapy agents (Drake, 2008).

1.8 B-cell chronic lymphocytic leukaemia (B-CLL/CLL)

CLL is the most common type of leukaemia in the Western world, representing 22-30% of all leukaemia cases, with a worldwide incidence between 1 and 5.5 / 100,000 people. The incidence of CLL increases considerably with age and is far more frequent in the elderly (Redaelli *et al.*, 2004). CLL is a B cell malignancy characterised by an accumulation of neoplastic B cells in the blood, bone marrow and peripheral lymphoid organs. The excess of neoplastic B cells is caused mainly by a failure of apoptosis rather than increased cell proliferation. CLL cells derive from antigen-experienced mature B lymphocytes that have functional competence and escape death due to interactions with factors produced by other cells, including T cells. This disease is defined by a unique phenotypic profile, which includes strong expression of CD19, CD5 and CD23, and weak expression of CD22 and CD79b (Keating *et al.*, 2003).

CLL is a dynamic disease with a heterogeneous clinical course. Patients can be divided into those with a rapid disease progression and those with a more stable indolent course of disease that may not require treatment. Several studies have demonstrated these two subtypes can be defined by somatic mutations present in rearranged Ig variable region genes, IgV_H. Ig variable gene rearrangements occurs during B cell development in bone-marrow. Somatic hyper-mutations of Ig variable regions occur in the germinal centres of lymphoid follicles. A worse clinical outcome has been correlated in patients carrying unmutated IgV_H genes as they tend to show evidence of advanced, progressive disease and resistance to therapy (Damle *et al.*, 1999; Hamblin *et al.*, 1999).

Surface expression of CD38, cytoplasmic expression of ζ -associated protein 70 (ZAP-70) and the presence of chromosomal abnormalities are also associated with a more progressive clinical course and shorter survival duration, and are frequently correlated with

other important poor prognosis markers, including unmutated IgV_H genes (Damle *et al.*, 1999; Van Bockstaele *et al.*, 2009). The most common chromosomal abnormalities in CLL patients are deletions of chromosomes 13q, 11q, 17p13 and trisomy 12. Aberrations in 17p and 11q occur almost exclusively in the unmutated subset, and have been considered to have the strongest adverse prognostic factors (Van Bockstaele *et al.*, 2009).

Elevated numbers of circulating T cells with a reduced CD4/CD8 ratio have been frequently reported in CLL, particularly in the more advanced stages of the disease (Dianzani *et al.*, 1994; Platsoucas *et al.*, 1982; Totterman *et al.*, 1989). Despite the associated expansion of both neoplastic B cells and T cells, CLL patients have an abnormal T cell function. T cell dysfunction can be explained by several aspects, including poor antigen presenting function of CLL cells, increased expression of CTLA-4 on T cells (Rossmann *et al.*, 2003), impaired immune synapse formation between CLL cells and T cells (Ramsay *et al.*, 2008), secretion of immunosuppressive cytokines, and expression by CLL cells of molecules like CD200 that actively suppress cytotoxic T cell responses and promote the induction of Tregs (Kretz-Rommel *et al.*, 2007; Pallasch *et al.*, 2009). An abnormal maturation and function of DCs resulting in inability to stimulate an effective T cell-mediated immune response has also been observed in CLL patients (Ravandi & O'Brien, 2006). All these factors undeniably change the ability of T cells to respond to tumour-specific antigens and form a tolerised environment in which the tumour is able to grow.

Furthermore, T cells of patients with poor prognosis (CD38+/ZAP-70+) showed significantly shorter telomeres than those from patients with good prognosis, implying that T cell expansion is occurring in response to malignant cell growth and is probably playing a role in the growth and survival of the tumour cells (Roth *et al.*, 2008). T cells, as well as stromal cells, appear to have a function in amplifying a microenvironment that favours the extended survival and proliferation of malignant B cells. CLL cells from lymph nodes and bone marrow are active players in shaping the microenvironment by secreting a chemokine (CCL22)

that attracts activated T cells expressing its receptor CCR4. T cells with their CD40L bind to CD40 on the CLL cells, providing signals that in turn induce proliferation and progressive accumulation of neoplastic cells (Ghia *et al.*, 2002).

There is considerable interest in the identification of tumour antigens in CLL not only for their prospective use in immunotherapy, but also as means of monitoring tumour specific T cell responses during the course of treatment and disease. In contrast to several solid tumours, there are relatively few tumour-associated antigens that have been identified for CLL. The mutated V_H genes found in malignant B cells result in surface expression of unique clonotypic Ig receptors (idiotype). The Ig idiotype is the best characterised antigen in CLL (Trojan *et al.*, 2000; Zirlik *et al.*, 2006), but a problem in using idiotype vaccination strategies is that idiotypes are poorly immunogenic. Furthermore, use of idiotypes requires personalised tumour vaccines and individualised monitoring of idiotype of immune endpoints (Ramsay & Gribben, 2008). Other tumour antigens have also been suggested as tumour antigens based on their expression in B-CLL, including CD23 (Bund *et al.*, 2007), CD229 (Bund *et al.*, 2006), fibromodulin (Mayr *et al.*, 2005), MDM2 (Mayr *et al.*, 2006), hTERT (Kokhaei *et al.*, 2007), and survivin (Schmidt *et al.*, 2003).

The most common treatment for patients with aggressive disease is chemotherapy, such as fludarabine (purine analogue) and chlorambucil (alkylating agent). Results from a randomised trial comparing the use of fludarabine and chlorambucil as initial treatment for CLL showed that fludarabine has a higher complete remission rate (20%) than chlorambucil (4%) (Rai *et al.*, 2000). Monoclonal antibodies are emerging as attractive agents in the treatment of CLL. Alemtuzumab, a humanized anti-CD52 antibody, is already approved for use in fludarabine-refractory CLL (Keating *et al.*, 2002). In addition, rituximab is a monoclonal antibody against CD20 and has been incorporated in combination regimens. Regimens combining chemotherapeutic agents with monoclonal antibodies have achieved higher complete remission rates. For example, in previously untreated patients, the

combination of fludarabine, cyclophosphamide and rituximab treatment resulted in a complete remission rate of 70% (Keating *et al.*, 2005). However, a main side effect of using these therapies is the depletion of normal lymphocytes and thereby contributing to an increased risk of infections (Auer *et al.*, 2007).

Autologous and allogeneic stem cell transplantation (SCT) is considered as another treatment options for patients with CLL. Results of autologous SCT in CLL showed that it is safe and feasible procedure but there is a high incidence of subsequent relapse. As for allogeneic SCT, initial results showed high treatment-related mortality, but did demonstrate that cure was possible in some patients. A consensus opinion suggests that allogeneic SCT is a procedure with evidence-based efficacy in poor-risk CLL, and a reasonable treatment option for younger patients with non-response or early relapse after purine analogues, relapse after achieving a response with combination therapy or autologous transplantation, and patients with p53 abnormalities requiring treatment (Gribben, 2007). Recently, it was demonstrated that an immunomodulatory drug, lenalidomide, with antitumour activity is clinically active in CLL. Lenalidomide has a T cell stimulatory effect and has been shown to enhance immune synapse formation between T cells and autologous CLL cells (Ramsay *et al.*, 2008).

Although these regimes can control CLL, the disease remains incurable and new adjunct treatments are needed. There is strong rationale for the use of T cell based immunotherapy as a potential adjunct treatment for leukaemia. A variety of vaccine strategies have been studied in CLL patients, including vaccination with DCs, oxidized CLL cells or modified autologous leukaemia cells. In addition, attempts to circumvent the T cell defects clinically, for example, by modulating CD40:CD40L interactions or vaccination have also been studied in CLL. Clinical trials using these vaccine strategies have so far provided very encouraging results, demonstrating anti-leukaemia immune responses, but failed to show significant clinical benefits to patients (Ramsay & Gribben, 2008). There is limited knowledge of tumour antigens expressed by CLL, and further identification and

characterisation will be of particularly importance for the development of successful immunotherapeutic strategies for CLL.

1.9 Apoptosis

Apoptosis, or programmed cell death, is an essential component of the development and health of multicellular organisms. It plays an important role in development, morphogenesis, proliferation/homeostasis, regulation and function of the immune system and in the elimination of damaged, altered or malignant cells. Apoptosis is a form of death in which a cell initiates a suicide programme and characteristic morphologic alterations are observed in dying and dead cells. These changes include chromatin condensation, nuclear disruption, cytoplasmatic contraction, and membrane blebbing, leading to phagocytosis of the cell. The decision for apoptosis can come from the cell itself, from the surrounding tissue or from a cell that is part of the immune system, and may be initiated in various circumstances, such as induction of DNA damage by radiation or toxic chemicals, the activation of a stress response, the lack of growth factors, and the triggering of specific signalling responses (Jin & El-Deiry, 2005).

1.9.1 Pathways

There are two major pathways that can trigger apoptosis, the extrinsic pathway and the intrinsic pathway, both of which culminate in a common death program, the activation of a set of proteolytic enzymes, called caspases (Figure 1.7). The extrinsic pathway, also known as the death receptor pathway, is initiated through the triggering of cell surface receptors of the TNF-receptor family. In contrast, the intrinsic pathway, also called mitochondrial pathway, is initiated through specialised proteins that induce mitochondrial leakiness, leading to release of death-inducing proteins that are normally sequestered within mitochondria. The induction of cell death is generally linked to the activation of caspases. The p53 tumour suppressor protein can also promote the expression of various pro-apoptotic gene products that initiate the

extrinsic and intrinsic pathways. Signals from both pathways activate initiator caspases, which cleave and activate effector caspases, which in turn cleave various proteins that are crucial for cellular integrity and also induce enzymes that promote the death of the cell. Both pathways use caspases 3, 6 and 7 as effector caspases (Jin & El-Deiry, 2005).

1.9.1.1 Extrinsic pathway

In the extrinsic pathway, ligands bind to transmembrane death receptors on the target. The death receptors are members of the TNF receptor family, such as Fas, TNF-receptors and TRAIL-receptors, and contain a conserved domain known as the death domain (DD) in the cytoplasmic side of the receptor. For example, CTL can also induce apoptosis via the interaction of FasL with Fas expressed on the target cell. The binding of the death receptor to its ligand on the target cell recruits an adaptor protein known as Fas-associated death domain protein (FADD), which interacts with the DD of the death receptor. FADD, in turn, recruits caspase-8, an initiator protein, to form the death inducing signal complex (DISC). Through the recruitment of caspase-8,10 to DISC, caspase-8,10 are activated and able to directly activate caspase-3, an effector protein, to initiate degradation of the cell. When TNF-receptors are stimulated by their ligands, the adapter protein, TNFR-associated death domain (TRADD) is recruited to the receptor. When TRADD binds to FADD, the pathway proceeds to apoptosis as described above. In other cells, TNF-receptor signalling promotes the induction of pro-inflammatory responses (Jin & El-Deiry, 2005). Although extrinsic pathways can induce apoptosis without requiring mitochondrial changes, death receptor signalling is amplified in some cell types by positive feedback mechanism involving mitochondria. In these cells, caspase-8 can also cleave a pro-apoptotic BH3-only protein (Bid), which acts as a signal to the mitochondrial pathway via Bak and Bax (Khosravi-Far & Esposito, 2004).

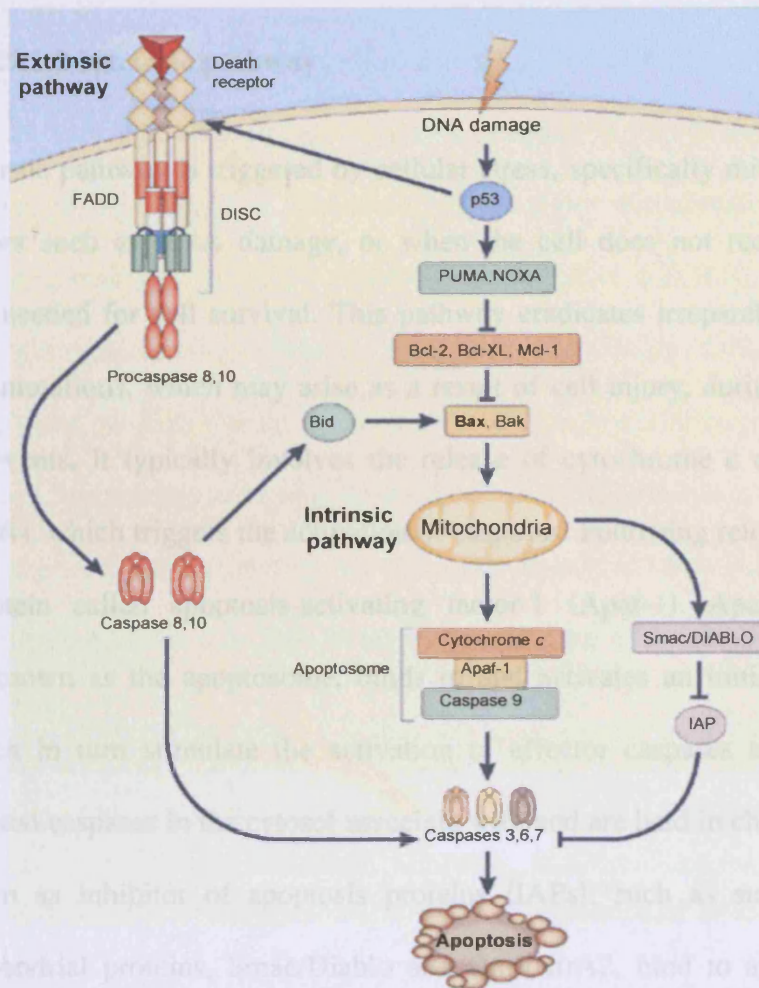


Figure 1.7 Extrinsic and Intrinsic apoptotic pathways (adapted from Ashkenazi, 2008). The two pathways can initiate apoptosis: extrinsic pathway acts through cell-surface death receptors and intrinsic pathway acts through intracellular Bcl-2 proteins. Extrinsic pathway: The ligand binding induces death receptor clustering and recruitment of the adaptor protein FADD and the initiator caspases 8 and 10 as pro-caspases, forming the complex DISC. This triggers activation of the caspases, which in turn activate the effector caspases 3, 6 and 7; Intrinsic pathway: cellular stress activates the p53 tumour-suppressor protein. p53 initiates the intrinsic pathway by upregulating Puma and Noxa, which in turn activate Bax and Bak. Bax and Bak permeabilise the outer mitochondrial membrane, resulting in release of cytochrome *c*, which binds to the adaptor Apaf-1 to recruit the initiator procaspase 9 into a signalling complex termed the apoptosome. Activated caspase 9 then cleaves and activates the effector caspases 3, 6 and 7 to trigger apoptosis. The mitochondrial protein Smac/DIABLO augments apoptosis by binding inhibitor of apoptosis proteins (IAPs) and reversing their activity on several caspases. Cross-talk between the pathways: p53 mainly stimulates the intrinsic pathway, but it also upregulates some of the pro-apoptotic receptors and augments extrinsic signalling. Extrinsic-pathway activation leads to caspase 8-mediated processing of Bid; truncated Bid subsequently stimulates Bax and Bak to engage the intrinsic pathway.

1.9.1.2 Intrinsic pathway

The intrinsic pathway is triggered by cellular stress, specifically mitochondrial stress, caused by factors such as DNA damage, or when the cell does not receive extracellular signals that are needed for cell survival. This pathway eradicates irreparably damaged cells with dangerous mutations, which may arise as a result of cell injury, during cell division or recombination events. It typically involves the release of cytochrome c and other proteins from mitochondria, which triggers the activation of caspases. Following release, cytochrome c binds to a protein called apoptosis-activating factor-1 (Apaf-1). Apaf-1-cytochrome c complex, also known as the apoptosome, binds to and activates an initiator caspase, pro-caspase 9, which in turn stimulates the activation of effector caspases as in the extrinsic pathway. Activated caspases in the cytosol associate with and are held in check by a family of inhibitors known as inhibitor of apoptosis proteins (IAPs), such as survivin. The other released mitochondrial proteins, Smac/Diablo and Omi/HtrA2, bind to and antagonise the activity of the IAPs and thus promote caspase activity and the induction of apoptosis (Jin & El-Deiry, 2005).

The intrinsic pathway is tightly regulated by interactions between members of the Bcl-2 family. The Bcl-2 family of proteins can be divided into two groups, the anti-apoptotic and pro-apoptotic proteins. These proteins are characterised by the presence of one or more Bcl-2 homology (BH) domains. The anti-apoptotic Bcl-2 family members are important regulators of apoptosis and promote cell survival. Multidomain anti-apoptotic proteins, including the Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bfl1/A-1, and Bcl-B, typically contain four BH domains. These contribute to mitochondrial stability and block the release of cytochrome c. The precise mechanism is not completely understood but they may act by directly blocking the effects of the pro-apoptotic members (Wong & Puthalakath, 2008) .

Many pro-apoptotic proteins are single domain BH3-only proteins, such as Bid, Bim, Puma and Noxa, and are essential initiators of the intrinsic pathway by acting as antagonists

of anti-apoptotic proteins and/or as agonists of pro-apoptotic multidomain proteins Bax and Bak. They may directly interact with pro-apoptotic Bax and Bak proteins in the cytoplasm, cause them to change conformation, translocate to the outer mitochondrial membrane and oligomerise. Multidomain pro-apoptotic proteins, such as Bax and Bak, contain three BH domains and are the executioner proteins, the downstream mediators of the intrinsic pathway. When activated, these proteins translocate to the outer mitochondrial membrane, where they become anchored, oligomerise and generate an increase in mitochondrial permeability. The loss of mitochondrial membrane integrity results in the release of key mitochondrial inducers of apoptosis and subsequently activation of caspases that leads to the death of the cell (Wong & Puthalakath, 2008).

1.9.2 Pro-apoptotic Bax protein and its dysregulation in cancer

One of the hallmarks of cancer is the evasion of cellular signals to undergo apoptosis (Hanahan & Weinberg, 2000). Various human malignancies overexpress anti-apoptotic Bcl-2 family proteins or have dysregulated or loss of pro-apoptotic proteins (Figure 1.8). The expression of Bax or Bak is considered to play a key role in suppressing cancer development, and thus loss of Bax or Bak would accelerate tumourigenesis. Indeed, decreased Bax levels in malignant cells can lead to resistance to apoptosis, and have been associated with resistance to cancer therapy. Instability of Bax has been observed in a number of cancers, such as leukaemia, prostate cancer, cervical cancer and primary melanoma (Fecker *et al.*, 2006; Li & Dou, 2000; Magal *et al.*, 2005). Low levels of Bax protein was significant associated with poor prognosis in prostate cancer and primary melanoma (Fecker *et al.*, 2006; Li & Dou, 2000). Decrease of Bax has also been reported as a negative prognostic marker for CLL, ovarian, pancreatic and breast cancer (Friess *et al.*, 1998; Krajewski *et al.*, 1995; Tai *et al.*, 1998).

Overexpression of Bcl-2 is one of the main causes of resistance of CLL cells (and other malignancies) to therapy. However, low levels of Bax have been observed in advanced

CLL and also play an important role in chemoresistance in CLL (Pepper *et al.*, 1997; Pepper *et al.*, 1998; Starczynski *et al.*, 2005). Several studies have revealed that Bax protein has a shortened half-life in cancer cells due its abnormally increased degradation by the proteasome (Agrawal *et al.*, 2008; Li & Dou, 2000; Liu *et al.*, 2008). Bax protein has been reported as a target for the ubiquitin-proteasome pathway of protein degradation in CLL and PCa. Enhanced degradation of Bax is particularly observed in advanced stages of disease, which correlates with very low levels of Bax protein being detected in patients with highest grade of cancer and thus with poor prognosis (Agrawal *et al.*, 2008; Li & Dou, 2000).

The proteins or protein patterns that have a crucial role in cancer development or suppression may represent model targets for therapeutic purposes. These features are important for all cancers, and thus therapies based on targeting these would be broadly relevant to most, if not all cancers. In this regard, it has been proposed that proteins that regulate apoptosis may be widely applicable targets for immunotherapy. Various regulators of apoptosis, such as Bcl-2 and survivin, have been described as tumour antigens in a large number of different cancers (Andersen *et al.*, 2005). Strategies designed to target regulators of apoptosis have gained considerable interest for the treatment of cancer.

1.1.8 Aims of project

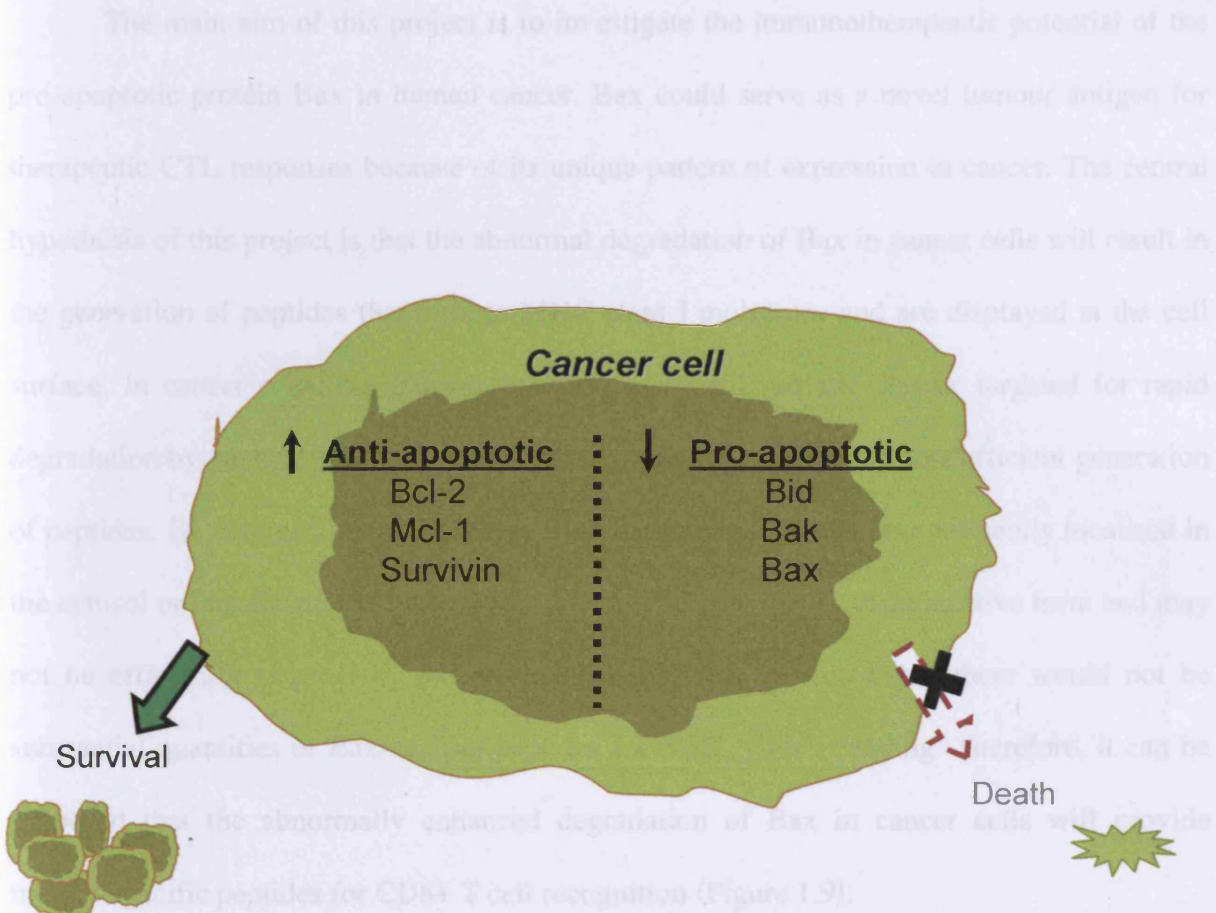


Figure 1.8 Cancer cells and dysregulation of anti-apoptotic and pro-apoptotic proteins. Cancer cells frequently exhibit defects in apoptosis. Impaired apoptosis can result from overexpression of anti-apoptotic proteins, such as survivin, Bcl-2, and Mcl-1, and decreased expression of pro-apoptotic proteins such as Bax, Bak and Bid. These dysregulations contribute to the survival of cancer cells and prevention of their cell death.

1. Investigate T cell responses against Bax peptides in cancer patients and healthy donors.
 - a) Define novel Bax specific T cell epitopes
 - b) Generate a T cell line/biome which specifically recognises the defined Bax epitopes
 - c) Test Bax specific T cells against a panel of human cancer cells
2. Analyse whether Bax expression in cancer cells is regulated by proteasomes.
3. Study CLL as a model system for generating human tumour-specific T cell responses.

1.10 Aims of project

The main aim of this project is to investigate the immunotherapeutic potential of the pro-apoptotic protein Bax in human cancer. Bax could serve as a novel tumour antigen for therapeutic CTL responses because of its unique pattern of expression in cancer. The central hypothesis of this project is that the abnormal degradation of Bax in cancer cells will result in the generation of peptides that bind to MHC class I molecules and are displayed at the cell surface. In cancer cells, we propose that Bax is short-lived and can be targeted for rapid degradation by the ubiquitin-proteasome pathway and thus there is a more efficient generation of peptides. By contrast, in healthy tissue, Bax has a long half-life, predominantly localised in the cytosol or loosely attached to outer mitochondrial membranes in an inactive form and may not be efficiently targeted by the proteasome. So, it is expected that there would not be substantial quantities of Bax-derived peptides for MHC class I binding. Therefore, it can be proposed that the abnormally enhanced degradation of Bax in cancer cells will provide tumour specific peptides for CD8⁺ T cell recognition (Figure 1.9).

This is a novel concept because definition of tumour antigens usually relies on stable over-expression of proteins in cancer cells. Here, it is proposed that a protein with low or unstable expression can be a candidate tumour antigen. At the onset of this project, T cell responses against Bax had not been reported. Therefore, the main objectives of this project are:

1. Investigate T cell responses against Bax peptides in cancer patients and healthy donors.
 - a) Define novel Bax specific T cell epitopes
 - b) Generate a T cell line/clone which specifically recognises the defined Bax epitopes
 - c) Test Bax specific T cells against a panel of human cancer cells
2. Analyse whether Bax expression in cancer cells is regulated by proteasomes.
3. Study CLL as a model system for generating human tumour-specific T cell responses.

Chapter 2

Materials & Methods

2.1 Tissue culture basics

2.1.1 Tissue culture media and buffers

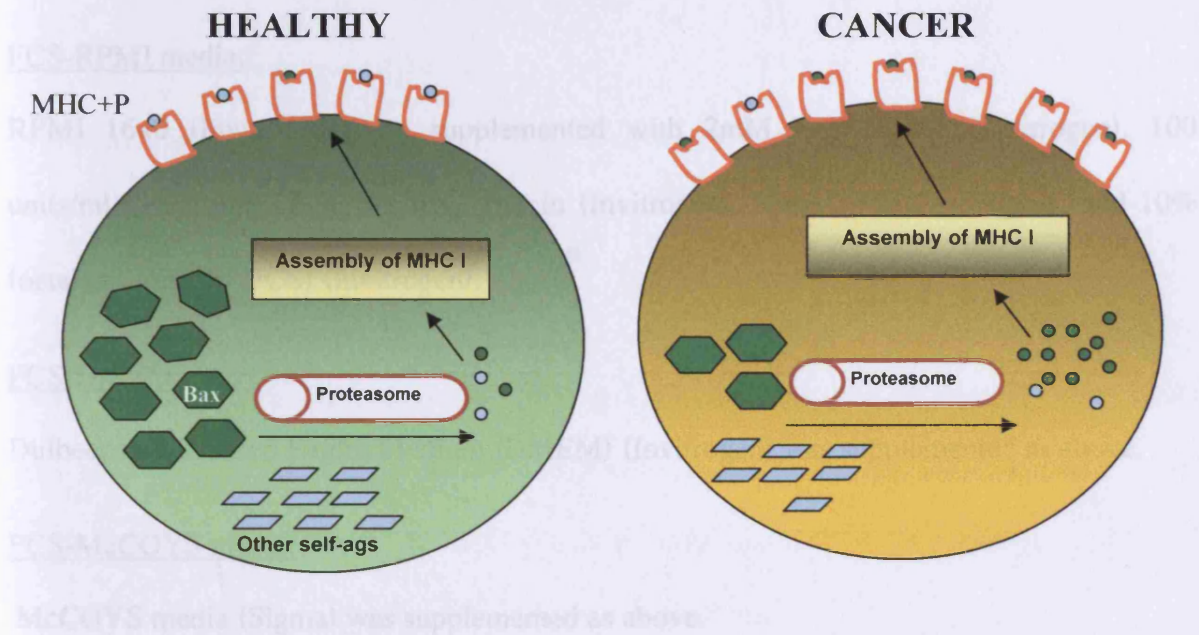


Figure 1.9 Schematic overview of the main hypothesis of the project: Degradation of Bax protein in healthy & cancer cells.

Chapter 2

Materials & Methods

2.1 Tissue culture basics

2.1.1 Tissue culture media and buffers

FCS-RPMI media:

RPMI 1640 (Invitrogen) was supplemented with 2mM L-glutamine (Invitrogen), 100 units/ml penicillin, 100µg/ml streptomycin (Invitrogen), 25mM HEPES (Sigma), and 10% foetal calf serum (FCS) (Invitrogen).

FCS-DMEM:

Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen) was supplemented as above.

FCS-McCOYS media:

McCOYS media (Sigma) was supplemented as above.

Serum free media:

RPMI 1640 was supplemented as above but without FCS.

AB media:

RPMI 1640 (Sigma) was supplemented with 10% human AB serum (Welsh Blood Transfusion Service), 2mM L-Glutamine, 100units/ml Penicillin, 100µg/ml streptomycin, and 25mM HEPES Buffer.

Freezing Mix

40% RPMI 1640, 10% Dimethyl Sulphoxide (DMSO) (Sigma), and 50% FCS.

Red cell lysis buffer

8.3g ammonium chloride (Sigma), 1g potassium bicarbonate (Sigma), 37mg potassium EDTA (Sigma) into 1 litre of distilled H₂O (pH adjusted to 7.4 on ice).

MACs buffer

0.5% BSA (Sigma), 2mM EDTA (Sigma), GIBCO PBS (Invitrogen). Sterile filtered using a 0.2µm bottle top filter.

FACS buffer

PBS+ 1%FCS

2.1.2 Tissue culture plastics

All tissue culture flasks (T25 and T75) and plates (24, 48 and 96 wells) were obtained from Nunc, Greiner, Fisher or Falcon.

2.1.3 Cell viability counting

Cells were examined for viability by mixing 15µl of trypan blue (Sigma) with an equal volume of well mixed cell suspension. Cells were counted using a haemocytometer. Non-viable cells are shown as distinctive blue colour under microscope. The percentage of total cells counted as white represents the viability of the culture.

2.1.4 Routine maintenance of cell lines

Culturing established cell lines

Non-adherent cell lines and adherent cell lines were cultured in 25cm³ (T25) or 75cm³ (T75) flasks in FCS-RPMI media or FCS-DMEM. Cells were routinely split 1:3 or 1:5 when confluent (every 3-4 days). Adherent cells were washed with sterile GIBCO PBS (Invitrogen) and incubated at 37°C with either 1mls in T25 or 5mls in T75 EDTA-trypsin (Invitrogen). After cells detached from the flasks, the cells were split between new flasks. FCS-RPMI or FCS-DMEM media was added to the cells and flasks were incubated at 37°C.

Thawing cryopreserved cells:

Cells were thawed using a 37°C water bath and then added to FCS RPMI media. Cells were centrifuged at 1500rpm for 5 minutes and resuspended in the appropriate serum containing RPMI media (10% FCS or 10% AB serum).

Table 2.1 Description of established cell lines used

Cell Line	Description	Maintenance
T2	Human T- and B-lymphoblast hybrid cell line that does not express HLA-DR & is MHC class II negative. It expresses only the HLA-A*0201 allele and is defective in endogenous antigen-processing which enhances the effectiveness of exogenous peptide loading. (Salter <i>et al.</i> , 1985)	Cultured in FCS-RMPI and split 1:3, 2-3 times a week
PC3	Human Caucasian prostate carcinoma adherent cell line. It was derived from a bone metastasis of grade IV prostatic adenocarcinoma and is not hormone sensitive. (Kaighn <i>et al.</i> , 1979)	Cultured in FCS-RPMI and split 1:3, 1-2 times a week
LNCaP	Human Caucasian prostate carcinoma adherent cell line. It was derived from a biopsy of the left supraclavicular lymph node of a male with metastatic prostate carcinoma. (Horszewicz <i>et al.</i> , 1983)	Cultured in FCS-RMPI and split 1:3, 2-3 times a week
DU145	Human Caucasian prostate carcinoma adherent cell line. It was derived from brain and is not detectably hormone sensitive. (Stone <i>et al.</i> , 1978)	Cultured in FCS-RMPI and split 1:3, 2-3 times a week
CA-HPV10	Human Caucasian prostate carcinoma adherent cell line. It was derived from a prostatic adenocarcinoma of Gleason grade 4/4. It is HPV-18 transfected. (Weijerman <i>et al.</i> , 1994)	Cultured in FCS-RMPI and split 1:3, 2-3 times a week
CaSki	Human cervical carcinoma adherent cell line. It was derived from a small bowel metastasis in a 40 year old Caucasian female with cervical carcinoma. It contains between 60 and 600 copies of HPV16 genome per cell integrated at various sites in the Caski cell genome. (Baker <i>et al.</i> , 1987)	Cultured in FCS-RMPI and split 1:3, 2-3 times a week
SiHa	Human cervix squamous carcinoma adherent cell line. It contains 1 to 2 copies of HPV16 genome integrated into the cell genome. (Baker <i>et al.</i> , 1987; Friedl <i>et al.</i> , 1970)	Cultured in FCS-DMEM and split 1:5, 2-3 a week
SiHa-A2	SiHa cells that were stably transfected with a recombinant expression vector pcDNA3 conferring neomycin resistance (Invitrogen), cloned with HLA-A*0201. (generated in the lab (Youde <i>et al.</i> , 2005))	Cultured in FCS-DMEM supplemented with 400µg/ml G418 (Sigma) and split 1:5, 2-3 a week
MS751	Human caucasian cervical carcinoma adherent cell line. It was isolated from established lymph node metastasis in a 47 year old Caucasian female with cervical carcinoma. It contains HPV45 sequences. (Pater & Pater, 1985)	Cultured in FCS-DMEM and split 1:3, 2-3 times a week
C33A	Human cervix carcinoma adherent cell line. It was derived from a cervical cancer biopsy. These cells are negative for human papillomavirus sequences. (ATCC HTB 31)	Cultured in FCS-DMEM in split 1:5, 2-3 times
C33A-HPV16	C33A transfected with a recombinant plasmid containing the HPV16 genome. (kindly provided by Prof. J. Dillner, Sweden)	Cultured in FCS-DMEM supplemented with 400µg/ml G418 and split 1:5, 2-3 a week
TK143 (143b)	Human bone osteosarcoma adherent cell line derived from a 13 year old Caucasian female.	Cultured in FCS-DMEM and split 1:5, 2-3 a week
Saos-2	Human osteosarcoma adherent cell line isolated from the	Cultured in FCS-

	primary osteogenic sarcoma of an 11-year-old Caucasian female.(ECACC, Catalogue No: 89050205)	McCOYS and split 1:3, 2-3 times a week
U2OS	Human osteosarcoma adherent cell line established from a moderately differentiated sarcoma of the tibia of a 15 year old Caucasian female. (ECACC, Catalogue No: 92022711)	Cultured in FCS-McCOYS and split 1:3, 2-3 times a week
HepG2	Human Caucasian hepatocyte carcinoma adherent cell line. It was derived from a liver biopsy of a 15 year old male with a well differentiated hepatocellular carcinoma. (ECACC, Catalogue No: 85011430)	Cultured in FCS-DMEM and split 1:3, 1-2 times a week
BJAB	EBV negative human cell line similar to Burkitt's lymphoma. (kindly provided by Prof. Martin Rowe)	Cultured in FCS-RMPI and split 1:5, 2-3 times a week
BV173	Human B cell precursor leukaemia line. (kindly provided by Dr. Paul Brennan)	Cultured in FCS-RMPI and split 1:5, 2-3 times a week
Daudi	Human negroid Burkitt's Lymphoma cell line, EBV positive with defects in HLA class I. (kindly provided by Prof. Martin Rowe)	Cultured in FCS-RMPI and split 1:5, 2-3 times a week
DG75	Human B cell lymphoma cell line, EBV negative. (kindly provided by Prof. Martin Rowe)	Cultured in FCS-RMPI and split 1:5, 2-3 times a week
MDA231	Human breast adenocarcinoma cell line isolated from a pleural infusion in a 51-year-old female with breast cancer.	Cultured in FCS-RMPI and split 1:3, 2-3 times a week
K562	Human chronic myelogenous cell leukemia cell line. It is easily killed by NK cells.	Cultured in FCS-DMEM with 400µg/ml G418 and split 1:3, 2-3 a week
SK29mel	Human melanoma adherent cell line that expresses Melan-A/MART-1. (kindly provided by Dr.V. Cerundolo)	Cultured in FCS-DMEM and split 1:5, 2-3 a week

2.2 Peptide Antigens

2.2.1 Positive peptide pool (PPP)

PPP contained common recall antigens for both CD8+ T cells and CD4+ T cells and it was used as positive control for T cell responses detected by ELISpot (Table 2.2) (Smith *et al.*, 2005). It was added to cultures at 10µg/ml. Several different antigen sources (Tetanus, Influenza, CMV, and EBV) were included. Each peptide synthesized (Severn Biotech) was >90% pure. Individual peptide stocks were made by dissolving each peptide in DMSO. The

PPP mixture was made by combining aliquots taken from each peptide stock, with each peptide being present at 10µg/µl. Peptide stocks and PPP aliquots were stored at -20°C.

Table 2.2 Positive Peptide Pool (PPP)

Sequence	Antigen source	Residue Position	HLA Restriction
PKYVKQNTLKLAT	Influenza A HA	306–324	DR4/DR7/DR11
QYIKANSKFIGITEL	Tetanus toxoid	830–844	Multiple DR
FNNFTVSFWLRVPKVSASHLE	Tetanus toxoid	947–967	Multiple DR
TSLYNLRRGTALA	EBV EBNA1	515–527	DR1*0701
AGLTLSELLVICSYLFISRG	EBV BHRF1	171–181	DR15(2)
IVTDFSVIKAIEEE	EBV EBNA3c	416–429	A11
LTKGILGFVFTLTVPSEGR	Influenza A M1	55–73	A2
IQNAGLCTLVAMLEE	EBV BMLF1	276–290	A2
RPFFHPVGEADYFEY	EBV EBNA1	403–417	B35
QEFFWDANDIYRIFA	CMV pp65	511–525	B44
EENLLDFVRF	EBV EBNA3c	281–290	B44
RKTPTVTGGGAMAGA	CMV pp65	415–429	B7
RPQKRPSIGCKGT	EBV EBNA1	71–85	B7
RKCRAKFQLLQHYR	EBV BZLF1	187–201	B8
CTELKLSDY	Influenza A NP	44–52	A1
ILRGSAVHK	Influenza A NP	265–273	A3

2.2.2 MART-1/Melan-A peptide

MART-1 (ELAGIGILTV) is a melanoma peptide analogue that is HLA-A*0201 restricted and highly immunogenic. It shows better HLA*0201 binding properties and antigenicity than the natural MART-1 peptide (27-35) AAGIGILTV (Valmori *et al.*, 1998). MART-1 peptide was synthesized by ProImmune with >70% purity. This peptide was dissolved in DMSO to give a working stock of 10mg/ml. Peptide stocks were stored at -20°C.

2.2.3 Bax peptide pools

Bax pool 1-15

Bax pool 1-15 consists of 9-and 10-mer peptides generated from Bax- α protein (192aa). Using two predictive computer algorithms (BIMAS and SYFPEITHI) for HLA binding, fifteen peptides have been identified for HLA binding. Ten are predicted to bind to HLA-A*0201, three to HLA-A3 and two to HLA-B7 (Table 2.3). The peptides were synthesized with purity greater than 40% (Mimotopes). Individual peptides were dissolved in DMSO to give stock solutions of 10mg/ml and 50mg/ml. Total concentration of this peptide pool is 10mg/ml. All individual peptide and Bax pool stocks were stored at -20°C.

Table 2.3 Bax peptide pool 1-15

Number	Sequence	Residue Position	HLA restriction	BIMAS score	SYFPEITHI score
1	KLSECLKRI	58	A2	344	24
2	ALFYFASKL	112	A2	300	25
3	IMGWTLDFL	136	A2	163	22
4	WIQDQGGWV	151	A2	129	19
5	ALCTKVPEL	124	A2	49	30
6	PVPQDASTK	49	A3	30	30
7	VLKALCTKV	121	A2	23	23
8	IMKTGALLL	19	A2	22	22
9	ALFYFASKLV	112	A2	257	21
10	VVYNAFSLRV	209	A2	126	17
11	KLVLKALCTK	119	A3	135	28
12	VVALFYFASK	110	A3	27	27
13	VPQDASTKKL	50	B7	80	21
14	KPPHPHHRAL	163	B7	80	22
15	LVLKALCTKV	120	A2	22	22

Bax pool 601-23

Bax pool 601-23 consists of 9-and 10-mer peptides also generated from Bax- α protein (192aa) but were identified using five/six predictive computer algorithms (BIMAS, SYFPEITHI, SMM, MHCpep, NET MHC, EpiJen) for HLA binding. This pool includes 23 peptides for HLA binding, fourteen of which are predicted to bind to HLA-A*0201, one to HLA-A3, one to HLA-B7, two to HLA-A1, two to HLA-B8 and three to HLA-B44 (Table 2.4). The peptides were synthesized with purity greater than 40% (Mimotopes). Individual peptides were dissolved in DMSO to give stock solutions of 10mg/ml and 50mg/ml. Total concentration of this peptide pool is 10mg/ml. All individual peptide and Bax pool stocks were stored at -20°C.

Table 2.4 Bax peptide pool 601-23

Number	Sequence	Position	HLA Restriction	Databases	% Purity
601	RMGGEAPEL	37	A2	6/6	73
602	QIMKTGALL	18	A2	4/6	37
603	LLSYFGTPT	161	A2	4/6	77
604	LLQGFIQDRA	26	A2	3/6	83
605	GLLSYFGTPT	160	A2	3/6	79
606	FVAGVLTASL	176	A2	3/6	67
607	ELQRMIAAV	75	A2	5/6	69
608	ALDPVPQDA	46	A2	4/6	65
609	LLLQFIQDR	25	A2	4/6	83
610	TIMGWTLDFL	135	A2	3/6	51 / 95.7
611	FLRERLLGWI	143	A2	3/6	76
612	KLSECLKRI	58	A2	6/6	36
613	IMGWTLDFL	136	A2	6/6	68 / 96.4
614	ALCTKVPEL	124	A2	6/6	64
615	DELDSNMEL	68	B44	3/3	51
616	MELQRMIAA	74	B44	3/3	77
617	SEQIMKTGAL	16	B44	3/3	68
618	CLKRIGDEL	62	B8	2/3	49
619	ASKLVLKAL	117	B8	2/3	37
620	GGWDGLLSY	156	A1	2/5	59
621	DTDSPREVF	84	A1	4/5	84
622	VALFYFASK	111	A3	5/5	78
623	SPREVFVRV	87	B7	3/3	84

2.3 Peptide binding assay

T2 cell line was used to determine the binding affinity of peptides. This cell line is an antigen processing mutant and expresses unstable class I molecules on the surface. Expression can be stabilised by the addition of exogenous peptide providing an indication of MHC allele specific binding of test peptides (Cerundolo *et al.*, 1990). 1×10^5 T2 cells were resuspended at 1×10^6 /ml in serum-free media and 100µl added/well of a 96-well plate. Peptide was added at 50µg/ml in DMSO and incubated overnight at 37°C. The appropriate controls were also included, a non A2-binding peptide and a known HLA-A2 binding peptide – the Flu matrix 1 (Flu M1 58-66) peptide GILGFVTFLL. The other negative control consisted of untreated cells. The cells were then stained with MA2.1 (an HLA-A2 antibody) as described in section 2.14.2. HLA-A2 expression was quantified as % increase according to the formula: % Increase = [(mean fluorescence with peptide – mean fluorescence without peptide) / mean fluorescence without peptide x 100].

2.4 Blood origin and preparation

2.4.1 Blood donors

Healthy donors used in this project were both male and female members of laboratory staff aged between 20 and 60 years or anonymous buffy coats from Welsh Blood Service. Blood from the laboratory staff was taken with informed consent. Since donors were healthy volunteers, no ethical approval was required. CLL patients studied in this project were attending clinics at Llandough hospital and Heath hospital. These patients are part of a cohort not requiring treatment. Blood samples were collected with informed consent as part of an ongoing LRF funded project (Drs Pepper and Fegan), and ethical approval was in place. Most of the blood samples used to generate short term T cell cultures were stained for HLA-A*201 using HLA-A2 specific monoclonal antibodies.

2.4.2 Isolation of peripheral blood mononuclear cells (PBMC)

Fresh human blood was collected into heparinised (10 units/ml) Falcon tubes. PBMC were isolated by Ficoll-Hypaque (Histopaque-1077, Sigma) density gradient centrifugation. Blood was layered gently onto Histopaque at a 1:1 ratio and centrifuged at 2000 rpm for 20 minutes without the use of a brake. PBMC formed a monolayer at the plasma/histopaque interface and were carefully harvested using sterile Pasteur pipettes and washed three times in serum-free RPMI 1640 media. The first wash was at 1800 rpm for 10 minutes and the next two at 1200rpm for 5 minutes. If the pellet was contaminated with a large proportion of red blood cells, it was resuspended in 1- 2mls of red blood cell lysis buffer and incubated for 10 minutes at 4°C. Viable cells were then counted by trypan-blue-exclusion and resuspended in an appropriate volume of media.

2.5 Primary PBMC /T cell cultures

2.5.1 Short-term *in vitro* culture of PBMC/T cells with peptides

PBMC/T cells were cultured in a 24 well plate at $2-2.5 \times 10^6$ cells/ml of AB-RPMI (1ml/well) in presence of peptide. MART-1, positive peptide pool (PPP), and Bax peptides were used at a total concentration of 10µg/ml where indicated. 1µg/ml of CD28 antibody and 0.5µg/ml CD49d antibody (Serotec) were also added to the wells. It has been shown that the use of these costimulatory antibodies enhanced peptide specific IFN γ production (Waldrop *et al.*, 1998). On day 2-3, 1ml of AB-RPMI media supplemented with IL-2 (40u/ml) (Proleukin) was added to each well. On day 7, some of the PBMC cultures were tested for the presence of peptide-specific T cells in the IFN γ ELISpot assay.

2.5.2 Cryopreservation of PBMC

A fraction of PBMC was resuspended in freezing mix to give $2-10 \times 10^6$ cells per ml depending on the intended use, and 1ml aliquots were dispensed per cryovial. These were then placed in a Nalgene 5100 cryo freezing container (Merck Laboratory Supplies) for at least 24 hours at -70°C and then transferred to liquid nitrogen. Frozen PBMC were later used as APC to restimulate T cells in culture or in the IFN γ ELISpot assay.

2.5.3 Restimulation of PBMC/T cell cultures

PBMC/T cell cultures were re-stimulated with the appropriate peptides every week. The cultured PBMC were harvested, counted, and re-plated at $1.5-2 \times 10^6$ cells/well in 0.5ml of AB-RPMI media supplemented with 40 U/ml IL-2. Autologous cryopreserved PBMC were used as APC. These cells were irradiated in FCS-RPMI at 3,000 rads, washed in serum-free RPMI and then added to wells in 0.5ml AB-RPMI at a ratio of 2 irradiated-APC: 1 cultured PBMC/T cells. Peptide or peptide pools were then added to the wells at $10\mu\text{g/ml}$. After 2-3 days, 1ml of AB-RPMI supplemented with IL-2 (40u/ml) and IL-7 (10ng/ml) (R&D systems) was added to each well. PBMC/T cell cultures were re-stimulated during 2-4 weeks where indicated.

2.5.4 Enrichment of CD8+ T cells from PBMC using specific microbeads

CD8+ T cells were enriched using magnetic microbeads specific for CD8 ($30-50 \times 10^6$ input cells). Cells were washed in PBS and spun twice at 1500rpm in a 15ml Falcon tube. Supernatant was removed with a pipette and pellets were resuspended in 80 μl cold MACs buffer and 20 μl anti-CD8 microbeads (MACs, Miltenyi) per 10^7 cells. Cells were incubated for 15 minutes at 4°C in a rotator. Pellets were then washed (in 20x the incubation volume) with cold MACs buffer and spun at 1300rpm for 10 minutes. Supernatant was removed and

cells resuspended in 500µl cold MACs buffer. This was then loaded onto a magnetic MS column (Miltenyi) pre-washed with 500µl MACs buffer. Unlabelled cells passed through the column, which was washed 3 times with 500µl MACs buffer. These cells were collected as the CD8 depleted fraction. To collect the CD8-enriched fraction, the column was removed from the magnetic field and 1ml MACs buffer was applied to the column. A syringe plunger was then used to force the buffer and bead-labelled cells through. An aliquot of the pre-sort, positive and negative fraction was taken for purity analysis by flow cytometry. CD8⁺ T cells were cultured as described in section 2.5.1.

2.6 Detection of peptide-specific T cells

2.6.1 IFN γ ELISpot assay

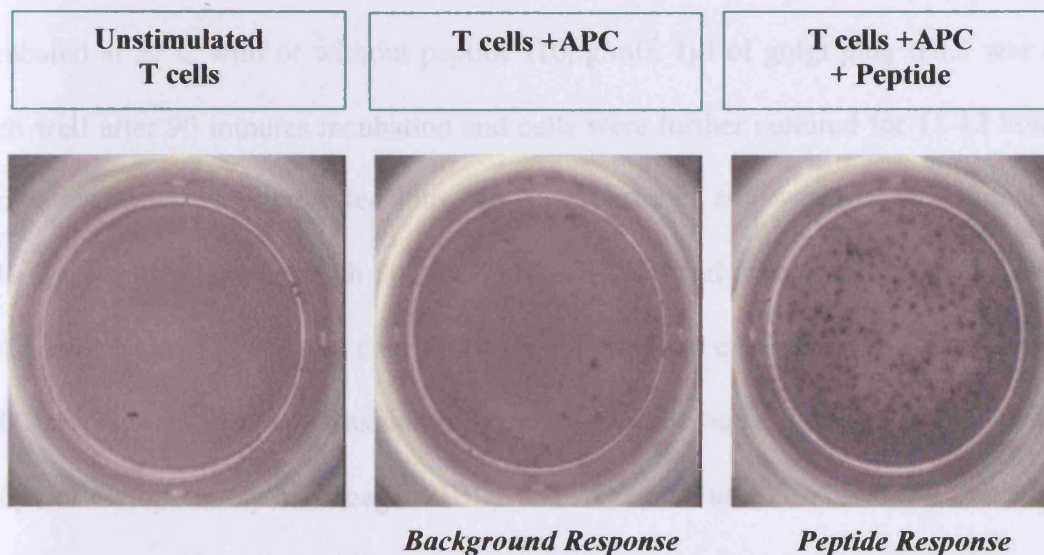
ELISpot wells were set up either in triplicate or duplicate. Most experiments included wells containing T cells alone, T cells + APC, T cells + APC + peptide, T cells+mitogens (positive control). Firstly, mouse anti-human IFN γ antibody 1-DIK (Mabtech) was diluted to 10µg/ml with PBS, and 50µl was added to each well of an ELISpot plate (Millipore). Plates were either incubated overnight at 4°C, or for 3 hours at 37°C. Plates were washed thoroughly 5 times with 150µl PBS/well, before blocking with 100µl of FCS-RPMI media for 1 hour at room temperature. The responder cells (either cultured PBMC or T cells) were added to the wells in 50µl of AB-RPMI media. The number of cells/well ranged from 1×10^3 - 1×10^5 . The APC (PBMC/T2) were then added in 50µl of AB-RPMI media to the appropriate wells at a ratio 1 APC: 1 responder. The peptide was added to appropriate wells in 50µl of AB-RPMI media. T cells + phytohaemagglutinin (PHA) (Biostat) 2µg/ml, ionomycin (Calbiochem) 1.5µg/ml, phorbol myristate acetate (PMA) (Calbiochem) 100ng/ml and Concavalin A (Sigma) 20µg/ml were added to the positive control wells. Control wells that did not require

peptide/APC received 50-100µl of the media alone, so that the total volume is 150µl. The plate was then wrapped in silver foil and incubated at 37°C for 16-18 hours.

Plates were washed 3 times with 150µl PBS and then incubated with 100µl sterile water for 10 minutes at room temperature, therefore lysing the remaining bound cells. The plate was then washed a further 2 times with 150µl/well PBS. The secondary biotinylated antibody 7-B6-1-Biotin (Mabtech) was diluted 1:1000 with PBS. This was added at 50µl/well and incubated in the dark at room temperature for 2 hours. The plate was washed 5 times with PBS, and 50µl of Streptavidin-Alkaline phosphatase (diluted 1:1000 with PBS) was added to each well. The plates were incubated for 2 hours in the dark at room temperature, followed by washing 5 times with PBS.

Developing solution was made freshly using 25xAP colour development buffer (Bio-Rad), AP conjugate substrate A and B solutions (Bio-Rad), and sterile water. The solution was added at 50µl/well and left to develop in the dark between 10 to 50 minutes, until the spots were clearly visible. To stop the developing reaction, tap water was used to wash the plates 4 times. Plates were then air dried in the dark before counting.

Spots were counted by eye using a stereomicroscope (magnification 3.2x) (Leica MZ6) and some were also counted using an automated ELISpot counting system (AID ELISpot reader, Cadama Medical) to confirm results. A response was considered positive if the mean number of spots from the test wells (T cells + APC + peptide) was greater than 20 spots/ 1×10^5 cells, after subtraction of the background response from control wells (T cells + APC). This criterion is used in order to reduce the possibility of detecting false positives.



$$\text{Positive ELISpot Response} = \text{Peptide response} - \text{Background response}$$

Figure 2.1 Detection of peptide specific T cells by IFN γ ELISpot assay.

ELISpot wells were set up in triplicate with T cells, + APC, and + APC + peptide. Each spot represents an IFN γ -secreting T cell. Background response (non-specific IFN γ secretion by T cells and/or APC) must be subtracted from Peptide response (specific IFN γ secretion by T cells recognising peptide + background response) in order to determine the peptide specific response. The ELISpot well images shown above were captured using an AID ELISpot reader. (Positive ELISpot response = ≥ 20 spots/ 1×10^5 cells)

2.6.2 IFN γ -Intracellular Cytokine Staining (ICS) analysis

T cells were plated out in 0.5ml AB-RPMI at 5×10^5 cells/well in a 48 well culture plate. APC were added to the wells in 0.5ml AB-RPMI at $0.5-1 \times 10^6$ /well, and the cells were incubated at 37°C with or without peptide (10 μ g/ml). 1 μ l of golgi plug (BD) was added to each well after 90 minutes incubation and cells were further cultured for 11-13 hours. After incubation, cells were harvested into 15ml Falcon tubes and washed twice with PBS. Cell pellets were then labelled with 5 μ l antibody anti-CD8 and incubated for 20 minutes at 4°C. Cells were washed twice with cold ICS sample buffer and centrifuged at 4°C for 4 minutes at 1500rpm. The supernatant was carefully removed and each cell pellet was resuspended in 250 μ l of Cytoperm/Cytofix reagent (BD) and incubated at 4°C in the dark for 20 minutes. Cells were washed twice with 1x perm/wash solution (BD), with the supernatant being carefully removed by pipette to avoid cell loss. The cell pellets were resuspended in 50 μ l perm/wash + 2 μ l of mouse anti-human IFN γ antibody (Caltag/Invitrogen) and incubated in the dark for 30 minutes at 4°C. Cells were then washed twice with perm/wash. The cells were resuspended in 200 μ l of ICS sample buffer and analysed by flow cytometry.

2.6.3 CD107 assay

A CD107 assay was carried out to identify and enrich Bax specific T cells following peptide stimulation. This assay allows the direct detection of degranulation by activated T cells. CD107 is present in the membrane of cytotoxic granules. During T cell degranulation the granule fuses with the T cell plasma membrane and CD107 can be detected on the cell surface. If the effector cells were frozen, they were thawed the day before and plated out at $1-2 \times 10^6$ / well in AB-RPMI containing 10ng/ml IL-7. Targets were pulsed with peptide or left unpulsed (control) for 1hour in serum-free media at 37°C. 1×10^5 T cells were incubated in 100 μ l AB-RPMI with 100 μ l containing target cells in a 96 well plate, normally at 1:2

ratio, otherwise stated in the results. The CD107a-FITC antibody was added to each well at 2µl/ml and golgi stop (BD) was added at 1µl/ well (diluted 1:5 in serum-free media). Cells were spun at 1300rpm for 2 minutes to increase cell to cell contact and incubated at 37°C for 4 hours, after which they were harvested and washed (in FACS buffer) before analysing by flow cytometry.

2.6.4 CD137 assay

A CD137 assay was also carried out to identify and enrich Bax specific T cells after peptide stimulation. This assay allows the detection of antigen-specific activated CD8⁺ T cells. CD137 is a member of TNRF family that is expressed on activated T cells and helps T cells to proliferate and survive (Wolfl *et al.*, 2007). If the effector cells were frozen, they were thawed the day before and plated out at $1-2 \times 10^6$ / well in AB-RPMI containing 10ng/ml IL-7. Targets were pulsed with peptide or left unpulsed (control) for 1 hour in serum-free media at 37°C. 1×10^5 T cells were incubated in 500µl AB-RPMI with 500µl containing target cells in a 24 well plate at 1:1 ratio. Cells were spun at 1300rpm for 2 minutes to increase cell to cell contact and incubated at 37°C for 24 hours. The cells were then harvested and washed (in FACS buffer) and incubated with anti-human CD137-FITC and CD8-PE before analysing by flow cytometry.

2.7 Expansion of T cells using an allogenic feeder system

This expansion method was used to generate large numbers of T cell lines (antigen-independent protocol). T cells with numbers up to 1×10^6 were seeded into a T25 tissue culture flask with 25mls of AB-RPMI supplemented with 20 U/ml IL-2, 0.5µg/ml PHA, and 10×10^6 irradiated allogeneous PBMC feeders (from 3 donors). The flask was placed into a 37°C incubator tilted at 45°, so as to enhance cell-cell contact. On day 5 of the expansion,

half the media was replaced with the same volume of AB-RPMI media supplemented with 20 U/ml IL-2. Cells were resuspended and incubated for a further 2 days in upright position. Cells were then harvested, counted and rested in fresh AB-RPMI supplemented with 20U/ml IL-2 at 2×10^6 cells/well in 24 well plates. Every 2-3 days when media turned yellow, half the media was replaced with AB-RPMI + 100 U/ml IL-2. The cells were then used in experiments or frozen until further use.

2.8 Enrichment of peptide specific T cells using CD107 or CD137 assay

Activation of CD8⁺ T cells after encounter with APC pulsed with Bax peptide pool was determined by cell surface expression of CD107a or CD137 as previously described in section 2.6.3 and 2.6.4. All samples were stained with an anti-CD8-PE antibody. The T cell cultures were then washed once with warm PBS and resuspended in 0.5ml AB-RPMI. A CD8⁺ CD107a⁺ or CD8⁺ CD137⁺ population containing peptide specific T cells was sorted using a MoFlo cell sorter (Dako). The cells were then expanded and used in further experiments or frozen until being used.

2.9 Enrichment of IFN γ -secreting T cells using magnetic beads

Firstly, target cells (T2 cells) were irradiated the day before the enrichment and left in the fridge overnight. Cells were washed and added in presence of peptide/peptide pool (10 μ g/ml) on top of T cells at 10^6 cells/100 μ l in 96 well plates. Cells were incubated for 4 hours at 37°C. After incubation, a dead cell removal kit (Miltenyi Biotec) was used prior to the cell separation in order to reduce any dead cell contamination in the sample. Cells were harvested into a 15ml falcon tube and centrifuged. Supernatant was completely removed and the cell pellet was resuspended in 100 μ l of Dead Cell Removal Microbeads / 10^7 total cells, mixed well and incubated for 15 minutes at room temperature. Meanwhile, a MS column was

prepared by rising with 500µl 1x Binding buffer. Cell were washed and resuspended in 500µl 1x Binding buffer. The live cells passed through the column, which was rinsed 4 times with 500µl 1x Binding buffer. Effluent was collected as the live cell fraction.

The cells were washed twice with 10mls of cold MACs buffer, and centrifuge at 1300rpm for 10 minutes. The pellet was incubated on ice for 5 minutes with 80µl cold serum-free RPMI and 20µl of IFN γ catch reagent/ 10^7 cells. This catch reagent is a mouse anti-human IFN γ antibody conjugated to a mouse anti-human CD45 antibody. Therefore, all leukocytes are labelled with an antibody capable of binding secreted IFN γ . The cells were then diluted with warm AB media according to the expected number of IFN γ secreting cells (<5% : $1-2 \times 10^6$ cells/ml; 5-20%: $1-2 \times 10^5$ cells/ml), thus restarting the IFN γ secretion process. Cells were incubated at 37°C for 45 minutes whilst being gently rotated using a MACsmix tube rotator (Miltenyi). During this period, the secreted IFN γ was captured onto the surface of the cell that produced it. The IFN γ secretion was terminated by topping up the tube with cold MACs buffer and placing the falcon on ice for 2 minutes. Cells were centrifuged and the pellet was resuspended in 80µl of cold MACs buffer, 20µl of IFN γ detection antibody ($/10^7$ cells). After 10 minutes incubation on ice, 10mls of cold MACs buffer was added to the tube and the cells were centrifuged. A small aliquot was taken for later analysis by flow cytometry. Supernatant was removed and the pellet was resuspended in 80µl cold MACs buffer and 20µl of anti-PE magnetic beads, mixed thoroughly and incubated at 4°C for 15 minutes. Cells were washed in 10mls of cold MACs buffer and the pellet was resuspended in 500µl cold MACs buffer. The remaining cells were loaded onto a prepared MS magnetic column (Miltenyi). The unlabelled and labelled fractions were collected as described in section 2.5.4, with one additional step in which the positive fraction was then reloaded onto a second MS column to ensure a high purity of IFN γ -secreting cells in the enriched population.

Aliquots of the pre and post enrichment fractions were analysed by flow cytometry. 7-AAD was added to the appropriate samples to allow exclusion of dead cells by negative gating during flow cytometric analysis.

2.10 Generating peptide-specific T cell lines

2.10.1 Culture of enriched peptide-specific T cell population

Enriched peptide-specific T cells using CD107, CD137 or IFN γ protocol, were expanded using the antigen-independent protocol as described in section 2.7. After expansion, the T cells were allowed to rest in fresh AB-RPMI supplemented with 20U to 100U/ml IL-2 at 2×10^6 cells / well in 24 well plates.

2.10.2 Generation of T cell lines from peptide specific T cells under limiting dilution conditions

The IFN γ enriched cells were cultured under limiting dilution conditions. The T cells were diluted such that 1, or 10 cells/well and added in 50 μ l AB-RPMI to the 60 innermost wells of 96 well, round-bottomed culture plates. Six plates were seeded with 1 cell/well, one plate with 10cells / well, and half a plate only with feeders. 50 μ l of a cloning mix was added to the appropriate wells. The cloning mix in each well consists of AB-RPMI, 40U/ml IL-2, 2 μ g/ml PHA, 5×10^4 irradiated allogenic PBMC (from 3 donors). On day 7, cells were fed with 100 μ l of fresh AB-RPMI + 40U/ml IL-2. On day 14, 100 μ l was removed from each well and replaced with 100 μ l cloning mix with a final concentration of 20U/ml IL-2, 1 μ g/ml PHA, and 5×10^4 irradiated autologous PBMC (from 3 donors). Feeding and re-stimulating were alternated for 2 further weeks. T cell lines that had grown to a large pellet size, were plated out into new plates, split 1:2 or 1:3 and fed with AB-RPMI + IL-2 (20 IU/ml). After 3-4

weeks, these cultures had grown sufficiently to be tested for recognition of Bax peptides by ELISpot.

2.10.3 Selection of peptide-specific T-cell lines from cloning plates

The T cell lines isolated from the cloning protocol in the previous section were tested for recognition of the Bax pool in an IFN γ ELISpot assay. Because the precise number of T cells in each well was not known, 4 different culture wells were harvested and counted and an average of 1.0×10^5 cells/well was calculated. 50 μ l of each well, including on average $2-3.0 \times 10^4$ cells, was taken from each line and added to each of two wells of an ELISpot plate. T2 cells were used as APC and these were added at 3×10^4 cells/well in 50 μ l AB-RPMI. Due to the number of responder cells, only 2 wells were set up, one of which was a control for spontaneous IFN γ secretion (T-cell +T2) and the other contained Bax pool (T-cell + T2+Bax pool). Bax pool was added (at a final concentration of 1 μ g/ml) in 50 μ l AB-RPMI media to one well, and 50 μ l of AB-RPMI media alone was added to the control well.

The most specific lines were selected according to the estimated number of spots in the peptide well (excluding lines that generated high background in control wells) and the size of the cell pellet in each culture well. These T cell lines were then expanded using the protocol described in section 2.7.

2.11 Chromium-51 (^{51}Cr) release cytotoxicity assays

Functional assays to determine Bax specific CTL killing of cancer cell lines were carried out by ^{51}Cr release cytotoxicity assay. The target cells were labelled with ^{51}Cr , which is released if the cell is killed by CTL. Some assays were carried out using target cells that had been pre-treated with IFN γ or proteasome inhibitors (described in sections 2.12 and 2.13).

Effector cells used in this assay, were either from T cells in culture or from cryopreserved stocks, which were thawed and incubated overnight at a density of 2×10^6 cells per well in a 24 well plate in 10% AB media containing 10ng/ml IL-7. On the day of assay, target cells were washed and labelled with ^{51}Cr (Perkin Elmer) ($500 \mu\text{Ci}/10^7$ cells of a 5mCi/ml stock) for 1 hour at 37°C . The cells were then washed, resuspended in 1ml FCS-RPMI. Peptide was added normally at $10 \mu\text{g/ml}$ to the appropriate target, but this varied for peptide dose response experiments. All targets were incubated for 1 hour at 37°C to allow peptide binding and unincorporated ^{51}Cr to leach into the media.

Meanwhile, effector cells were harvested, resuspended in FCS-RPMI media and counted. The number of cells determined the top Effector:Target (E:T) ratio, but a top E:T ratio varied according to the experiment. The effectors were plated out and double diluted in a 96 well plate so as to give a range of E:T ratios and $100 \mu\text{l/well}$.

After leaching, target cells were washed and resuspended at $1-2 \times 10^5$ cells/ 10ml in FCS-RPMI. 1000 - 2000 targets/ $100 \mu\text{l}$ was added to the wells of a 96 well plate which already contained effector cells in triplicate. Control wells containing only target cells in FCS-RPMI (minimum: spontaneous release) and wells containing targets with 5% Triton-X100 (Sigma) (maximum release) were also set up. Assay was incubated at 37°C for 4 hours.

After the 4 hour incubation, $20 \mu\text{l}$ of supernatant from each well was harvested and transferred to flexible 96 well counting plates (Wallac). $150 \mu\text{l}$ of scintillation fluid (Optiphase scint, Wallac) was added to each well. The amount of ^{51}Cr released was determined by measuring beta particle events per minute (Beta-plate counter, Wallac).

% specific lysis was calculated as follows:

$$\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100.$$

2.11.1 Cold-target competition assay

For competition assays, an E:T ratio of 40:1 or 50:1 was used. Along with the labeled targets (T2, T2+Bax p613, tumour cell), nonlabeled T2, T2 + irrelevant peptide, T2 + Bax p613 and the same tumour cell type were included into the cytotoxicity assay at a competitor (cold target): target (hot target) (C:T) ratios of 20:1, 10:1, 5:1. Control lysis in the absence of competitor cells was also determined.

Firstly, the competitors T2 were pulsed with Bax p613 or Flu M1 peptide at 10µg/ml for 1 hour. Meanwhile, the hot targets were labeled with ⁵¹Cr and pulsed with peptide as described in the previous session. While hot targets are leaching, the competitors were washed and seeded at different C:T ratios. These cells were double diluted in a 96 well plate so as to give the chosen C:T ratios stated above and 50µl/well of FCS-RPMI. Effectors (with a fixed ratio of 25:1 or 50:1) were added on top of the competitors in 100µl/well.

After leaching, hot target cells were washed and resuspended at 1-2x10⁵ cells/ 5ml in FCS-RPMI. 1000 - 2000 targets/50µl was added to the wells of a 96 well plate which already contained competitor and effector cells in triplicate. Control wells for spontaneous and total release containing only hot target cells with or without competitors were set up. The rest of this assay was carried using the method described before.

2.11.2 Antibody-blocking assay

HLA-restriction of CTL-mediated killing was achieved by incubating the targets with HLA-specific antibodies for 30 minutes at 37°C before addition of a fixed number of effector cells. T2 and T2+peptide were included as controls. The targets were labeled with ⁵¹Cr as previous described. After labeling and leaching, the targets were plated out in 50µl/well with different volumes (25, 50 or 100µl) of Ma2.1 antibody for HLA-A2.1, L243 antibody for HLA-DR (negative control) and anti-HLA-A23/24 antibody at a concentration of 10µg/ml or 20µg/ml.

After 30 minutes incubation, the effector cells were added to the wells in 50µl/well (normally an E:T of 40:1 or 50:1). The rest of the assay was performed as described before.

2.12 IFN γ treatment of target cells

Target cells were split 48 hours prior to assay and 200units/ml of IFN γ (Roche) was added to the flasks. After 48 hours, the cells were harvested and used as targets in cytotoxic assays.

2.13 Treatment of cells with proteasome inhibitors

Clasto-Lactacytin β -lactone (Calbiochem) or bortezomib, also known as Velcade, (Millenium) were used as proteasome inhibitors. Established cell lines were split 48 hours prior to assay, and then 18 hours before the assay proteasome inhibitor was added to the flasks. For PBMC cultures, cells were incubated overnight with proteasome inhibitor in 15ml falcon at a concentration of approximately 2×10^6 /ml. Thereafter, the cells were harvested, counted and used in further experiments.

2.14 Flow cytometric analysis and monoclonal antibodies

Cells were analysed using a BD FACSCalibur flow cytometer or a BD FACSCanto II cytometer (Becton Dickinson & Co) and analysis was performed using CellQuest Pro analysis software or FlowJo analysis software. Analysis of PBMC by flow cytometry involved the construction of a live lymphocyte gate which was based upon the forward and side scatter profiles of these cells. For immunophenotyping, compensation was done automatically using an anti-Mouse Ig/Negative control compensation particles set and FACSDiva software (BD). Three drops of CompBeads anti-mouse Ig and CompBeads negative control were added per 700µl of FACS buffer, from which 100µl was transferred

into FACS tubes. Fluorochrome-conjugated monoclonal abs (with the same concentration added to the cell samples) were added per tube and incubated for 5 minutes at room temperature in the dark. After this incubation, 900µl of FACS buffer was added to each tube. These were then used as single-colour compensation controls. On the dot plot for each single-stained control, a gate corresponding to a single population was set up and the negative and positive populations on a histogram were adjusted. Compensation was then automatically calculated using FACSDiva software for each tube based on the corresponding single-stained control.

2.14.1 Monoclonal antibodies

The monoclonal antibodies used in this study are shown in Table 2.5 and 2.6. Table 2.6 shows the panels of antibodies used for the immunophenotyping study in the last chapter of results.

Table 2.5 Monoclonal antibodies

Antibody Specificity	Supplier	Description
CD3-PE	Serotec	Mouse IgG1, clone UCHT1
CD4-PE	Serotec	Mouse IgG1, clone RPA-T4
CD8-FITC	Serotec	Mouse IgG1, clone LT8
CD8-PE	Serotec	Mouse IgG1, clone LT8
CD19-FITC	Serotec	Mouse IgG1, clone LT19
CD107a-FITC	BD	Mouse IgG1, clone H4A3
CD137-FITC	Serotec	Mouse IgG1, clone 4B4-1
HLA-A2 (Ma2.1)	ATCC	HB-54: B lymphocyte hybridoma; Mouse IgG2
HLA-DR (L243)	ATCC	HB-55: B lymphocyte hybridoma; Mouse IgG2a
HLA-A2-FITC	Serotec	Mouse IgG2b, clone BB7.2
HLA-A23/24	One Lambda	Mouse IgG2b
IFN γ -FITC	Caltag	Mouse IgG1, clone B27
TCR V β 2	Serotec	Mouse IgG1, Clone MPB2D5
TCR V β 3	Serotec	Mouse IgGM, Clone CH92
TCR V β 5.1	Serotec	Mouse IgG2a, Clone IMMU157
TCR V β 5.2	Serotec	Mouse IgG1, Clone 36213
TCR V β 7	Serotec	Mouse IgG2a, Clone ZOE
TCR V β 8	Serotec	Mouse IgG2a, Clone 56C5
TCR V β 9	Serotec	Mouse IgG2a, Clone FIN9
TCR V β 11	Serotec	Mouse IgG2a, Clone C21
TCR V β 12	Serotec	Mouse IgG2a, Clone VER2.3.2
TCR V β 13.1	Serotec	Mouse IgG2b, Clone IMMU222

TCR V β 13.6	Serotec	Mouse IgG1, Clone JU-74
TCR V β 14	Serotec	Mouse IgG1, Clone CAS1.1.3
TCR V β 16	Serotec	Mouse IgG1, Clone TAMAYA1.2
TCR V β 17	Serotec	Mouse IgG1, Clone E17.5F3
TCR V β 18	Serotec	Mouse IgG1, Clone BA62
TCR V β 20	Serotec	Mouse IgG1, Clone ELL1.4
TCR V β 22	Serotec	Mouse IgG1, Clone IMMU546
TRC V β 23	Serotec	Mouse IgG1, Clone AF-23
FITC/PE (negative)	Dako	Mouse IgG1/FITC; Mouse IgG2a/RPE
FITC/PE (negative)	Serotec	Mouse IgG1/FITC; Mouse IgG1/RPE
FITC	Dako	Rabbit α -mouse IgG

Table 2.6 Panels of monoclonal antibodies for immunophenotyping

Panels	Fluorochrome	Supplier	Description	Volume added/ $10^6(\mu\text{l})$
General				
CD56	FITC	Serotec	Mouse IgG2a, clone MEM-188	5
CD200	PE	Serotec	Mouse IgG1, clone OX-104	10
CD5	PE-Cy5.5	Invitrogen	Mouse IgG1, clone CD5-5D7	5
CD19	PE-Cy7	Ebioscience	Mouse IgG1, clone HIB19	10
CD16	APC	Serotec	Mouse IgG1, clone 3G8	2.5
CD8	APC-Cy7	Invitrogen	Mouse IgG1, clone SK1	5
CD4	Pacific Blue	BD	Mouse IgG1, clone RPA-T4	2.5
CD3	AmCyan	BD	Mouse IgG1, clone SK7	2.5
T cell subsets				
CD57	FITC	Serotec	Mouse IgM, clone TB01	5
CCR7	PE	R&D systems	Mouse IgG2a, clone 150503	10
CD28	percp-cy.5.5	BD	Mouse IgG1, clone L293	10
CD27	PE-CY7	Ebioscience	Mouse IgG1, clone O323	10
CD45RO	APC	Invitrogen	Mouse IgG2a, clone UCHL1	2.5
CD8	APC-CY7	Invitrogen	Mouse IgG1, clone SK1	5
CD4	Pacific Blue	BD	Mouse IgG1, clone RPA-T4	2.5
CD3	AmCyan	BD	Mouse IgG1, clone SK7	2.5
PD-1, TCR GD, CD200R				
CD279	FITC	Serotec/BD	Mouse IgG1, clone MIH4	10
CD200R	PE	Serotec	Mouse IgG1, clone OX-108	10
CD19	PE-Cy7	Ebioscience	Mouse IgG1, clone HIB19	5
TCR $\gamma\delta$	APC	BD	Mouse IgG1, clone B1	10
CD8	APC-CY7	Invitrogen	Mouse IgG1, clone SK1	5
CD4	Pacific Blue	BD	Mouse IgG1, clone RPA-T4	2.5
CD3	AmCyan	BD	Mouse IgG1, clone SK7	2.5
Tregs				
Foxp3	FITC	Ebioscience	Rat IgG2a, clone PCH101	10
CD25	PE	Miltenyi Biotec	Mouse IgG2b, clone 4E3	8
CD45RA	APC	BD	Mouse IgG2b, clone HI100	10
CD4	Pacific Blue	BD	Mouse IgG1, clone RPA-T4	2.5
CD3	AmCyan	BD	Mouse IgG1, clone SK7	2.5

2.14.2 Single colour immunofluorescence staining

Cells were pelleted in wells of a 96 well round bottomed plate or in FACS tubes at $1-2 \times 10^5$ cells/ well or tube. The pellets were washed twice with 100 μ l cold FACS buffer (or 500 μ l if tubes) and centrifuged at 2000rpm at 4°C for 2 minutes. Supernatant was removed and the appropriate cells were resuspended in 2-3 μ l of commercially available antibody, or 20 μ l of the supernatants L243 and Ma2.1. Cells were incubated for 15-30 minutes at 4°C in the dark. Cells were washed with 100 μ l cold FACS buffer (or 500 μ l if tubes) and centrifuged at 2000rpm for 2 minutes at 4°C. Supernatant was removed and the wash process repeated twice. The cells were resuspended in FACS buffer and were either fixed with paraformaldehyde (PAF) or analysed immediately. For fixation, cells were resuspended in 100 μ l FACS buffer + 100 μ l 4% PAF, giving a final concentration of 2% PAF, and incubated at 4°C in the dark until analysis could be performed. Where un-conjugated antibodies were used, cells were subsequently incubated with the secondary rabbit anti-mouse-FITC antibody (Dako). Cells were incubated for 15-30 minutes at 4°C in the dark, before being washed 2 times with FACS buffer. Cells were then either fixed or analysed immediately. Negative control wells were included in all experiments. For directly conjugated antibodies a mouse IgG-FITC/PE (Dako) negative control was used. The control for un-conjugated antibodies was incubation with the rabbit α -mouse-FITC alone. Unstained cells were also included as additional controls.

2.14.3 Multicolour immunofluorescence staining

The method varied according to whether the primary antibodies were conjugated or not. For directed conjugated antibodies (except for Tregs panel), staining was performed in a single step by adding conjugated antibodies to pelleted cells for 15-30 minutes at 4°C in the dark, before being washed twice with FACS buffer. Cells were then either fixed or analysed

immediately. In the immunophenotypic study, usually $1-1.5 \times 10^6$ cells were used per tube and were analysed immediately after staining without fixation. The antibodies that were used for immunophenotyping (see Table 2.6) were optimized by using neat, $\frac{1}{2}$ dilution and $\frac{1}{4}$ dilution of the recommended concentration by the manufacturer. The panels were also optimized by replacing antibodies that did not work together.

In the absence of a FITC-conjugated antibody, rabbit anti-mouse FITC antibody was added to cells after washing off the unconjugated primary antibody. The cells were incubated for 15-30 minutes at 4°C in the dark and washed twice with FACS buffer. The directed conjugated antibody was then added for 15-30 minutes at 4°C in the dark. The cells were again washed twice and then either fixed or analysed immediately.

2.14.4 Human Foxp3 staining

For Tregs panel, cells were stained using a FITC anti-human Foxp3 staining set (eBioscience) that preferentially stains $\text{CD4}+\text{CD25}^{+}$. Cells were resuspended at $1 \times 10^6/100\mu\text{l}$ /tube in FACS buffer and stained for 30 minutes on ice with the surface cell markers: CD25-PE , CD45RA-APC , CD4-Pacific blue , and CD3-AmCyan (see Table 2.6). Cells were washed twice with $500\mu\text{l}$ cold FACS buffer. Fix/perm buffer was made by diluting the fixation/permeabilization concentrate (1 part) into the fixation/ permeabilization diluent (3 parts). The cells were resuspended in $500\mu\text{l}$ of fix/perm buffer and incubated for 40-60 minutes on ice. This step fixes the cells. Meanwhile, perm wash buffer was made by diluting the 10x permeabilization buffer in distilled water to a 1x solution. The cells were washed with 1-2ml of perm wash buffer and centrifuge twice at 1500 rpm for 5 minutes. The cells were resuspended in $30\mu\text{l}$ of perm wash buffer with 2% normal rat serum and incubated for 15 minutes on ice. This step permeabilises the cells. Without washing, $10\mu\text{l}$ of Foxp3-FITC was added to the cells and incubated for 30 minutes on ice in the dark. After incubation, the cells were washed and centrifuged twice with perm wash buffer. The cells

were washed again with FACS buffer, resuspended in approximately 250 μ l FACS buffer and analysed immediately.

2.15 Western blotting

Detection of Bax protein in cells was carried out by Western blotting. This technique involves the separation of proteins (based on MW) by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transfer onto a polyvinylidene difluoride (PVDF) support membrane followed by immunostaining.

2.15.1 Buffers

2x gel sample buffer: 0.1M Tris/HCl (pH 6.8), 4% SDS, 20% glycerol, 0.1 % bromophenol blue, 0.4M sodium 2-mercaptoethanesulfonate (MESNA) (Sigma) made up to 100ml of distilled water.

Running buffer: 50mls of 20x NuPAGE MOPS (Invitrogen), 950mls distilled water, made up to 1L.

Blotting buffer: 30g Tris, 144g Glycine, 2L methanol, 8L distilled water.

Casein blocking solution (IBT): 0.2% I-Block, 0.1% Tween-20, 0.002% sodium azide made up in PBS.

Alkaline Phosphatase (AP) buffer: 11ml Diethylamine (Tropix-Applied Biosystems), 80ml distilled water, pH corrected to 9.5 before adding 0.2g $MgCl_2$. Made up to 100ml with distilled water.

MESNA stripper: 6.25ml 1M Tris pH6.8, 0.82g MESNA, 2.0g SDS, made up to 100ml of distilled water.

2.15.2 Generation of total cell lysates

Cells were counted on a haemocytometer and resuspended in 50 μ l 1x PBS per 5×10^5 - 1×10^6 cells. An equal volume of 2x gel sample buffer was added. Cells were then frozen at $-20^\circ C$.

Prior to use samples were sonicated for 1-2 minutes on continuous setting (W0385 sonicator, Heatsystems-Ultrasonics Inc.), and then heated at 100 °C for 5 minutes.

2.15.3 SDS-PAGE

Equal amounts of cell lysates (10-20 μ l) were loaded onto pre-cast polyacrylamide gels (NuPAGE 4%-12% Bis-Tris gels 10 wells 1.5mM, Invitrogen) and subjected to SDS-PAGE using the Invitrogen XCell system (Invitrogen). Invitrogen Xcell Surelock Mini-cell was filled with 800-900mls of 1x Running buffer. The gel was removed from storage buffer, comb was taken out and adhesive strip removed from foot of gel. Wells were washed 2-3 times with running buffer, ensuring that all air bubbles were removed. Gels were inserted into the tank with wells facing inwards and locked into place. 10-20 μ l of samples and 10 μ l pre-stained marker (See blue plus2, Invitrogen) were loaded with gel loading pipette tips. The tank was connected to power supply and run at 200volts for approximately 50-60minutes. Once the gels have finished running, the gels were removed from the gel cassettes using the Gel knife.

2.15.4 Transfer onto PVDF membranes

PVDF membranes, cut to size, were soaked in methanol for 1 minute, and then in blotting buffer. Pads and filter papers were soaked in blotting buffer. The gels were removed from cassette using a piece of pre-wet filter paper to assist gel handling. The gel/membrane sandwich was assembled by layering, if only one gel, 2 pads on the cathode core of the blot module, then filter paper, gel, membrane, filter paper, 2 pads. If two gels, the assembly was done by layering 2 pads, filter paper, first gel, membrane, filter paper, 1 pad, filter paper, second gel, membrane, filter paper, 2 pads. This transfer stack was placed into Invitrogen Xcell II blot module, taking care to avoid air bubbles between gel and membrane. Blotting buffer was added to the central chamber, enough to just cover the gel/membrane sandwich. Water was added to the outside chamber to help diffuse any heat generated during transfer.

The tank was connected to power supply and run at 30v 250mA, if one gel, for 60 minutes, and, if two, for 80 minutes.

2.15.5 Immunodetection of protein on PVDF blots

After transfer, the membranes were washed with 0.2% PBS-Tween-20 for 10 minutes on a rocking platform. The blots were blocked for a minimum of 1 hour using IBT. The membranes were removed from the blocking reagent and cut into two. The top part was incubated overnight with anti-human Actin (mW 42kDa, Sigma) and bottom part with monoclonal anti-human Bax (mW 21kDa, R&D systems). Bax antibody was made up as a 1/1000 dilution in IBT, and Actin was made up as 1/10000 dilution.

The membranes were removed from primary antibody and washed in PBS-Tween 3 times for a period of 10 minutes. After washing, the membranes were probed for 1 hour with the secondary antibody, made up as a 1/10000 dilution in IBT. Goat anti-rabbit IgG-AP antibody and goat anti-mouse IgG-AP antibody were used as secondary antibodies for Actin and Bax detection, respectively. The membranes were removed from secondary antibody and washed 3 times for 10 minutes. The membranes were incubated with AP buffer for 5 minutes, and subsequently incubated with 500µl of substrate CDP-star (ready-to-use, Tropix-Applied Biosystems) to enable detection of the protein of interest using chemiluminescence. In a darkroom the blots were exposed to photographic film (Kodak, GE-Healthcare Life sciences) in an autoradiography cassette and developed using a Compact X4 automated developer (Xograph Imaging Systems).

2.16 Statistical analysis

All statistical analysis were performed with Graphpad Prism software. Non-parametric Mann-Whitney test was used for comparison of two independent groups.

Chapter 3

Generation of CD8+ T cell Responses against Bax derived peptides in healthy donors

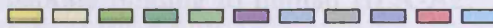
The central hypothesis for this thesis predicted that T cells specific for Bax would be able to recognise and kill human tumour cells. To test this prediction, T cells against Bax were required. However, there had been no previous reports that Bax could be immunogenic. In an attempt to define novel peptide epitopes from Bax protein that prime specific CTL, a reverse immunology approach was used (Figure 3.1). First, candidate peptides were predicted using computer algorithms and Bax derived peptides were synthesized. Binding of HLA-A2 predicted peptides was analysed in a T2 binding assay. This study was primarily focused on HLA-A2 because it is widely expressed (30-50%) in the Caucasian population. However, peptides predicted to bind other HLA types were included in an effort to increase the possibility of generating a Bax specific T cell response, as T cells might not be restricted to HLA-A2 but to other HLA. Subsequently, Bax derived peptides were used to stimulate primary CD8+ T cells from the peripheral blood of HLA-A2 donors. In order to generate short-term peptide specific cultures, CD8+ T cells were cultured for 2-4 weeks in the presence of IL-2 and a Bax peptide pool, using autologous PBMC as APCs. T cell immunogenicity for the Bax-derived peptides was tested after 2 and 4 weeks of stimulation using IFN γ ELISpot assays.

3.1 Defining CD8+ T cell responses against Bax pool 1-15

3.1.1 Peptide binding to HLA_{A2} on the T2 cell line**BAX protein (192 a.a)**

MDGSGEQPRGGPTSSSEQIMKTGALLLQGFQIDRAGRMGGEAPELADVPQDASTKKLSECLKRIGDELDNSNMLQRMIAAVDTDSPREVFRR
VAADMFSQGNFNNWGRVVALFYFASKLVLKALCTKVPELRTIMGWTLDFLRERLLGWQDQGGWGLLSYFGTPTWQTVTIFVAGVLTASLTWKK

Used computer algorithms to predict candidate peptides

Candidate peptides

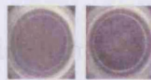
Bax-derived peptides synthesised

Peptide binding assay

Tested HLA-A2 peptides for MHC binding using T2 cells

In vitro priming of T cells with Bax peptides

Used HLA-A2 healthy donors to test immunogenicity of peptides:
Stimulated CD8+ T cells with autologous PBMC + Bax peptide pool

IFN γ ELISpot assay

Detected Bax specific IFN γ -secreting T cells

Enrichment of Bax specific T cells

Used CD107 assay or IFN γ secretion assay

Generation of highly specific T cell lines / clones for BAX peptides

Expanded T cells using the antigen-independent protocol

Test against tumour cells

Figure 3.1 General plan for the generation of Bax specific T cells.

3.1 Defining CD8+ T cell responses against Bax pool 1-15

3.1.1 Peptide binding to HLA-A2 on the T2 cell line

There are several steps that determine processing and presentation of peptides to T cells. These include degradation of proteins by the proteasome, transport of peptides by TAP and binding of peptide to MHC class I molecules. A binding assay using the T2 cell line was used to analyse peptide binding to human HLA-A2. The T2 cell line is TAP deficient and cannot load endogenous peptide onto HLA-A2 molecules, prior to transport to the cell surface. This results in the production of unstable “empty” HLA-A2 molecules and therefore low expression of HLA-A2 at the surface of the T2 cells. HLA-A2 molecules on the T2 cells can be stabilised by addition of appropriate exogenous peptide, which results in an increase in HLA-A2 expression. The increased number of HLA-A2/peptide complexes are detectable on the cell surface by flow cytometry (Nijman *et al.*, 1993). Therefore, upregulation of HLA-A2 expression is an indication of peptide binding, which is assessed in relation to a negative control (no peptide sample).

Fifteen candidate epitopes (9- to 10-mers) were selected from Bax on the basis of scores generated by two computer algorithms (BIMAS and SYFPEITHI) (see Table 2.3). Ten were predicted to bind to HLA-A2, three to HLA-A3, and two to HLA-B7. However, when the binding of the predicted HLA-A2 peptides was tested in T2 binding assays, only 3 out of 10 peptides showed measurable binding capacity to HLA-A2 molecules (Figure 3.2). These results do not exactly correlate with the scores from the computer algorithms as some of the highest scored peptides (e.g. Bax 2, 6 and 9) do not bind at all or bind weakly (Bax 1) to HLA-A2. Bax 5 (ALCTKVPPEL) was the strongest HLA-A2 binding peptide, with 123% increase in HLA-A2 expression, followed by Bax 3 (IMGWTLDFL) with 106% increase.

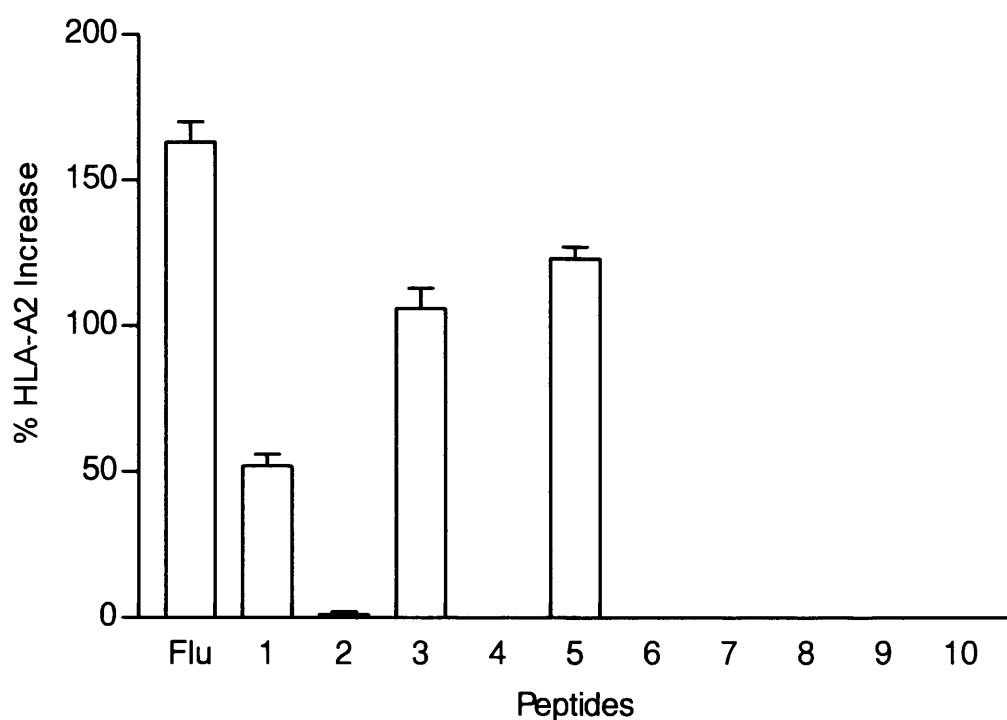


Figure 3.2 Binding of candidate peptides from Bax (pool 1-15) to HLA-A2. Binding was determined by flow cytometric measurement of HLA-A2 expression on the TAP-deficient T2 cell line. Peptides were added into wells containing T2 cells and, after overnight culture, cells were stained with an anti-HLA-A2.1 monoclonal antibody. Peptides that bind to HLA-A2 up-regulate HLA-A2 expression. HLA-A2 expression was quantified as percentage increase, according to the formula: % Increase = [(mean fluorescence with peptide – mean fluorescence without peptide) / mean fluorescence without peptide x 100]. The positive control is a known HLA-A2 binding peptide, Flu M1₅₈₋₆₆ (GILGFVFTL), from Influenza A matrix 1 protein. This graph shows the results from triplicate experiments.

These peptides exhibited a binding capacity that was comparable to the positive peptide control (163%), Flu M1₅₈₋₆₆, which is a well-known strong HLA-A2 binding peptide. In contrast, Bax 1 (KLSECLKRI) exhibited 52% upregulation of HLA-A2, indicating that this peptide is a weaker binding peptide. Therefore, three Bax peptides were potential candidate epitopes for T cells based on HLA-A2 binding.

3.1.2 Donor responses

The previous results identified three candidate peptides that could bind to HLA-A2. However, to maximise the possibility of obtaining T cell responses, the entire peptide pool (containing the 15 peptides) was used to stimulate T cells. This was done for two reasons: T2 binding is not an absolute guarantee of immunogenicity and there is the possibility to generate non-HLA-A2 restricted responses. Twelve healthy volunteers were tested for T cell responses to Bax pool 1-15. Six of the twelve donors (donors 1, 4, 7, 8, 9 and 11) responded to Bax peptides, with responses ranging from 24 to 139 spots/10⁵ cells. Bax specific responses in healthy donors are illustrated in Figure 3.3.

Three restimulation steps were necessary as only weak responses (24 to 37 spots) were detected in three donors after two weeks stimulation (Donor 4, 7 and 8). However, due to limited number of cells from donors 7 and 8, no further stimulations were possible. Donor 1, 9, and 11 responded to Bax after 4 weeks stimulation. The frequency of Bax-specific T cells was greatest for donors 1 and 11, with an average number of spots of 131 and 139 per 10⁵ cells, respectively. However, only T cells from donor 1 could be grown and used in further experiments.

The remaining six donors (donors 2, 3, 5, 6, 10 and 12) did not elicit a Bax specific T cell response after 2 or 4 weeks but were capable of responding to mitogens (PHA, PMA, concavalin, ionomycin) in an ELISpot assay (>200 spots) (data not shown).

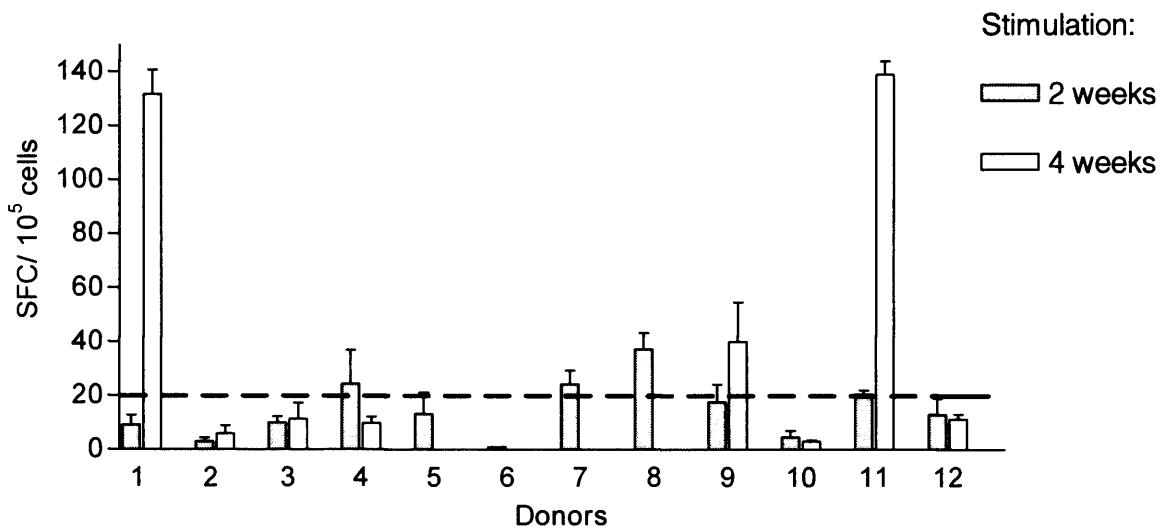


Figure 3.3 Bax T cell responses from healthy donors after 2 to 4 weeks of stimulation (Bax pool 1-15). CD8⁺ T cells were purified using immunomagnetic beads and cultured with irradiated autologous PBMC and the Bax pool 1-15. Cultures were restimulated weekly with the Bax pool. After 2-4 weeks of culture, the number of Bax specific T cells was determined by using an IFN γ ELISpot assay. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown. Background responses have been subtracted from the data. SFC= Spot Forming Cells.

As previously stated, a positive response was detected from donor 1 after 4 weeks of stimulation with Bax peptides. T cells from this donor were restimulated for seven more days with the Bax peptide pool using irradiated autologous PBMC as APCs. This additional period of restimulation was included in an attempt to further increase specificity for the Bax peptides. This T cell culture was shown to maintain specificity, with an average of 150 spots/ 10^5 cells (Figure 3.4), however, cell numbers were low. In an attempt to generate larger numbers of T cells for further experiments, the resulting T cells were expanded using the antigen-independent protocol (section 2.7). This protocol produced a 48-fold increase in cell number (24×10^6 from 5×10^5 cells), but simultaneously there was a decrease in CD8+ T cells from 98% to 19%. In order to validate the immunogenicity of Bax epitopes, attempts were made to generate long term, stable CD8+ T cell lines, as described in the next section.

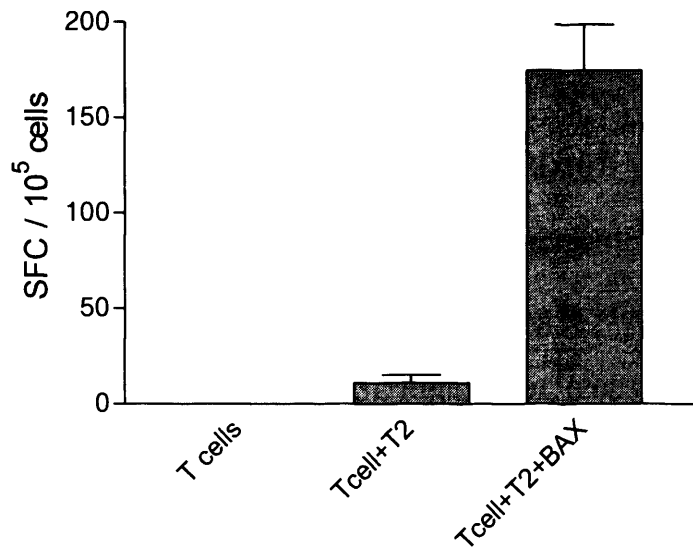


Figure 3.4 Bax T cell response from donor 1 after 5 weeks of stimulation. CD8⁺ T cells from donor 1 were purified using immunomagnetic beads and cultured with irradiated autologous PBMC and the Bax pool 1-15. Culture was restimulated weekly with the Bax pool and autologous PBMC. After 5 weeks of culture, the number of Bax specific T cells was determined by using an IFN γ ELISpot assay. Background response has not been subtracted. Average and standard deviation of triplicate wells are shown. SFC= Spot Forming Cells.

3.2 Generation of a Bax-specific polyclonal T cell line using CD107 assay

In an attempt to isolate Bax-specific T cells, a CD107 activation assay was used because this assay had the advantage that it did not require knowledge of epitope specificity, and it allowed direct sorting of CD8⁺ cytotoxic T cells. During T cell killing, cytotoxic granules, which express LAMPs (CD107a, CD107b and CD63), are transported to cell membrane, releasing cytotoxic mediators, such as perforin and granzymes, into the immune synapse between the effector T cell and the target. These LAMPs are transiently exposed on the cell surface during degranulation. The CD107 assay allows direct detection of degranulation of activated T cells by flow cytometric measurement of CD107 surface expression. Several groups have used CD107 assay to identify antigen-specific CD8⁺ T cells (Betts *et al.*, 2003; Rubio *et al.*, 2003). Rubio *et al.* (2003) were able to directly identify and isolate viable populations of tumour-cytotoxic T cells from patient blood using CD107a as a marker after tumour stimulation.

Therefore, CD8⁺ T cells from donor 1 (after 5 weeks stimulation) were tested for their ability to secrete lytic granules in response to Bax peptides using the CD107 assay. This assay was used to identify and isolate polyclonal T cells specific for Bax peptides by flow sorting. It was possible to detect 1.6% CD107a+CD8⁺ T cells specific for Bax (Figure 3.5), with almost no detection when T cells were left unstimulated (0.04%) or stimulated with just the APC T2 alone (0.09%) (data not shown). Subsequently, 1.5×10^4 CD107a+CD8⁺ T cells were flow sorted (MoFlo) and propagated using the antigen-independent expansion protocol (section 2.7). An enriched T cell population against Bax was generated (10×10^6 cells), showing an enrichment to 32.9% CD107a+CD8⁺ T cells against Bax peptides (Figure 3.5), which is hereon referred to as the JSB line. In addition, either 1 cell/well or 10 cells/well were plated into the wells of 96 well plates using the MoFlo cell sorter (10 plates with 1 cell/well and 2 plates with 10 cells/well) (section 2.10.2). However, these cells proved difficult to grow and no further tests were done.

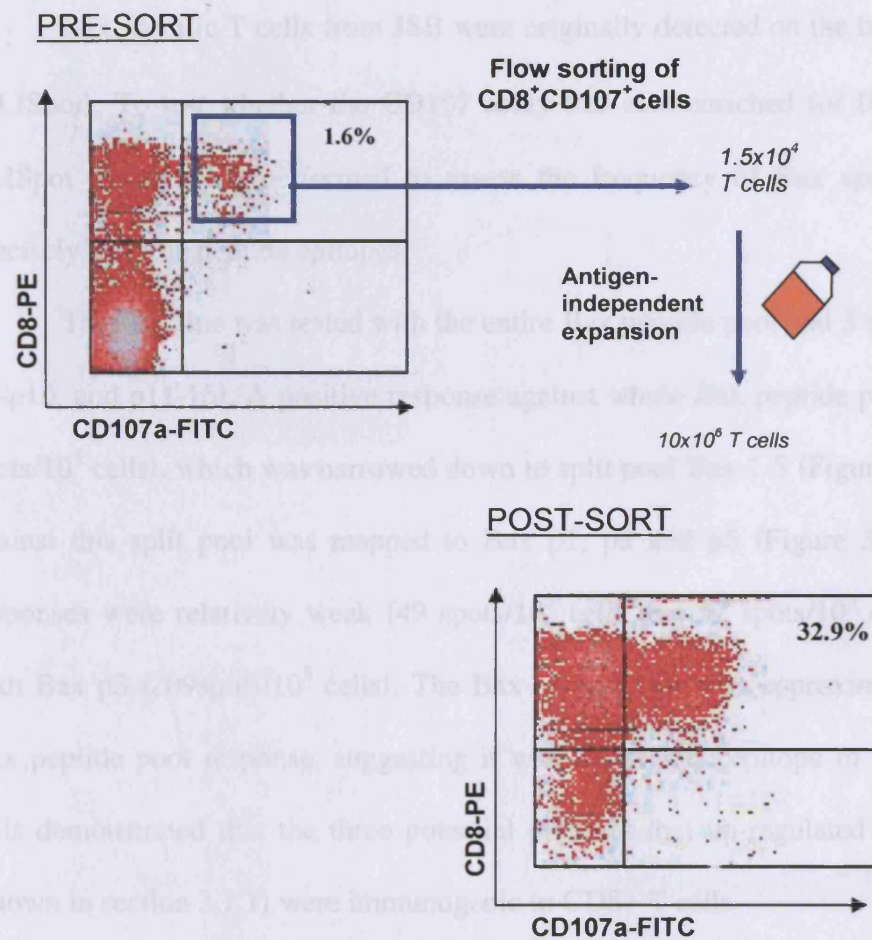


Figure 3.5 The approach used to derive a Bax peptide specific T cell line (JSB line) by surface expression of CD107a. Activation of CD8⁺ T cells after encounter with APC pulsed with Bax peptide pool 1-15 was determined by cell surface expression of CD107a. Flow sorting (MoFlo cell sorter) was used to generate a CD107a⁺CD8⁺ enriched population (1.5×10^4). This sorted population was expanded using the antigen-independent expansion protocol (10×10^6) before testing again for CD107a expression. This gave an enrichment from 1.6% to 32.9% for CD107a⁺CD8⁺ cells following peptide stimulation.

3.2.1 Mapping epitopes from Bax pool 1-15 responses using the JSB line

Bax specific T cells from JSB were originally detected on the basis of IFN γ secretion (ELISpot). To test whether the CD107 assay had also enriched for IFN γ -secreting T cells, ELISpot assays were performed to assess the frequency of Bax specific T cells, and to precisely map the peptide epitopes.

The JSB line was tested with the entire Bax peptide pool and 3 split pools (Bax p1-p5, p6-p10, and p11-15). A positive response against whole Bax peptide pool was detected (200 spots/ 10^5 cells), which was narrowed down to split pool Bax 1-5 (Figure 3.6A). The response against this split pool was mapped to Bax p1, p3 and p5 (Figure 3.6B). Bax p1 and p5 responses were relatively weak (49 spots/ 10^5 cells and 52 spots/ 10^5 cells) when compared with Bax p3 (209spots/ 10^5 cells). The Bax p3 response was approximately equal to that of Bax peptide pool response, suggesting it was a dominant epitope in the Bax peptide pool. This demonstrated that the three potential peptides that up-regulated HLA-A2 on T2 cells (shown in section 3.1.1) were immunogenic to CD8+ T cells.

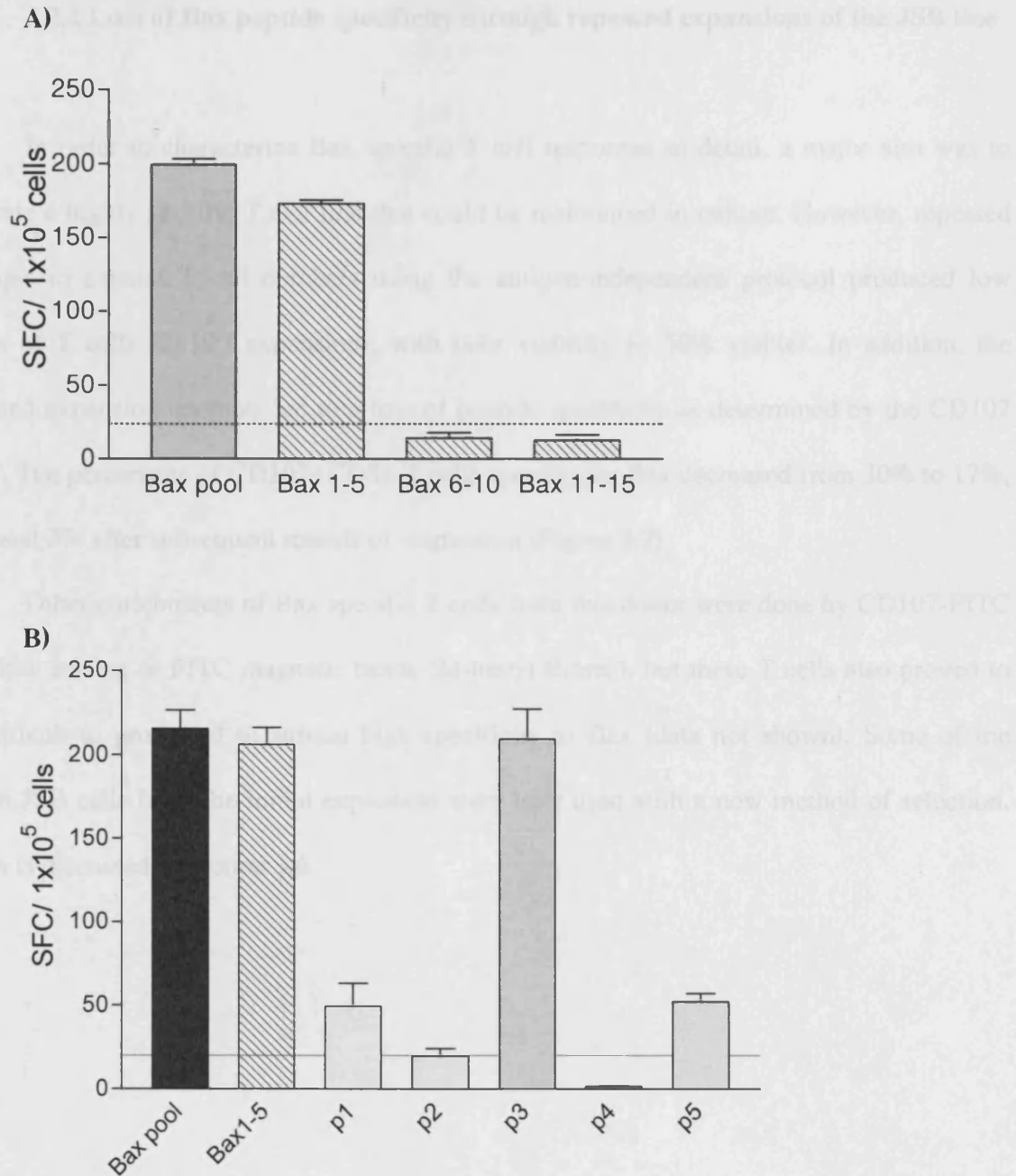


Figure 3.6 Mapping the Bax response from the JSB line to split pools and individual peptides.

The JSB line was tested by IFN γ ELISpot against the whole Bax pool, and split pools: Bax 1-5, Bax 6-10, and Bax11-15 (**A**). The JSB line was again tested by ELISpot against the whole Bax pool, split pool Bax1-5 and individual peptides Bax p1, p2, p3, p4 and p5 (**B**). Background responses were subtracted from this data. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown. SFC= Spot Forming Cells.

3.2.2 Loss of Bax peptide specificity through repeated expansions of the JSB line

In order to characterize Bax specific T cell responses in detail, a major aim was to generate a highly specific T cell line that could be maintained in culture. However, repeated attempts to expand T cell numbers using the antigen-independent protocol produced low yields of T cells (2×10^6 / expansion), with poor viability (< 50% viable). In addition, the repeated expansion attempts led to a loss of peptide specificity as determined by the CD107 assay. The percentage of CD107+CD8+ T cells specific for Bax decreased from 30% to 17%, 10% and 3% after subsequent rounds of expansion (Figure 3.7).

Other enrichments of Bax specific T cells from this donor were done by CD107-FITC and flow sorting or FITC magnetic beads (Miltenyi Biotec), but these T cells also proved to be difficult to grow and to sustain high specificity to Bax (data not shown). Some of the frozen JSB cells from the initial expansion were later used with a new method of selection, which is discussed in section 3.6.

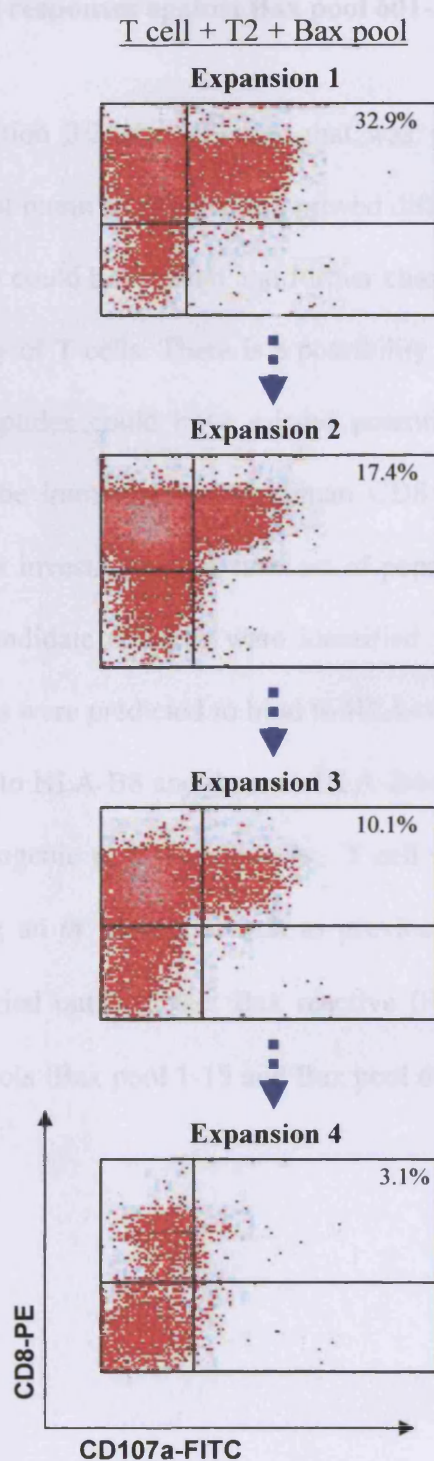


Figure 3.7 Demonstration of Bax specificity loss of the JSB line after repeated expansions using CD107 assay. The JSB line was expanded using the antigen-independent protocol. Specificity was determined by surface expression of CD107a after incubation of the JSB line with T2 cells in presence of the Bax peptide pool. T cells were gated based upon their forward and side scatter profile. Flow cytometric analysis was done using CellQuest software.

3.3 Defining CD8+ T cell responses against Bax pool 601-23

As described in section 3.2, the JSB line that was generated by flow sorting using CD107a expression did not retain specificity and proved difficult to grow. No T cells specific for Bax from other donors could be isolated and further characterised due to limited numbers and relative low frequency of T cells. There is a possibility that the selection process for the original set of fifteen peptides could have missed potential epitopes. Nevertheless, three peptides were shown to be immunogenic to human CD8+ T cells and therefore putative epitopes worthy of further investigation. A new set of peptides, which included those three peptides, and 20 more candidate epitopes were identified from the Bax protein (Bax pool 601-23). Fourteen peptides were predicted to bind to HLA-A2, one to HLA-A3, one to HLA-B7, two to HLA-A1, two to HLA-B8 and three to HLA-B44. The first step was to identify if the peptides were immunogenic to human T cells. T cell immunogenicity for Bax derived peptides was tested using an *in vitro* approach as previously done for the first Bax pool. ELISpot assays were carried out to detect Bax reactive IFN- γ -secreting T cells. Table 3.1 shows both sets of Bax pools (Bax pool 1-15 and Bax pool 601-23).

Table 3.1 Bax pool 1-15 (A) and Bax pool 601-23 (B)**A**

Bax pool 1-15	Sequence	Residue Position	HLA
2	ALFYFASKL	112	A2
4	WIQDQGGWV	151	A2
6	PVPQDASTK	49	A3
7	VLKALCTKV	121	A2
8	IMKTGALLL	19	A2
9	ALFYFASKLV	112	A2
10	VVYNAFSLRV	209	A2
11	KLVLKALCTK	119	A3
12	VVALFYFASK	110	A3
13	VPQDASTKKL	50	B7
14	KPPHPHHRAL	163	B7
15	LVLKALCTKV	120	A2

Highlighted rows correspond to the peptides that were used in both Bax peptide pools

B

Bax pool 601-23	Sequence	Residue Position	HLA
601	RMGGEAPEL	37	A2
602	QIMKTGALL	18	A2
603	LLSYFGTPT	161	A2
604	LLQGFIQDRA	26	A2
605	GLLSYFGTPT	160	A2
606	FVAGVLTASL	176	A2
607	ELQRMIAAV	75	A2
608	ALDPVPQDA	46	A2
609	LLLQFIQDR	25	A2
610	TIMGWTLDFL	135	A2
611	FLRERLLGWI	143	A2
615	DELDSNMEL	68	B44
616	MELQRMIAA	74	B44
617	SEQIMKTGAL	16	B44
618	CLKRIGDEL	62	B8
619	ASKLVLKAL	117	B8
620	GGWDGLLSY	156	A1
621	DTDSPREVF	84	A1
622	VALFYFASK	111	A3
623	SPREVFVRV	87	B7

3.3.1 Peptide binding to HLA-A2 on the T2 cell line

Twenty new candidate epitopes were selected from Bax on the basis of a computer score, using three to six computer algorithms (Table 2.4). Fourteen were predicted to bind to HLA-A2 and were therefore used in a T2 binding assay. From the fourteen potential HLA-A2 binding peptides, only the three original peptides that were considered immunogenic in the previous pool showed measurable binding capacity to HLA-A2 molecules (Figure 3.8). Again, Bax p614 (ALCTKVPEL = Bax p5 from old pool) was the strongest HLA-A2 binding peptide (150%) followed by Bax p613 (IMGWTLDFL = Bax p3) with 115% increase, which showed a binding capacity comparable to the positive peptide control (161%), Flu M1₅₈₋₆₆. Bax p612 (KLSECLKRI = Bax p1) exhibited a lower up-regulation than the other two peptides as previously shown. These three peptides were predicted to bind to HLA-A2 by 6/6 computer algorithms, but one peptide (Bax 601) that was also predicted by 6/6 computer algorithms did not actually bind to HLA-A2 according to this assay. Thus, based on the criteria of strong peptide binding, Bax peptides p614, p613 and p612 would be predicted to be likely T cell epitopes.

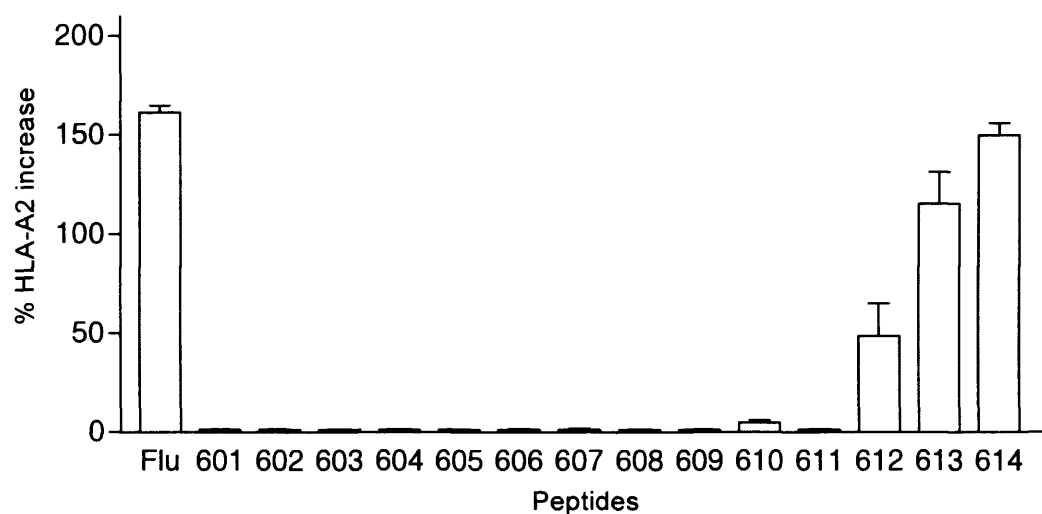


Figure 3.8 Binding of candidate peptides from Bax (pool 601-23) to HLA-A2.

Binding was determined by flow cytometric measurement of HLA-A2 expression on the TAP-deficient T2 cell line. Peptides were added to wells containing T2 cells and, after overnight culture, cells were stained with anti-HLA-A2.1. Peptides that bind to HLA-A2 up-regulate HLA-A2 expression. HLA-A2 expression was quantified as a percentage increase according to the formula: % Increase = [(mean fluorescence with peptide – mean fluorescence without peptide) / mean fluorescence without peptide x 100]. The positive control is a known HLA-A2 binding peptide, Flu M1₅₈₋₆₆ (GILGFVFTL), from Influenza A matrix 1 protein. HLA-A2- Bax peptides 615 to 623 were also tested as negative controls and no HLA-A2 up-regulation was seen for these (data not shown). This graph shows the results from triplicate experiments.

3.3.2 Donor responses

Five healthy volunteers were tested for T cell responses to Bax pool 601-23. As donor 11 showed a positive response for Bax pool 1-15 (Figure 3.3), cells from this donor were also tested against Bax pool 601-23. Unfortunately, the donor for JSB line was not available to donate more blood, so it was not possible to test this donor with the Bax peptide pool 601-23.

Positive but variable Bax T cell responses (23 to 160 spots/ 10^5 cells) were detected from all of the five donors after 4 weeks of stimulation with peptide. However, none of donors elicited a Bax specific T cell response after 2 weeks of stimulation (Figure 3.9). Donor 11 showed the highest Bax specific T cell response with an average count of 160 spots, followed by donors 13 and 15 with 130 and 99 spots, respectively. Donor 14 and 16 responded weakly to Bax peptides, with an average number of 32 and 23 spots, respectively. All of the donors were capable of responding to mitogens (PHA, PMA, concavalin, ionomycin), positive control in ELISpot (>200 spots/ 10^5 cells) (data not shown).

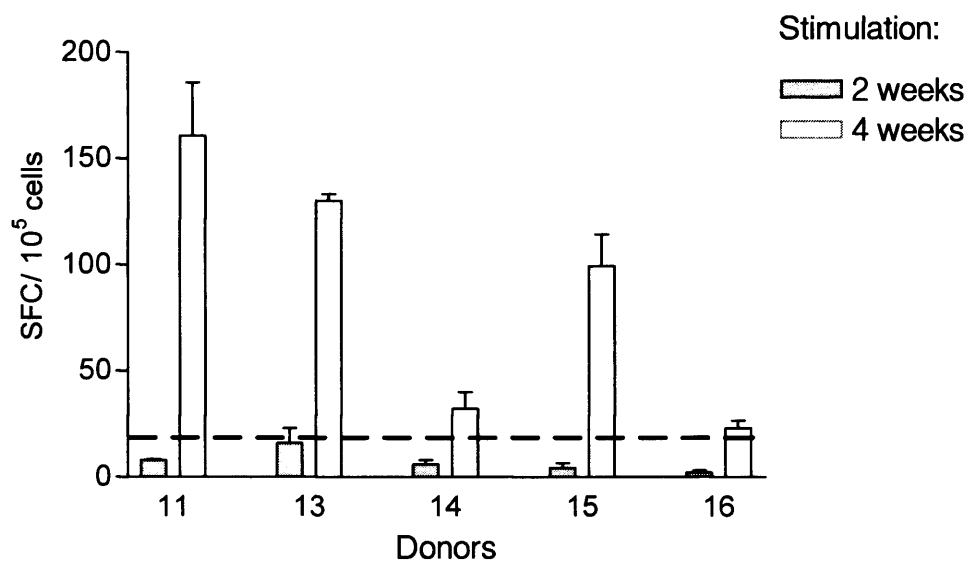


Figure 3.9 Bax T cell responses from healthy donors after 2 to 4 weeks of stimulation (Bax pool 601-23). CD8⁺ T cells were purified using immunomagnetic beads and cultured with irradiated autologous PBMC and Bax pool 601-23. Cultures were restimulated weekly with the Bax pool. After 2-4 weeks of culture, the number of Bax specific T cells was determined using an IFN γ ELISpot assay. The dashed line represents the 20 spot cut-off for a positive result and the standard deviation is also shown. Background responses have been subtracted from data. SFC= Spot Forming Cells.

3.4 Testing different protocols for the detection of peptide specific T cells

As demonstrated in sections 3.1.2 and 3.3.2, it was possible to generate short-term Bax peptide specific CD8⁺ T cell cultures. However, initial attempts to produce highly enriched peptide specific T cell lines and clones from these low frequency cultures failed. So, it was crucial to develop a consistent method for detecting and purifying peptide specific T cell lines and clones.

To address this, a test was made using Melan-A/MART-1 peptide because it is a model system for naïve T cell responses against HLA-A2 tumour antigens. It is possible to detect responses against Melan-A/MART-1 in a large proportion of healthy HLA-A2 donors (Pittet *et al.*, 1999). T cells were cultured as explained in section 2.5. Briefly, T cells were stimulated with peptide on days 0 and 7, and grown until day 14. The cultures were then tested for specificity using several different detection systems. T2 or autologous frozen PBMC were used as APC.

Several studies have shown that CD107 (Betts *et al.*, 2003; Rubio *et al.*, 2003), CD137 (Wolfl *et al.*, 2007) and IFN γ (Brosterhus *et al.*, 1999; Campbell, 2003; Oelke *et al.*, 2000) can be used to detect and isolate antigen-specific T cells. Therefore, all these methods were compared for detection of MART-1-specific T cells, in order to determine the optimum method for selection of Bax-specific T cells. The CD107 assay detects antigen-specific T cells by measurement of the surface expression of CD107a as a marker for degranulation of activated T cells (see section 3.2). The CD137 assay measures CD137 expression on specifically activated CD8⁺ T cells. CD137 (4-1BB) is a member of the TNFR-family with costimulatory function that promotes T cell proliferation and T cell survival. Antigen specific IFN γ secretion by CD8⁺ T cells was also investigated by ELISpot assay and flow cytometry based ICS.

Figure 3.10 illustrates all the methods used for detection of peptide-specific T cells. The ELISpot assay demonstrated a positive response against MART-1 peptide with an average of 290 spots/ 10^5 cells after subtracting background, which gives approximately 0.3% of positive cells against MART-1 (Figure 3.10A). As for the CD107 assay, no peptide-specific T cells were detected with this method as the percentage of CD107a+CD8+ T cells was similar when incubated with only T2 or with T2 + peptide (~1%) (Figure 3.10B). The CD137 assay showed 0.3% CD137+CD8+ T cells against MART-1 after background subtraction (2.3% T cells + T2; 2.6% T cells + T2 + MART-1) (Figure 3.10C). Finally, it was possible to detect a higher percentage of MART-1 specific T cells (1.5% after subtracting background) when using IFN γ ICS. Further to this, it was also noticeable that the expression intensity of IFN γ + peptide specific T cells was several fold higher than T cells + T2 without peptide, or CD137+ T cells (Figure 3.10D). This suggests that it should be easier to distinguish activated versus non-activated T cells for cell sorting purposes.

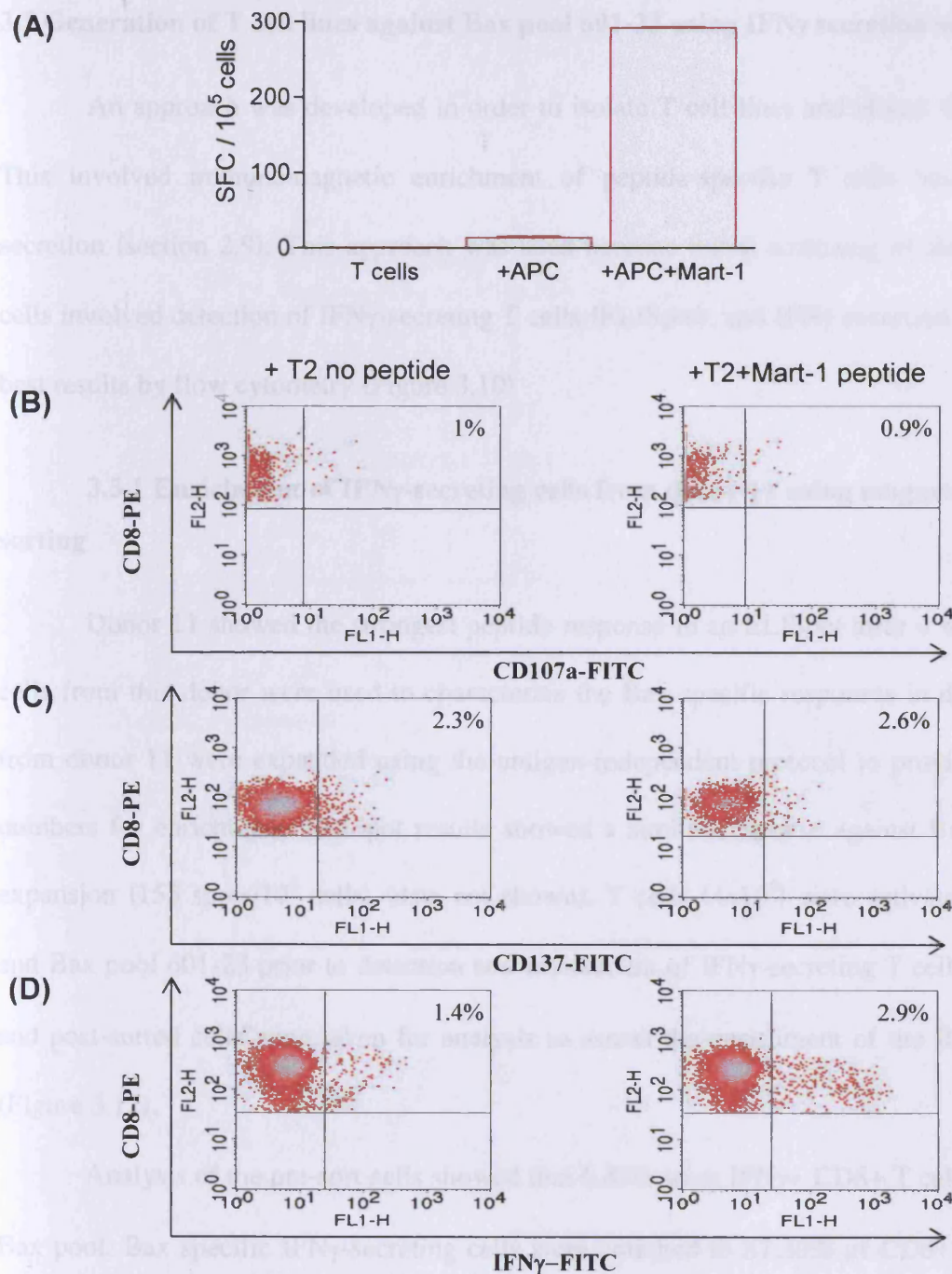


Figure 3.10 Comparison of methods for detection of MART-1 specific T cells. An ELISpot assay was set up in triplicate wells with T cells alone, T cells+APC, T cells+APC+MART-1. Background response has not been subtracted (A). Flow cytometric analysis of % CD107a⁺CD8⁺ cells (B), %CD137⁺CD8⁺ (C) % IFN γ -secreting CD8⁺ (D) within the lymphocyte gate after incubation with T2 with or without peptide. All samples were stained with an anti-CD8-PE antibody. T cells were gated based upon their forward and side scatter profile. Dead cells were excluded from analysis of d) plots using negative gating, based on 7-AAD staining. Flow cytometric analysis was done using CellQuest software.

3.5 Generation of T cell lines against Bax pool 601-23 using IFN γ secretion assay

An approach was developed in order to isolate T cell lines and clones (Figure 3.11). This involved immunomagnetic enrichment of peptide-specific T cells based on IFN γ secretion (section 2.9). This approach was used because initial screening of Bax specific T cells involved detection of IFN γ -secreting T cells (ELISpot), and IFN γ secretion provided the best results by flow cytometry (Figure 3.10)

3.5.1 Enrichment of IFN γ -secreting cells from donor 11 using magnetic-based cell sorting

Donor 11 showed the strongest peptide response in an ELISpot after 4 weeks, thus T cells from this donor were used to characterise the Bax specific responses in detail. T cells from donor 11 were expanded using the antigen-independent protocol to provide sufficient numbers for enrichment. ELISpot results showed a similar response against Bax as in pre-expansion (155 spots/ 10^5 cells) (data not shown). T cells (4×10^6) were activated with APC and Bax pool 601-23 prior to detection and enrichment of IFN γ -secreting T cells. Pre-sorted and post-sorted cells were taken for analysis to assess the enrichment of the IFN γ + T cells (Figure 3.12).

Analysis of the pre-sort cells showed that 6.45% were IFN γ + CD8+ T cells reactive to Bax pool. Bax specific IFN γ -secreting cells were enriched to 87.36% of CD8+T cells. This also showed that the CD8 negative T cells were lost with the enrichment. Furthermore, the mean fluorescence intensity (MFI) of the IFN γ + cells in the post-sort fraction was several fold higher than in the pre-sort fraction, suggesting that this method preferentially selects for cells that secrete high levels of IFN γ . The implementation of a magnetic-based cell sorting technique permitted the isolation of 1.2×10^5 enriched Bax-specific CD8+ T cells from a starting population of 4×10^6 cells. These enriched cells were then used in the generation of T cell lines containing high frequencies of Bax-specific T cells.

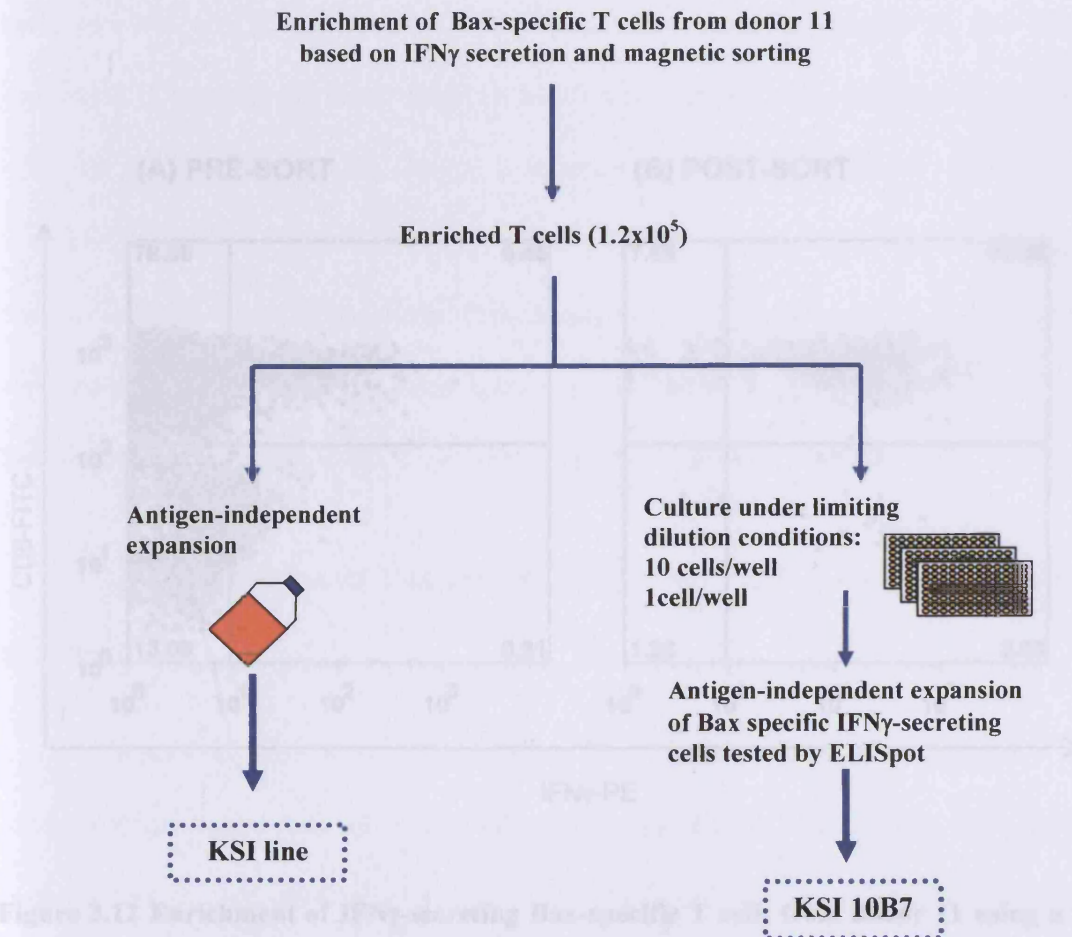


Figure 3.11 Overview of the process by which Bax-specific T cell lines were generated from donor 11.

Figure 3.11 illustrates the process of generating Bax-specific T cell lines from donor 11. The process begins with the enrichment of Bax-specific T cells from donor 11 based on IFN γ secretion and magnetic sorting. This results in enriched T cells (1.2×10^5). These cells are then divided into two main pathways. The left pathway involves antigen-independent expansion, leading to the KSI line. The right pathway involves culture under limiting dilution conditions (10 cells/well and 1 cell/well), followed by antigen-independent expansion of Bax-specific IFN γ -secreting cells tested by ELISpot, leading to the KSI 10B7 line. The figure also includes flow cytometry plots (A) PRE-SORT and (B) POST-SORT, showing the enrichment of IFN γ -secreting cells. The percentage of each population is shown in the plots, and the analysis was done using FlowJo software.

3.5.2 Generation of KSI line through the antigen-independent expansion of IFN γ -secreting T cells

To increase the likelihood of generating a highly specific T cell line from the enriched cells, two different methods were utilized: expansion using the antigen-independent protocol (section 2.2) and cloning under limiting dilution conditions in 96 well plates (section 3.1.2).

The first method involved the antigen-independent expansion of IFN γ -secreting cells. This

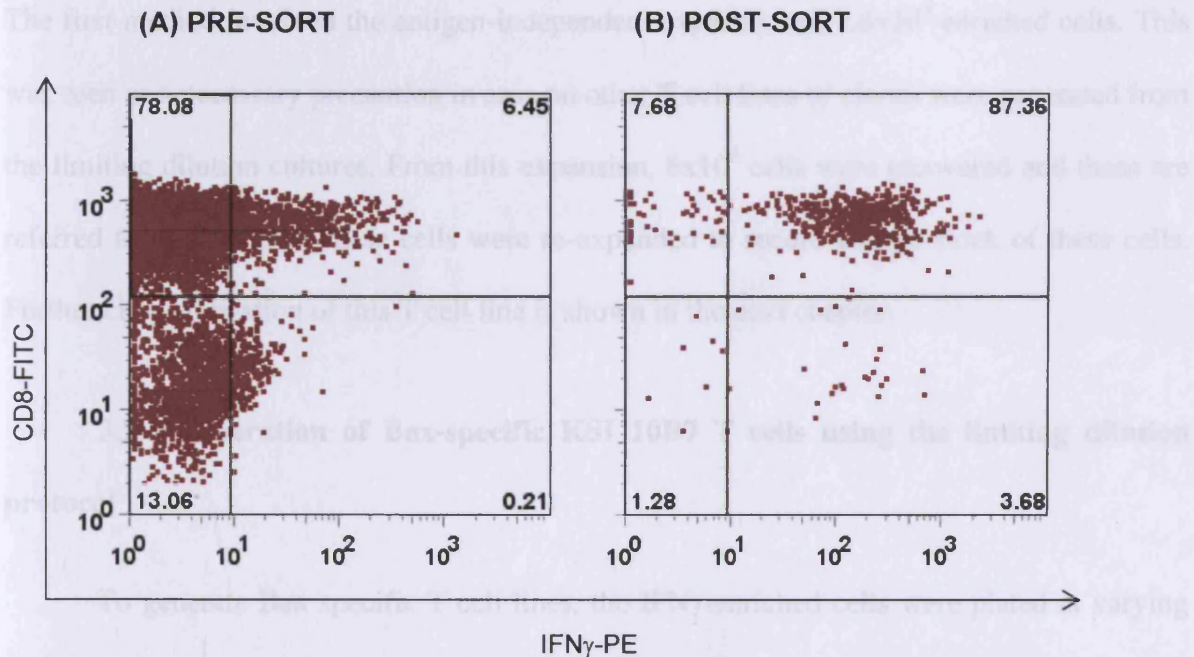


Figure 3.12 Enrichment of IFN γ -secreting Bax-specific T cells from donor 11 using a magnetic bead cell sorting system. After re-stimulation of T cells from donor 11 with Bax pool 601-23 and irradiated T2 for 4 hours, the IFN γ -secreting cells were stained with anti-IFN γ -PE antibody and were enriched using anti-PE magnetic beads. Cells were also stained with an anti-CD8-FITC antibody. The pre-sort (A) and post-sort (B) fractions were analysed by flow cytometry. T cells were gated based upon their forward and side scatter profile, and dead cells were excluded from analysis using negative gating, based on 7-AAD staining. The percentage of each quadrant is shown. Flow cytometric analysis was done using FlowJo software.

3.5.2 Generation of KSI line through the antigen-independent expansion of IFN γ -enriched T cells

To increase the likelihood of generating a highly specific T cell line from the enriched cells, two different methods were utilized: expansion using the antigen-independent protocol (section 2.7) and cloning under limiting dilution conditions in 96 well plates (section 2.10.2). The first method involved the antigen-independent expansion of 7.8×10^4 enriched cells. This was seen as a necessary precaution in case no other T cell lines or clones were generated from the limiting dilution cultures. From this expansion, 8×10^6 cells were recovered and these are referred to as KSI line. These cells were re-expanded to secure a large stock of these cells. Further characterisation of this T cell line is shown in the next chapter.

3.5.3 Generation of Bax-specific KSI 10B7 T cells using the limiting dilution protocol

To generate Bax specific T cell lines, the IFN γ -enriched cells were plated at varying concentrations into seven 96 well cloning plates (section 2.10.2). After 3-4 weeks, 90-100 cultures had grown sufficiently to have at least two replicate wells. In total, 60 cultures were grown from plates with 10 cells/well and 40 cultures from 1 cell/well. These were tested by ELISpot assay with the Bax pool 601-23 to indicate the specificity of each culture (section 2.10.3). The purpose of the ELISpot was to help selection of the most specific cultures for further study. From these results (Figure 3.13) it can be seen that the vast majority of cultures recognised Bax peptides, although with varying magnitudes of response. Cultures B7 from 10 cells/well plates (Figure 3.13A), 3B2 and 4E9 from 1 cell/well plates (Figure 3.13B) provided the strongest responses. The spot counts for the 3 cultures selected for expansion were above 200 spots and B7 culture gave the strongest response against Bax (644 spots).

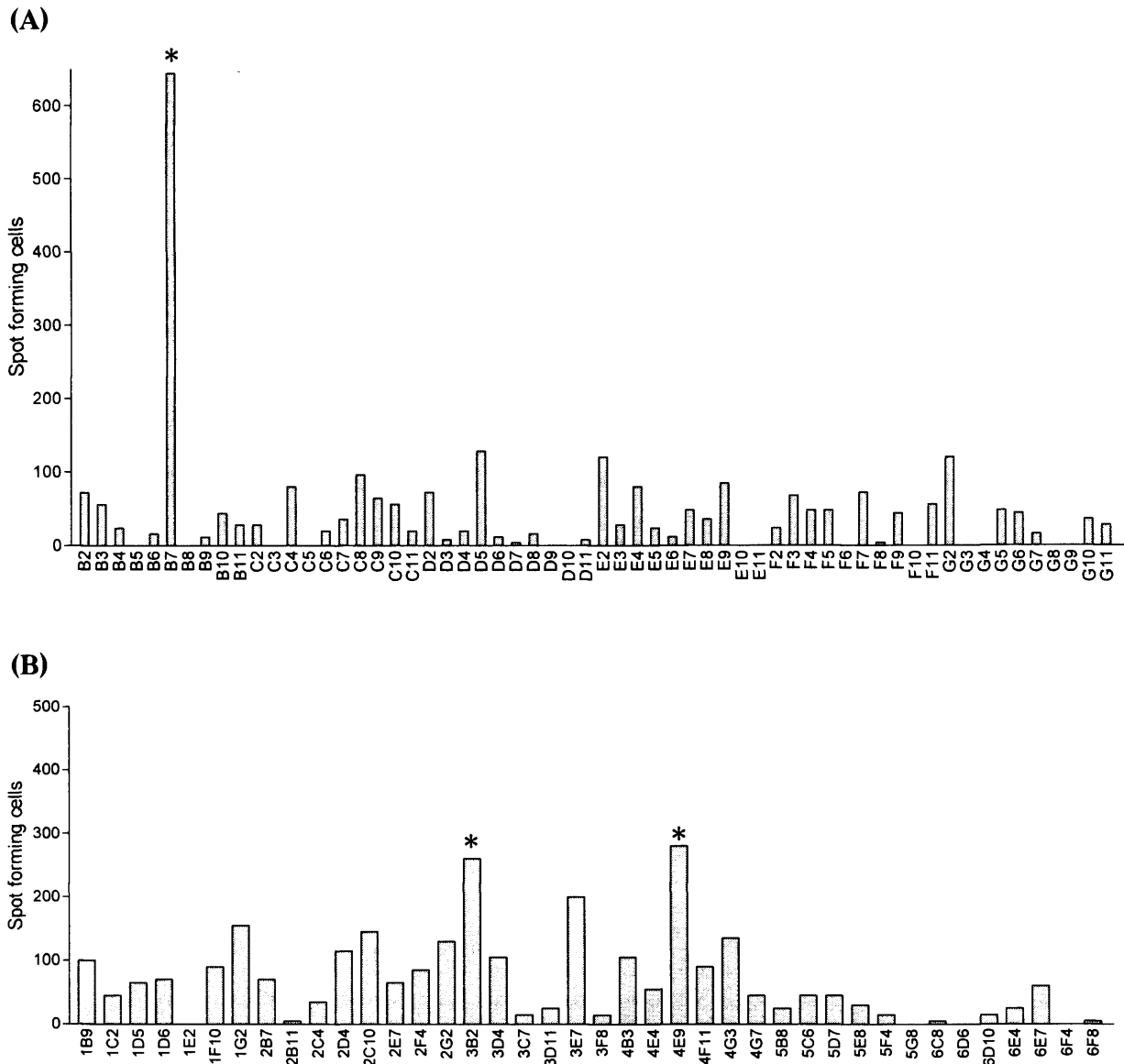


Figure 3.13 T cell lines (100 in total) generated using limiting dilution culture conditions were screened for recognition of Bax pool by ELISpot assay. To help estimate the average cell number in the cultures, 4 different cultures were counted. The cell counts ranged from 5×10^4 to 1.5×10^5 , with a mean average of 1.0×10^5 cells/well. On average $2-3 \times 10^4$ cells/well from each culture were plated out with 3×10^4 T2 cells in the presence and absence of Bax pool 601-23 ($10 \mu\text{g/ml}$) in an ELISpot plate. There was only one well for each condition. 60 lines were generated from 10 cells/well plates (A) and 40 lines generated from 1 cell/well (B). The actual frequency of Bax-specific T cells in each line could not be determined in this experiment as the exact number of cells added per well was unknown. The lines selected for expansion are highlighted (*). Background responses (+T2 without peptide) have been subtracted.

Cells from each culture were further propagated using the antigen-independent expansion protocol. For unknown reasons, cultures 3B2 and 4E9 did not expand well, and there were insufficient cells for further characterisation. Therefore, only B7 culture was used for further characterisation (described in the next chapter), which is hereon referred as KSI 10B7.

3.6 Generation of T cell lines from JSB cells using IFN γ secretion assay

Since this enrichment protocol allowed the successful generation of lines from a low frequency population, the JSB line that was previously generated by CD107 sorting (section 3.2) was “re-sorted” based on IFN γ secretion. These enriched T cells were then used to produce several T cell lines using the approach described previously for Donor 11 in Figure 3.11.

3.6.1 Enrichment of IFN γ -secreting cells from JSB cells using magnetic-based cell sorting

Bax p1, p3 and p5 were found to be immunogenic to JSB line (Figure 3.6) and so to increase the chance of getting more specific T cells, only these three peptides were used to activate T cells prior to sorting. Analysis of the pre-sort cells showed that 11.22% of JSB cells were reactive to Bax peptides, as measured by dual expression of CD8 and IFN γ . Bax specific IFN γ -secreting CD8⁺ T cells were enriched to 92.18% after sorting (Figure 3.14). An IFN γ ⁺ CD8⁻ T cell population with a lower expression intensity was also detected in the presort (11%) but virtually eliminated after sorting. The MFI of the IFN γ ⁺ cells in the post-sort fraction was several fold higher than in the pre-sort fraction, implying again that cells secreting high levels of cytokine are preferentially selected by this method. This technique permitted the isolation of 1.0×10^4 enriched Bax-specific CD8⁺ T cells.

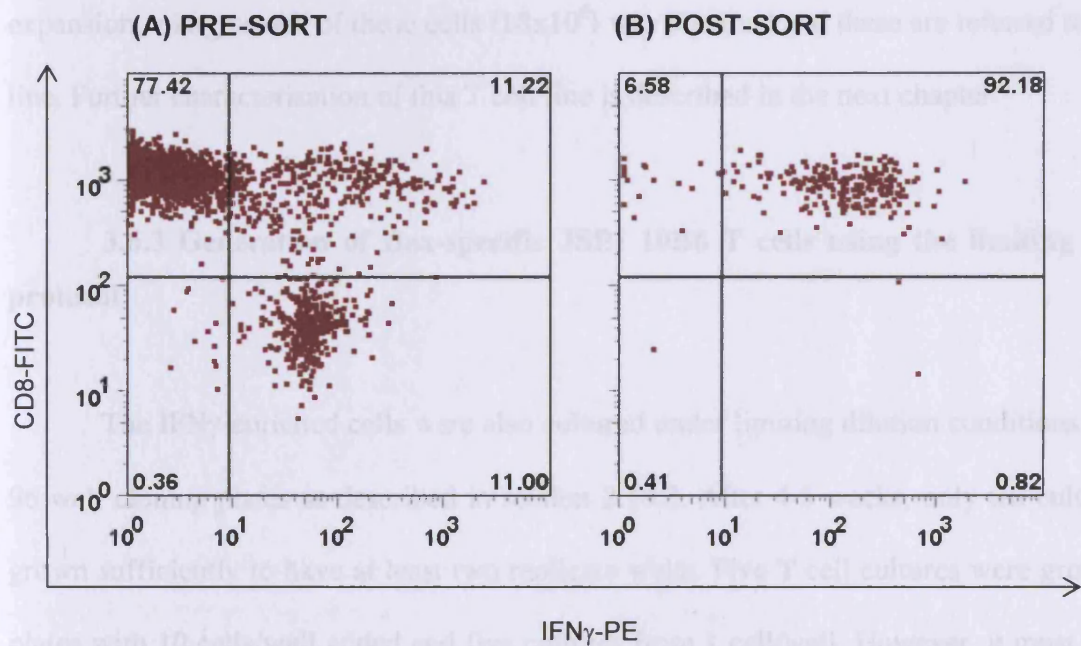


Figure 3.14 Enrichment of IFN γ -secreting Bax-specific T cells from JSB line using a magnetic bead cell sorting system. After re-stimulation of JSB line with Bax peptides p1, p3 and p5 and irradiated T2 for 4 hours, the IFN γ -secreting cells were stained with anti-IFN γ -PE antibody and were enriched using anti-PE magnetic beads. Cells were also stained with an anti-CD8-FITC antibody. The pre-sort (A) and post-sort (B) fractions were analysed by flow cytometry. T cells were gated based upon their forward and side scatter profile, and dead cells were excluded from analysis using negative gating, based on 7-AAD staining. The percentage of each quadrant is shown. Flow cytometric analysis was done using FlowJo software.

3.6.2 Generation of JSBI line through the antigen-independent expansion

As done for the generation of KSI line, some of the enriched cells were expanded using the antigen-independent protocol as a precaution in case no cells were grown from the limiting dilution cultures. In this case, because of the lower number of enriched cells, only 9.3×10^3 cells were expanded using the antigen-independent protocol. After two rounds of expansion, a large stock of these cells (18×10^6) was obtained and these are referred to as JSBI line. Further characterisation of this T cell line is described in the next chapter.

3.6.3 Generation of Bax-specific JSBI 10B6 T cells using the limiting dilution protocol

The IFN γ -enriched cells were also cultured under limiting dilution conditions in seven 96 well cloning plates as described in section 2.10.2. After 4-5 weeks, only ten cultures had grown sufficiently to have at least two replicate wells. Five T cell cultures were grown from plates with 10 cells/well added and five cultures from 1 cell/well. However, it must be taken into consideration that the cells from this donor were known to be difficult to grow from previous experiments when the T cells were sorted using CD107 (section 3.2.2).

These cultures were tested by ELISpot with Bax peptides p1, p3 and p5 to confirm the specificity of each culture (Figure 3.15). Almost all the cultures recognised Bax peptides, although with varying magnitudes of response. 10E6 and 3E6 cultures were selected for expansion, with spot counts of 480 and 200, respectively. Unfortunately, only 10E6 line was able to grow to sufficient number for further characterisation, which hereon is called JSBI 10E6. Further characterisation of JSBI 10E6 will be discussed in the following chapter.

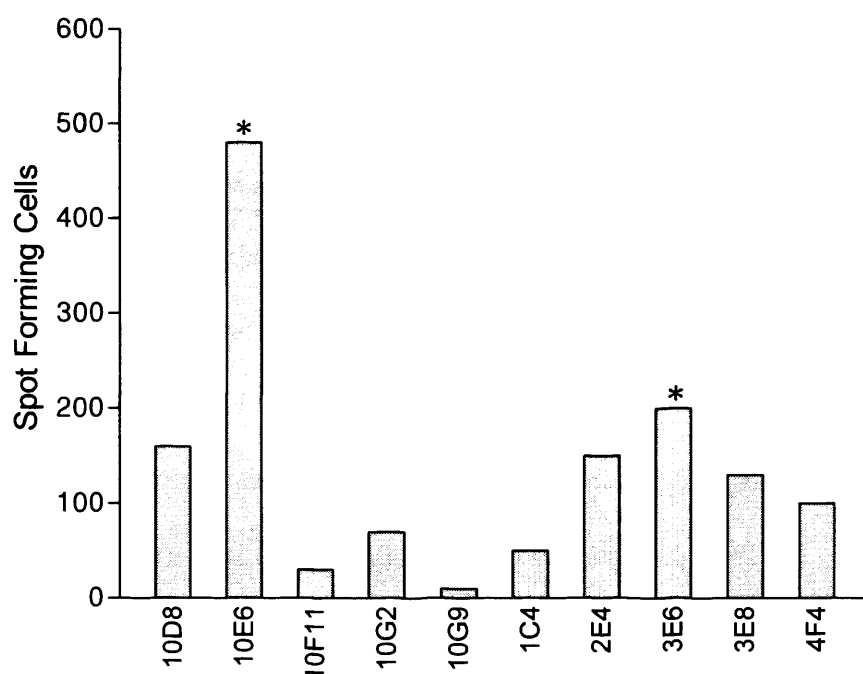


Figure 3.15 T cell lines (10 in total) generated using limiting dilution culture conditions were screened for recognition of Bax peptides by ELISpot assay. On average $2-3.0 \times 10^4$ cells/well from each culture were plated out with 3×10^4 T2 cells in the presence and absence of Bax peptides p1, p3 and p5 ($10 \mu\text{g/ml}$). There was only one well for each condition. Five lines were generated from 10cells/well plates and five lines generated from 1cell/wel. The actual frequency of Bax-specific T cells in each line could not be determined in this experiment as the exact number of cells added per well was unknown. The lines selected for expansion are highlighted (*). Background responses (+T2 without peptide) have been subtracted.

3.7 Discussion

In the first stage of this study, the ability of Bax-derived peptides to generate peptide-specific CD8⁺ T cells in healthy donors was investigated. This involved a reverse immunology approach, which consisted of candidate peptide selection based on computer algorithms, peptide binding assays to determine the actual binding of the predicted peptides to MHC molecules, *in vitro* stimulation of CD8⁺ T cells from healthy donors, generation of highly peptide specific T cell lines and clones, and ultimately testing the generated T cells towards tumour target cells endogenously expressing the antigen (discussed in chapter 4). A large number of CD8⁺ T cell epitopes have been identified from several human tumour antigens using this approach, such as hTERT (Minev *et al.*, 2000), PSCA (Kiessling *et al.*, 2002), STEAP (Rodeberg *et al.*, 2005), and survivin (Reker *et al.*, 2004).

Alternatively, a direct immunology approach could have been used in this study, which investigates the specificity of T cells that recognise tumour cells in order to define the epitope. The tumour-reactive T cells are used to screen cDNA libraries prepared from autologous tumour cells for expression of a potential antigen. This approach allowed the identification of the first tumour antigen MAGE-1 (van der Bruggen *et al.*, 1991). Another alternative approach, which also starts from T cell function, uses biochemical methods to purify naturally occurring peptides associated with MHC molecules on the tumour cells. After acid elution from the MHC molecules, these are fractionated by high-performance liquid chromatography (HPLC), and the fraction that is recognised by T cells is then analysed by mass spectrometry to identify the peptide epitopes (Cox *et al.*, 1994). However, this approach requires large numbers of cells (10^9 to 10^{10}) and highly sensitive analytical techniques due to the low number of tumour-specific peptides among thousands of other peptides. Such approaches have been most successful in identifying melanoma-associated antigens (Cox *et al.*, 1994; van der Bruggen *et al.*, 1991). Nevertheless, it has been complicated to employ this method for the identification of other cancer antigens because it is

difficult to isolate and grow tumour cell lines from most of other types of cancers, and large numbers of long-term stable T cell clones or lines for screening are required, which frequently cannot be achieved. Thus, the reverse immunology approach seemed to be the most appropriate and straightforward for the present study.

Initially, 15 candidate peptides were identified from Bax protein using two computer-based algorithms. Three peptides (Bax p1, p3 and p5) were shown to bind to HLA-A2 molecules in a T2 binding assay. Binding to HLA is a pre-requisite for T cell recognition. Efficient peptide binding to MHC class I is necessary for immunogenicity. However, immunogenicity is not assured by efficient peptide binding to MHC molecules, suggesting that other factors are involved (Feltkamp *et al.*, 1994). Furthermore, predictive algorithms are not 100% precise as not all high-scoring peptides bind to MHC molecules (e.g. Bax 2, 6 and 9) and some low-scoring peptides are epitopes.

Correlation between actual peptide binding and immunogenicity is not perfect. Peptides that bind with high affinity are not always epitopes, and peptides that bind with low affinity sometimes are epitopes, e.g. hTERT peptide with low affinity to HLA-A2 (Hernandez *et al.*, 2002). Peptide-MHC complex stability and immunogenicity are more closely correlated than peptide affinity and immunogenicity. Immunogenic peptides are characterised by a low dissociation rate, indicating that they induce stable MHC-peptide complexes. Peptides (including low or intermediate affinity peptides) that display low dissociation rates are immunogenic, while peptides with a high dissociation rate are probably non-immunogenic (van der Burg *et al.*, 1996). So, in order to increase the chance of finding immunogenic peptides, a Bax pool containing all predicted peptides was tested for its ability to trigger primary CD8⁺ T cells from the peripheral blood of HLA-A2 healthy donors. This study was focussed on HLA-A2 restricted T cell responses because the frequency of this allele in the Caucasian population (30-50%) and the availability of well-characterized reagents.

Twelve healthy donors were tested by ELISpot for T cell responses against Bax pool 1-15. Overall, six donors responded to Bax peptides with variable responses after two to four weeks of stimulation. Three restimulation steps were included in the *in vitro* culture protocol, so as to increase the frequency of peptide-specific T cells. This indicated the likelihood that these were naïve T cell responses. Several studies have demonstrated that healthy donors can be used to evaluate the immunogenicity of peptides from well-known antigens, such as PSA (Correale *et al.*, 1997; Elkord *et al.*, 2005), STEAP (Rodeberg *et al.*, 2005), PTHrP (Yao *et al.*, 2004), survivin (Schmitz *et al.*, 2000) and hTERT (Hernandez *et al.*, 2002; Minev *et al.*, 2000). For example, two hTERT peptides induced peptide-specific CTLs in 7-9 out of 10 healthy donors (Minev *et al.*, 2000). The data obtained here is comparable to those findings, by demonstrating that T cell precursors for self-antigens circulate in the peripheral blood of healthy donors.

It was possible that the selection of the peptides of Bax pool 1-15 using only two algorithms could have missed potential epitopes. Therefore, a new set of peptides was identified from Bax protein using three to six algorithms. Interestingly, the HLA-A2 binding peptides that were shown to be immunogenic in Bax pool 1-15, were also ranked highly by the additional algorithms. However, these algorithms did not predict any further peptides that were capable of binding to HLA-A2 in T2 binding assays. Therefore, epitope prediction efficiency was not increased by using additional algorithms.

Screening healthy donors for T cell responses using the Bax peptide pool 601-23 produced similar results to Bax peptides 1-15. A donor (donor 11) that had strong response against Bax pool 1-15, also had a strong response against Bax pool 601-23. Neither the magnitude nor the kinetics of the responses improved with the new 601-23 Bax peptide set. However, the data obtained here indicated that at least one peptide derived from Bax should

be immunogenic to human CD8⁺ T cells and that T cell precursors reactive to Bax are present in the peripheral blood of healthy donors.

The IFN γ ELISpot assay was used to detect low frequency peptide specific T cells in culture. This assay has been extensively used in the laboratory to monitor HPV specific responses in peripheral blood of healthy donors and patients (Gallagher & Man, 2007; Smith *et al.*, 2005). The ELISpot assay is a simple, reliable, cost effective and very sensitive technique, permitting enumeration of antigen-specific T cells at frequencies lower than 1 in 10,000 (Helms *et al.*, 2000). This assay is particularly useful when dealing with low cell numbers for analysis (Shacklett *et al.*, 2003). The ELISpot is less expensive, uses less number of cells for analysis than ICS. Furthermore, this assay is more suitable to use when detecting low frequency T cell responses than ICS due to its lower detection limit (Karlsson *et al.*, 2003). The criteria used in this thesis to define a positive response of ≥ 20 spots per 1×10^5 cells after subtraction of background is either similar or more stringent than that used by several other groups (Currier *et al.*, 2002; Godard *et al.*, 2004; Karlsson *et al.*, 2003)

The initial experiments using the Bax pool 1-15 allowed limited characterisation of T cells from a single donor (Donor 1). The first attempt to generate long term, stable CD8⁺ T cell lines/clones involved using the CD107 assay combined with high speed flow cell sorting. It allowed the demonstration that at least three of the HLA-A2 binding peptides were candidate T cell epitopes. However, it proved difficult to derive T cell lines or clones for further characterisation, and to address the central hypothesis.

Repeated expansions of JSB cells using the antigen-independent protocol produced low yields of T cells with poor viability and led to a loss of peptide specificity as verified by CD107 assay. This loss of Bax specificity could have occurred for a number of reasons. The antigen-independent expansion protocol uses non-specific stimuli (IL-2 and PHA) which support multiple rounds of T cell proliferation. During this process, there is the possibility

that the non-specific T cell population (which also included CD4⁺ T cells) expanded to a greater degree compared to the Bax-specific T cells. The CD107-based sorting may compromise cell viability to some extent, in part through the toxicity of the reagent GolgiStop (BD). An alternative explanation for poor viability or functionality of the T cells could be that the flow cell sort conditions are too harsh for these T cells. Given that cells are submitted to considerable stress by acceleration in the nozzle, vibration, illumination, decompression, charging and deflection. It is likely that some damage occurs to the sorted T cells. Besides that, the flow sorting procedure is laborious and requires a trained technician to run the apparatus.

Rubio *et al.* (2003) were able to identify and isolate viable populations of tumour cytolytic T cells from blood using CD107a and flow cell sorting. However, for this current study (and other studies in the laboratory) it was not possible to corroborate their findings. Therefore, it was necessary to develop a consistent method for detecting and purifying low-frequency peptide specific T cell lines and clones. Several methods have been reported for this purpose, including CD107 (Betts *et al.*, 2003; Rubio *et al.*, 2003), CD137 (Wolfl *et al.*, 2007) and IFN γ secretion (Brosterhus *et al.*, 1999; Oelke *et al.*, 2000) assays. These methods were compared using MART-1 peptide as model antigen and only IFN γ secretion demonstrated a distinct population of peptide-specific T cells.

The IFN γ secretion assay has an advantage over CD107 assay by not requiring the addition of GolgiStop, which may partly affect the viability of cells. Furthermore, unlike high-speed flow cell sorting, there was little physical stress during MACs sorting, favouring the recovery of viable cells. This assay allows the identification and isolation of viable cytokine-secreting cells with very low frequencies (down to 0.0001%) by MACs (Campbell, 2003). However, it is not suitable for isolation of high frequency of antigen-specific T cells, as the culture medium can become saturated with cytokine and all cells will be non-specifically labelled. This method requires a restimulation step with antigen to produce IFN γ

secretion. Due to the downregulation of TCR after antigen stimulation, the enriched T cell fraction cannot be tested directly after selection and needs a further culture period to permit re-expression of the TCR. This IFN γ based enrichment has been used by a number of groups to isolate antigen-specific T cells (Brosterhus *et al.*, 1999; Campbell, 2003; Oelke *et al.*, 2000). It has also been used successfully in the laboratory to generate CD4 $^{+}$ T cell lines/clones highly specific to a HPV peptide (Gallagher & Man, 2007).

Besides the methods explained above, soluble MHC-peptide tetramers have been widely used to enumerate and isolate antigen specific T cells, for example in melanoma and HIV patients (Dunbar *et al.*, 1998; Lee *et al.*, 1999; Yee *et al.*, 2002). The use of commercially made tetramers to enrich Bax specific T cells was not an option due to the high cost of production to engineer several different peptide-MHC complexes. On the other hand, the IFN γ based enrichment was selected for use because it is a rapid, cost-effective, and relatively simple technique that does not require knowledge of peptide epitopes. Moreover, the advantage of this technique is that it would select T cells based on their effector function, whereas tetramer staining does not reveal information about the functionality of the T cells and not all tetramer positive cells secrete IFN γ .

The IFN- γ based enrichment method had a higher success rate in isolating highly Bax specific T cell populations than the previous attempts made by other methods. This system has allowed the isolation of full repertoire of CD8 $^{+}$ T cells responding to Bax antigen in an epitope independent way by using the Bax pool 601-23. It has subsequently permitted the generation of T cell lines from two healthy donors. Lines containing high frequencies of Bax specific T cells were generated by culturing cells under limiting dilution conditions. However, only polyclonal/oligoclonal T cell populations were isolated suggesting that culture conditions used here may not be sufficient to allow growth of single cells. The conditions could have been improved by using other types of media supplemented with IL-2 and perhaps other cytokines that support the *in vitro* expansion of T cells, such as IL-7 and IL15.

Nevertheless, the derivation of stable T cell lines allows testing of the hypothesis, and the further characterisation of these lines will be described in the next chapter.

To sum up, this chapter has so far provided evidence for the recognition of Bax protein as an antigen by human CD8⁺ T cells from healthy donors. Overall, the approach used here has proved to be an efficient method for detecting immunogenicity of Bax peptides, and has thus provided basis for further analysis of Bax T cell responses. Consequently, implementing the technology of selecting T cells with low frequency based on their IFN γ secretion resulted in the successful purification of Bax-specific T cells. The present study also implies that this system could be applied to other candidate tumour antigens or rare T cell populations. Figure 3.16 shows a summary of the *in vitro* approach used to generate Bax-specific T cell lines.

For Bax antigen to be considered a tumour antigen, functional assays need to be performed that conclusively show specific tumour recognition by the Bax-specific T cells in a MHC-restricted manner. This will be addressed in the next chapter.

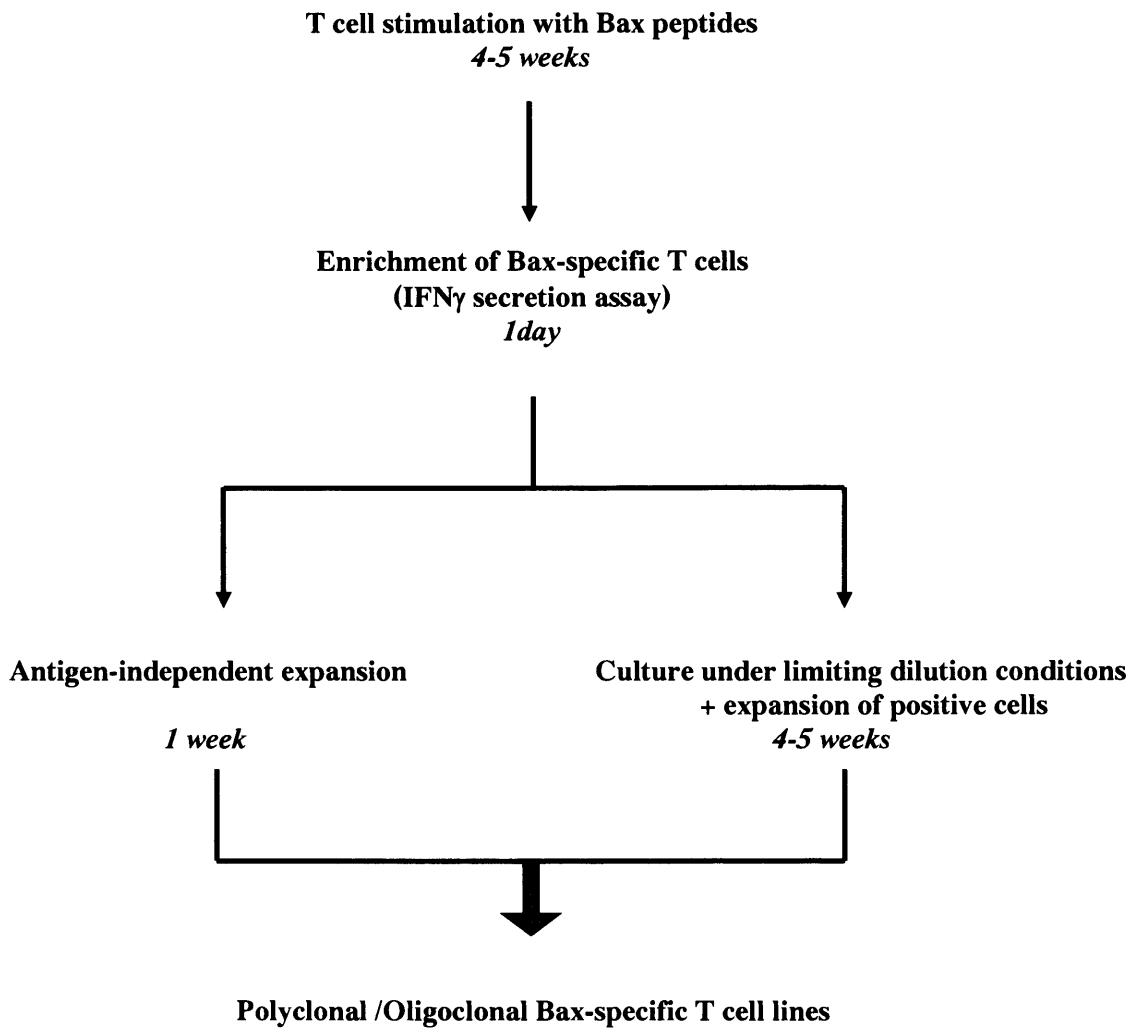


Figure 3.16 Summary of the *in vitro* approach used to generate Bax-specific T cell lines

Chapter 4

Characterisation of CTL generated against Bax derived peptides

In the chapter 3, several T cell lines were generated against Bax peptides from two HLA-A2+ healthy donors using the IFN γ secretion assay and magnetic sorting. This part of this study was focussed on the detailed characterisation of those Bax-specific T cells. In particular, the epitope specificity, clonality by TCR V β usage, avidity and tumour cell specificity of the T cells were determined.

4.1 Characterisation of Bax-specific T cells derived from the JSB line

As explained in section 3.6, two different strategies were used to generate highly specific T cell lines. First, the JSBI line was generated by expanding 9.3×10^3 of the IFN γ -enriched T cells from the JSB line using the antigen-independent protocol (section 3.6.2). Second, JSBI 10E6 was generated by culturing the enriched T cells under limiting dilution conditions in 96 well plates (section 3.6.3).

4.1.1 Epitope recognition by JSBI line using an ELISpot assay

A mixture of Bax peptides p1 (KLSECLKRI), p3 (IMGWTLDFL) and p5 (ALCTKVPEL) were used to enrich peptide-specific T cells and create the JSBI line (section 3.6.1). However, it was not known whether the antigen-independent expansion was able to maintain the peptide specificity of the T cells. This was tested by using an ELISpot assay, and demonstrated that specificity against the pool and each individual peptide was maintained (Figure 4.1). The peptide IMGWTLDFL (Bax p3) response was similar to the pool response (392 spots / 2×10^4 cells), with weaker responses against p1 and p5. Overall, this indicates the JSBI line is polyclonal, but has a dominant reactivity against the peptide IMGWTLDFL (Bax p3).

4.1.2 Epitope recognition by JSBI 10E6 using an ELISpot assay

JSBI 10E6 was also generated using the mixture of Bax p1, p3 and p5 peptides. To evaluate whether JSBI 10E6, which was cultured under limiting dilution conditions, had an enhanced specificity, it was also tested using ELISpot assays. JSBI 10E6 has different pattern of specificity and a higher magnitude of response than JSBI line (Figure 4.2). A dominant reactivity was seen against peptide IMGWTLDFL (Bax p3), as for the JSBI line, there was only a subdominant response against p3. Therefore, in this case culturing T cells under limiting dilution conditions had narrowed down the range of peptide specificities and enhanced the frequency of Bax p3-specific T cells.

4.1.3 Analysis of the TCR V β chains used by Bax-specific polyclonal T cell lines

The purpose of this experiment was to assess the number of different clonal T cell populations in each line, and whether the difference in peptide specificities between JSBI and JSBI 10E6 correlated with TCR V β usage. There are over 20 different groups of the TCR V β chain family that can be detected serologically. A panel of 19 PE-conjugated antibodies recognising different TCR V β chains was used to stain the T cell lines, which were subsequently analysed by flow cytometry.

TCR analysis of the JSBI line demonstrated that 85% of T cells expressed TCR V β 14, 12% TCR V β 12 and 2.4% TCR V β 3 (Figure 4.3A). Analysis of JSBI 10E6 was shown to be predominately TCR V β 14+ T cells (98.4%) (Figure 4.3C). The TCR V β usage was compared with the parental T cells (JSB line), from which both were generated. The JSB line (generated using a CD107 assay, section 3.2) was shown to express five different TCR V β , which are V β 3 (23.3%), V β 7 (12.5%), V β 14 (45%), V β 16 (7.5%), and V β 17 (6.7%) (Figure 4.3C).

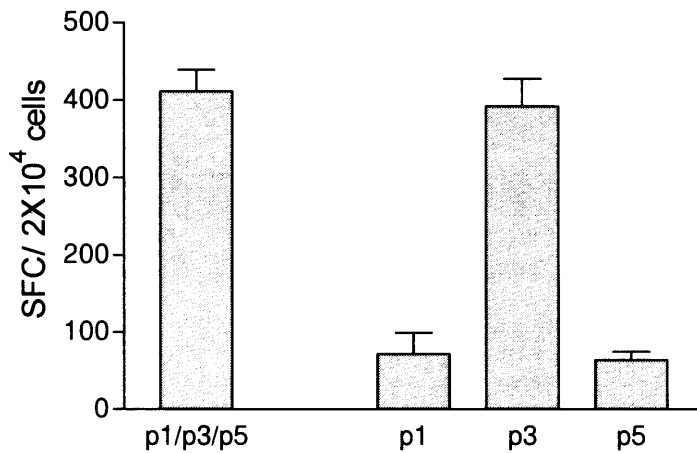


Figure 4.1 Epitope recognition by JSBI line using an ELISpot assay. The JSBI line was tested by IFN γ ELISpot against Bax p1 (KLSECLKRI), p3 (IMGWTLDFL) and p5 (ALCTKVPPEL) pooled together and individually. The JSBI line T cells were plated out at 2×10^4 cells/well in triplicate and incubated either alone, with T2 or with T2 + Bax peptides (10 μ g/ml) at a ratio of 1:1. An average of 411 spots/ 2×10^4 cells was positive for the mixture of peptides. An average response of 392 spots was detected against Bax p3. Average responses against Bax p1 and Bax p5 were 72 and 64 spots, respectively. Background responses have been subtracted from the data and the standard deviation is shown.

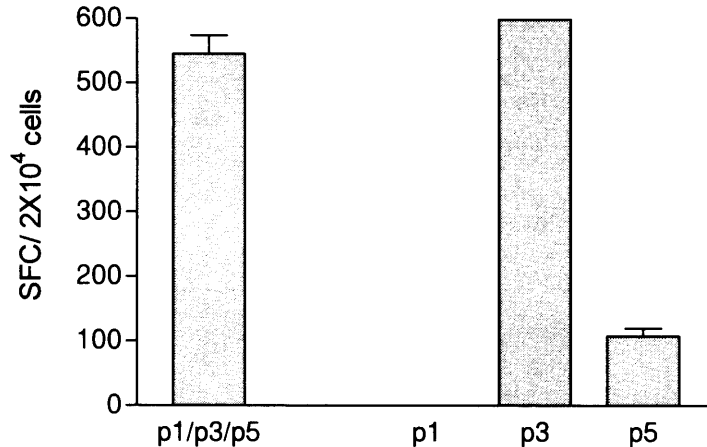


Figure 4.2 Epitope recognition by JSBI 10E6 using an ELISpot assay. The JSBI 10E6 was tested by IFN γ ELISpot against Bax p1 (KLSECLKRI), p3 (IMGWTLDFL) and p5 (ALCTKVPPEL) pooled together and individually. The JSBI 10E6 T cells were plated out at 2×10^4 cells/well in triplicate and incubated either alone, with T2 or with T2 + Bax peptides (10 μ g/ml) at a ratio of 1:1. An average of 411 spots/ 2×10^4 was positive for the mixture of peptides. A response of 107 spots was detected against Bax p5 and no response was detected against Bax p1. Background responses have been subtracted from the data and the standard deviation is shown except for Bax p3. Due to the numerous spots per well, only an estimation was made with approximately 600 spots / well containing Bax p3 and no standard deviation is shown for this result.

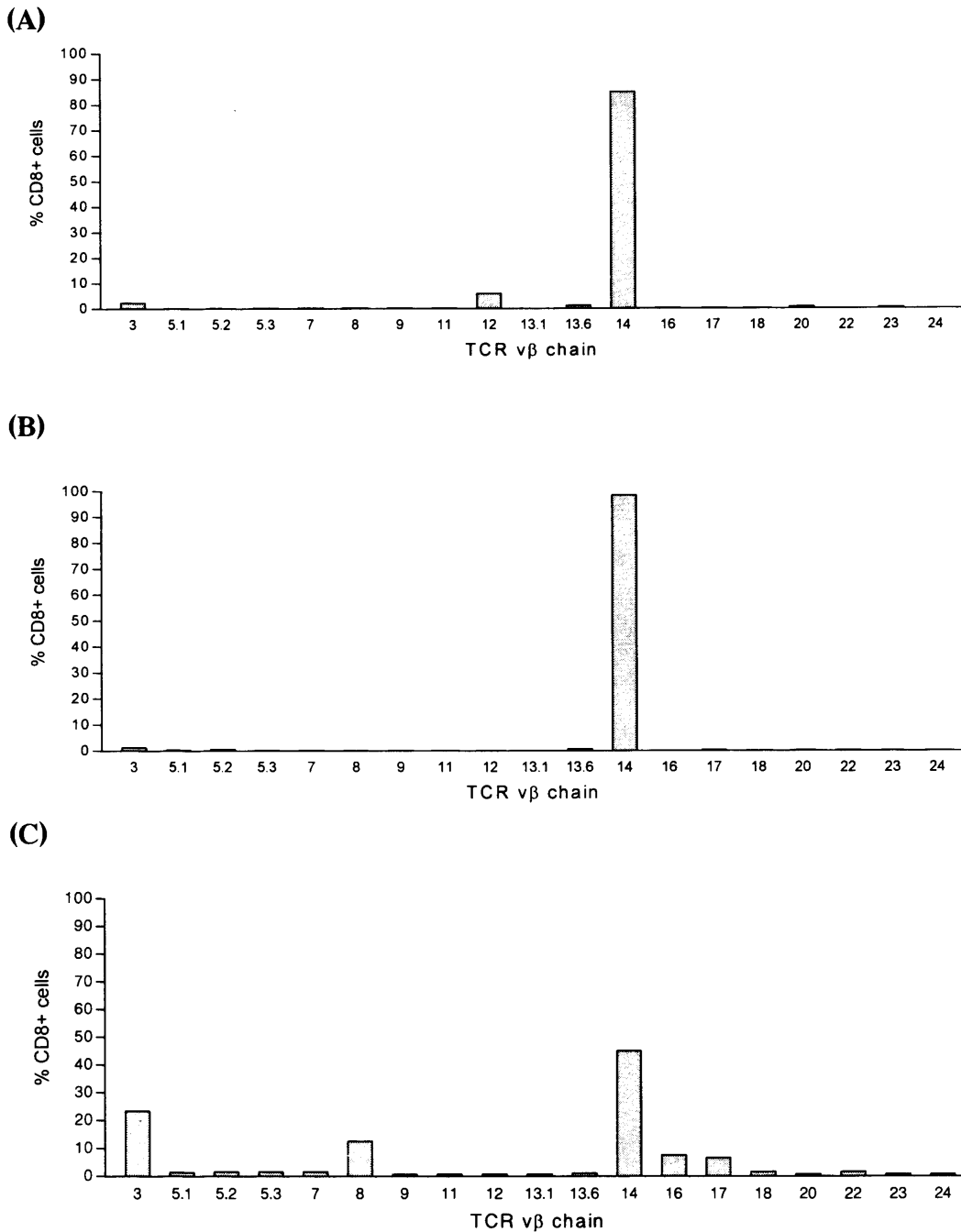


Figure 4.3 Staining of Bax-specific T cell lines (from donor 1) for TCR Vβ expression. The JSBI line **(A)**, JSBI 10E6 **(B)**, and the parental T cells (JSB line, CD107 sorted) **(C)** were co-stained with a CD8-FITC antibody and 19 different TCR Vβ chain-PE antibodies. Data shown represents the percentage of CD8+ cells expressing each Vβ chain. T cells were gated, based on their forward and side scatter profile. Data was analysed using CellQuest software.

Overall, these results indicate that a narrow range of TCR V β chains were used by the T cell lines. Furthermore, the percentage of cells expressing TCR V β 14 increased as the T cell lines became more specific for Bax peptides. Therefore, it seemed likely that TCR V β 14 was involved in Bax peptide recognition.

4.1.4 JSBI 10E6 is able to lyse peptide-pulsed targets

In order to assess whether JSBI 10E6 contains cytotoxic activity towards these Bax peptides, it was tested against peptide-pulsed T2 target cells using a ^{51}Cr release assay. JSBI 10E6 demonstrated peptide-specific killing against T2 cells pulsed with Bax pool p1/p3/p5 (Figure 4.4). These CTLs only recognised T2 cells loaded with Bax pool (70%) whereas they did not lyse unpulsed T2 (5%).

4.2 Characterisation of Bax-specific T cell lines derived from donor 11 using Bax pool 601-23

Because initial attempts to generate T cell lines/ clones failed with donor 1 (section 3.2.2), T cells from donor 11 were also used to maximise the likelihood of successful generation of Bax-specific T cell lines/clones. Similar to donor 1 (JSB), two different strategies were used to generate highly specific T cell lines from donor 11 using Bax pool 601-23 (section 3.5). The KSI line was generated by expanding 7.8×10^4 of the IFN γ -enriched T cells using the peptide-independent protocol (section 3.5.1). A second KSI 10E6 was generated by culturing the cells using the peptide-dependent protocol (section 3.5.3).

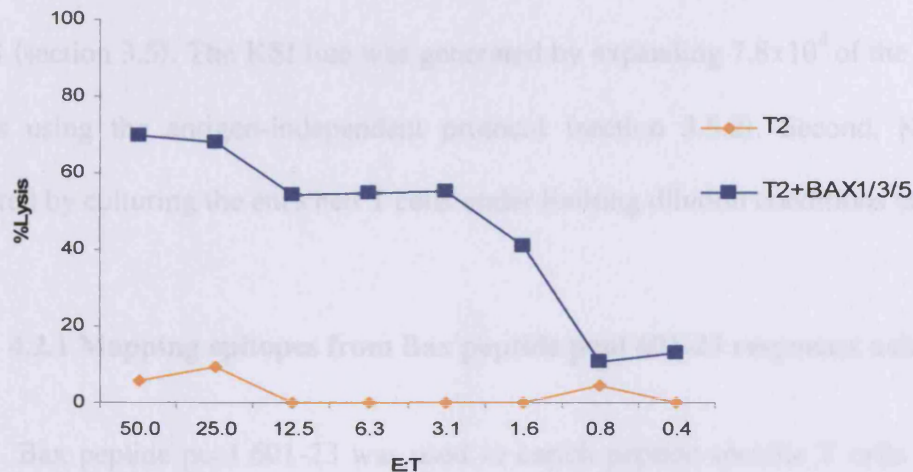


Figure 4.4 Ability of JSBI 10E6 to lyse target pulsed with Bax peptides. JSBI 10E6 was assayed against T2 which were pulsed with Bax p1/p3/p5 (■) or unpulsed (◆). Peptide-pulsed targets were pulsed with peptides at $10 \mu\text{g}/\text{ml}$ for 1 hour at 37°C after ^{51}Cr -labelling. Cytotoxicity was measured in a 4 hours ^{51}Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate samples. A positive result is considered $> 20\%$ lysis.

were detected against Bax p603, p619, p613, and p614. A cytotoxic response ($> 20\%$ lysis) was also detected against Bax p685, p611, and p635, (Table 4.2). 30% and 61% spots $> 2 \times 10^4$ cells respectively. Surprisingly, the KSI line derived from donor 11 (MLA-42, A24, Bw4, Bw6, Bw4, Bw6, Cw5, Cw6) was able to elicit a detectable response to Bax p613, which is predicted to bind to B44 (61 spots $> 2 \times 10^4$ cells), using T2 cell line (MLA-42) as APC. In general, these results demonstrated that the KSI line contained T cells with multiple peptide specificities. This suggests that for donor 11, at least 7 peptides within the Bax pool (23 peptides) were immunogenic.

4.2 Characterisation of Bax-specific T cell lines derived from donor 11 using Bax pool 601-23

Because initial attempts to generate T cell lines/clones failed with donor 1 (section 3.2.2), T cells from donor 11 were also used to maximise the likelihood of successful generation of Bax-specific T cell lines/clones. Similar to donor 1 (JSB), two different strategies were used to generate highly specific T cell lines from donor 11 using Bax pool 601-23 (section 3.5). The KSI line was generated by expanding 7.8×10^4 of the IFN γ -enriched T cells using the antigen-independent protocol (section 3.5.2). Second, KSI 10B7 was generated by culturing the enriched T cells under limiting dilution conditions (section 3.5.3).

4.2.1 Mapping epitopes from Bax peptide pool 601-23 responses using KSI line

Bax peptide pool 601-23 was used to enrich peptide-specific T cells and create the KSI line. In order to map the peptide specificity, the KSI line was tested by ELISpot with the whole Bax pool 601-23 and three smaller sub pools. As every peptide pool turned out positive (data not shown), individual peptides were tested. Figure 4.5 illustrates the identification of potential epitopes recognised by the KSI line. Seven peptides from Bax pool 601-23 were recognised from Bax pool by the KSI line. Peptide responses of > 100 spots were detected against Bax p603, p610, 613, and p614. A weaker response (< 100 spots) was also detected against Bax p605, p611, and p615, with 42, 50, and 61 spots / 2×10^4 cells respectively. Surprisingly, the KSI line derived from donor 11 (HLA-A2, A24, B44, B60, Bw4, Bw6, Cw5, Cw20) was able to elicit a detectable response to Bax p615, which is predicted to bind to B44 (61 spots / 2×10^4 cells), using T2 cell line (HLA-A2) as APC. In general, these results demonstrated that the KSI line contained T cells with multiple peptide specificities. This suggests that for donor 11, at least 7 peptides within the Bax pool (23 peptides) were immunogenic.

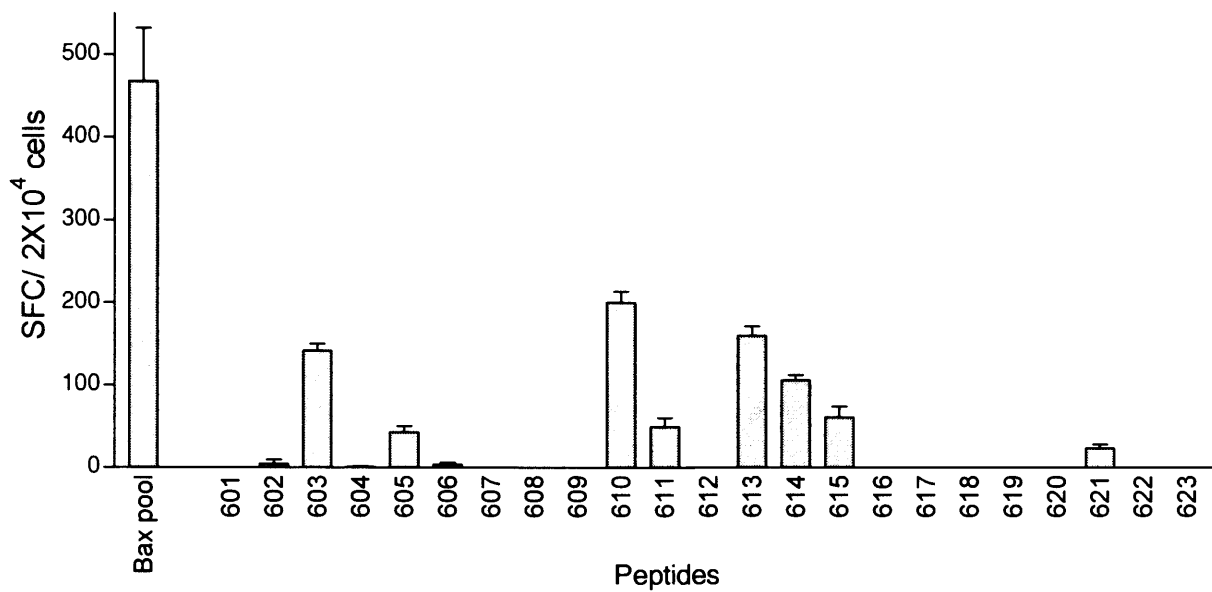


Figure 4.5 Mapping the Bax 601-23 response from KSI line to individual peptides. The KSI line generated from the enriched Bax-specific T cells was tested by IFN γ ELISpot against the Bax pool 601-23 and individual peptides. The KSI line T cells were plated out at 2×10^4 cells/well in triplicate and incubated either alone, with T2 or with T2 + Bax pool 1-23 (10 μ g/ml) or individual peptides at a ratio of 1:1. Background responses have been subtracted from the data and the standard deviation is shown. The number of spots detected (per 2×10^4 cells) for each peptide ranged from 42 to 200 after background subtraction. The spots/well for Bax pool were too numerous to count accurately, but 470 spots were estimated for against Bax 601-23.

4.2.2 Mapping epitopes from Bax peptide pool 601-23 responses using KSI 10B7

The KSI 10B7 was generated under limiting dilution conditions. Therefore, it was of interest to determine whether it had a more restricted peptide specificity than the KSI line. T cells were tested by ELISpot to map epitopes, first using three split pools (Bax p601-609, Bax p610-615 and Bax p616-623) to narrow down the response, followed by individual peptides to allow identification of potential epitopes. KSI 10B7 was able to strongly respond to split pool Bax p610-615 (Figure 4.6), but as spots / well in the ELISpot assay were too numerous to count accurately, only an estimation of the number of spots was made with approximately 600 spots. A comparable response to Bax pool 601-623 and split pool Bax p610-15 was detected against Bax p610 and p613. Again, the spots / well were too numerous to give an accurate count, therefore the number of spots were considered to be in the region of ≥ 600 spots. Interestingly, these two peptides are almost identical: Bax p610 is a 10-mer (TIMGWTLDFL) and Bax p613 is a 9-mer (IMGWTLDFL). These results showed that KSI 10B7 response is constrained to these two related peptides, and suggests that the limiting dilution culture had narrowed down the range of peptide specificities.

4.2.3 Analysis of the TCR V β chains used by Bax-specific polyclonal T cell lines

As mentioned in section 4.1.3, the purpose of this experiment was to identify the number of different T cell populations each line contained and to determine whether the enrichment for particular peptide recognition correlate with TCR V β usage. Donor 11 polyclonal T cells (section 3.3.2), from which Bax specific T cell lines were derived, were initially

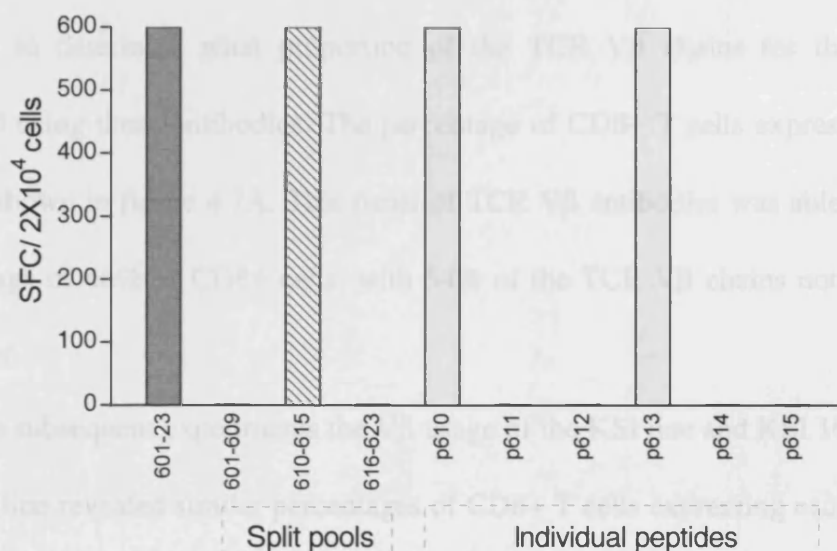


Figure 4.6 Mapping the Bax 601-23 response from KSI 10B7 to split pools and individual peptides. KSI 10B7 generated from the enriched Bax-specific T cells was tested by IFN γ ELISpot against the Bax pool 601-23, split pools (Bax p601-609, Bax p610-615 and Bax p616-623) and individual peptides from the positive split pool (Bax p610, p611, p612, p613, p614 and p615). The KSI 10B7 T cells were plated out at 2×10^4 cells/well in triplicate and incubated either alone, with T2 or with T2 + peptides at a ratio of 1:1. Background responses have been subtracted from the data. The positive responses were approximately ≥ 600 spots (*). No standard deviation is shown because no accurate count of number of spots was possible.

4.2.3 Analysis of the TCR V β chains used by Bax-specific polyclonal T cell lines

As mentioned in section 4.1.3, the purpose of this experiment was to identify the number of different T cell populations each line contained and to determine whether the enrichment for particular peptide specificities correlate with TCR V β usage. Donor 11 pre-sort T cells (section 3.3.2), from which Bax specific T cell lines were derived, were initially tested in to determine what proportion of the TCR V β chains for this donor could be identified using these antibodies. The percentage of CD8+ T cells expressing each TCR V β chain is shown in figure 4.7A. This panel of TCR V β antibodies was able to identify the V β chain usage of 46% of CD8+ cells, with 54% of the TCR V β chains not being detected for this donor.

In subsequent experiments the V β usage of the KSI line and KSI 10B7 was examined. The KSI line revealed similar percentages of CD8+ T cells expressing each TCR V β chain to the pre-sorted T cells (Figure 4.7B). On the other hand, analysis of KSI 10B7 showed that 66.4% of the T cells expressed TCR V β 5.1 and 29.9% expressed TCR V β 17 (Figure 4.7C). This data indicates that KSI 10B7 is oligoclonal, containing two main T cell populations, a V β 5.1+ and a V β 17+ population. By contrast, in the KSI line, only 1.8% of gated cells were TCR V β 5.1+ and 5.3% were TCR V β 17+.

Based on these results, the approach used for KSI 10B7 did lead to selective enrichment of Bax specific T cells. Furthermore, it seemed likely that TCR V β 5.1 or V β 17 was involved in Bax p610 and Bax p613 recognition. Because of the interesting results obtained with KSI 10B7, these cells were further sorted according to their TCR V β chains, described in the next section.

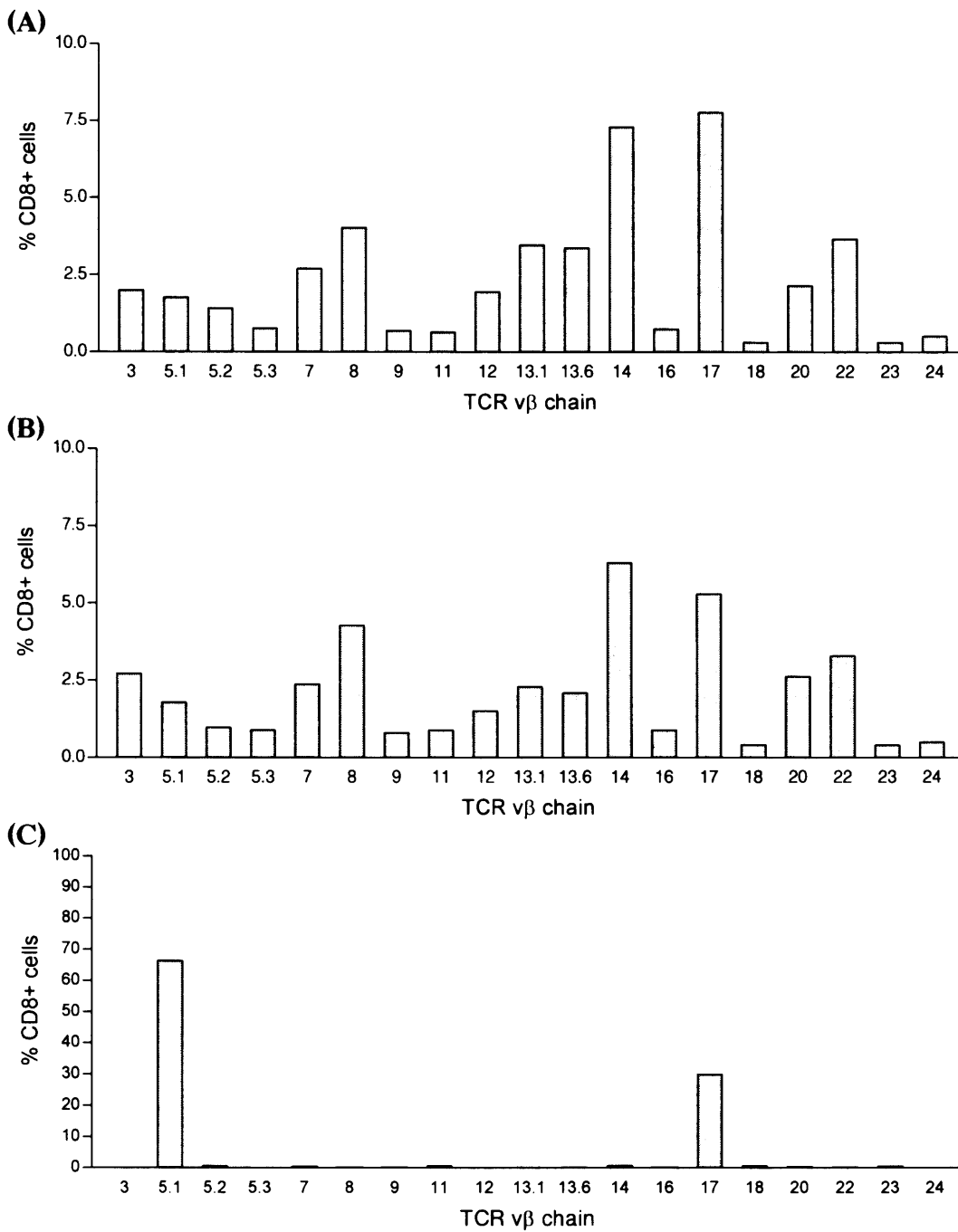


Figure 4.7 Staining of Bax-specific T cell lines (from donor 11) for TCR V β expression. Donor 1 pre-sorted T cells (from which the Bax specific T cell lines were generated) **(A)**, KSI line **(B)**, and KSI 10B7 **(C)** were co-stained with a CD8-FITC antibody and 19 different TCR V β chain-PE antibodies. Data shown represents the percentage of CD8+ cells expressing each V β chain. T-cells were gated, based on their forward and side scatter profile. Data was analysed using CellQuest software.

4.2.4 KSI line and KSI 10B7 are able to lyse target cells pulsed with Bax pool

601-23

It was not known whether both the KSI line and KSI 10B7 had cytotoxic activity against the Bax peptides. Therefore, these T cell lines were tested against T2 target cells pulsed with Bax pool 601-23 in a ^{51}Cr release assay. The KSI line was able to kill Bax-pulsed T2 cells (65% lysis at E:T 25:1), but not unpulsed T2 (10%) (Figure 4.8). KSI 10B7 effectively lysed T2 pulsed with Bax pool 601-23 (92% lysis at E:T 50:1), whereas no cytotoxicity was observed against unpulsed T2 (0.1%). These T cells demonstrated a higher lytic activity against Bax peptides than the KSI line, at multiple E:T ratios (Figure 4.9).

It was demonstrated that these generated T cell lines increased their specificity against Bax peptides after enrichment, particularly when cultured under limiting dilution conditions. These initial results indicate that Bax are immunogenic and able to induce a cytotoxic response to peptide-loaded target cells. Since KSI 10B7 showed the highest lytic activity against peptide-pulsed targets compared to the other T cell lines, only KSI 10B7 was used in subsequent experiments. A summary of Bax-specific T cell lines generated in this study is shown in table 4.1

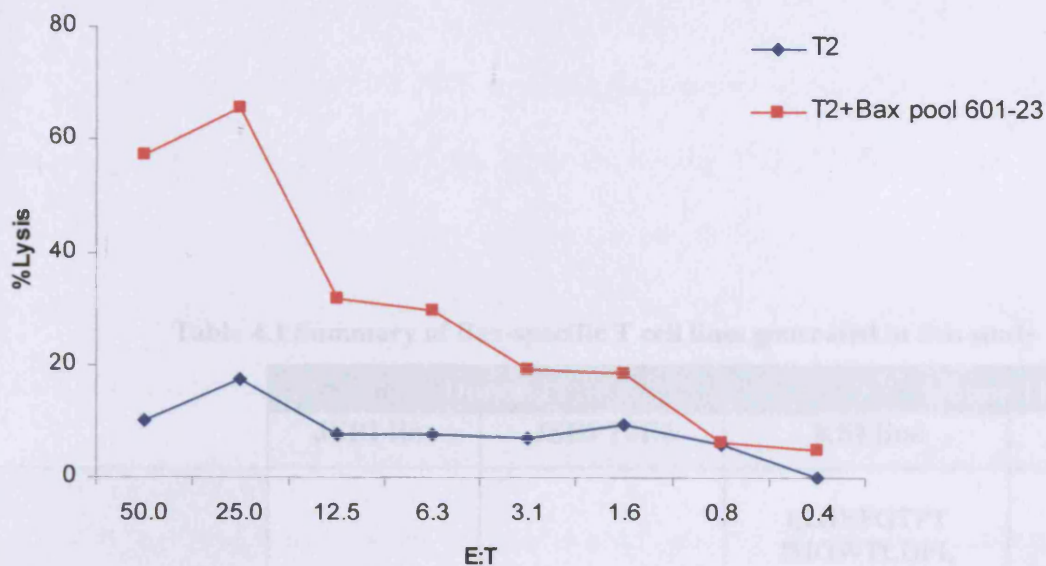


Figure 4.8 Ability of the KSI line to lyse target pulsed with Bax pool 601-23. The KSI line was assayed against T2 which were pulsed with Bax pool 601-23 (■) or unpulsed (◆). Peptide-pulsed targets were pulsed with peptides at 10µg/ml for 1 hour at 37°C after ^{51}Cr -labelling. Cytotoxicity was measured in a 4 hours ^{51}Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate samples. A positive result is considered > 20% lysis.

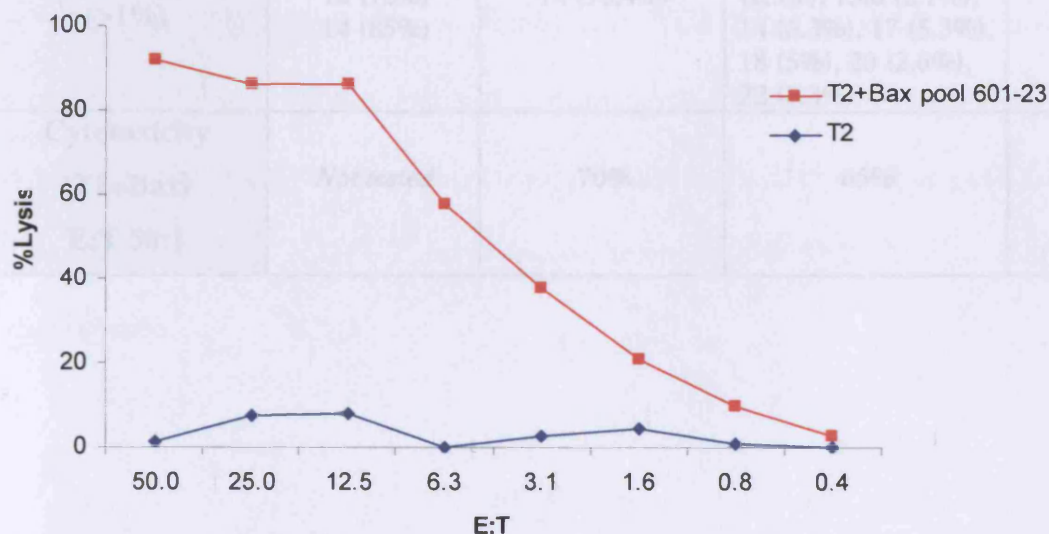


Figure 4.9 Ability of KSI 10B7 to lyse target pulsed with Bax pool 601-23. KSI 10B7 was assayed against T2 which were pulsed with Bax pool 601-23 (■) or unpulsed (◆). Peptide-pulsed targets were pulsed with peptides at 10µg/ml for 1 hour at 37°C after ^{51}Cr -labelling. Cytotoxicity was measured in a 4 hours ^{51}Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate sample. A positive result is considered > 20% lysis.

Table 4.1 Summary of Bax-specific T cell lines generated in this study

	Donor 1		Donor 11	
	JSBI line	JSBI 10E6	KSI line	KSI 10B7
Peptide specificity	IMGWTLDFL ALCTKVPEL KLSECLKRI	IMGWTLDFL ALCTKVPEL	LLSYFGTPT IMGWTLDFL TIMGWTLDFL ALCTKVPEL GLLSYFGTPT FLRERLLGWI DELDNMEL	IMGWTLDFL TIMGWTLDFL
TCR $\nu\beta$ (>1%)	3 (2.4%) 12 (12%) 14 (85%)	14 (98.4%)	3 (2.7%), 5.1 (1.8%), 7 (2.4%), 8 (4.3%), 12 (1.5%), 13.1 (2.3%), 13.6 (2.1%), 14 (6.3%), 17 (5.3%), 18 (5%), 20 (2.6%), 22 (3.3%)	5.1 (66.4%) 17 (29.9%)
Cytotoxicity (T2+Bax) E:T 50:1	<i>Not tested</i>	70%	65%	92%

4.3 Generation of TCR V β 5.1+ and V β 17+ T cells from KSI 10B7

Characterisation of KSI 10B7 revealed the presence of two distinct T cell populations, one expressing TCR V β 5.1 and the other expressing TCR V β 17. In order to effectively investigate Bax peptide recognition by these sub-populations, they were separated using high speed flow sorting. To generate a TCR V β 5.1+ and V β 17+ T cell populations, KSI 10B7 T cells were stained with a TCR V β 17-PE antibody and sorted based upon positive (V β 17+) and negative (V β 17-) PE staining using a MoFlo cell sorter. After sorting cells were cultured overnight in media containing IL-2 before expanding using the antigen-independent protocol. Expansions yielded $>15 \times 10^6$ cells for both T cell populations. Flow cytometric analysis revealed the KSI V β 5.1+ T cells were 98.37% positive for V β 5.1 expression and the KSI V β 17+ T cells were 99.1% positive for V β 17 (Figure 4.10).

Both enriched populations were tested in a ^{51}Cr release assay against T2 pulsed with either Bax p610 or Bax p613 to confirm whether TCR V β 5.1 or V β 17 were involved in the recognition of these peptides. Figure 4.12 demonstrated that KSI V β 17+ T cells, but not KSI V β 5.1+ T cells, were able to recognise target pulsed with Bax p613 or p610 (Figure 4.11). Therefore, only KSI V β 17+ T cells (hereon referred as KSIVB17) were used for further experiments.

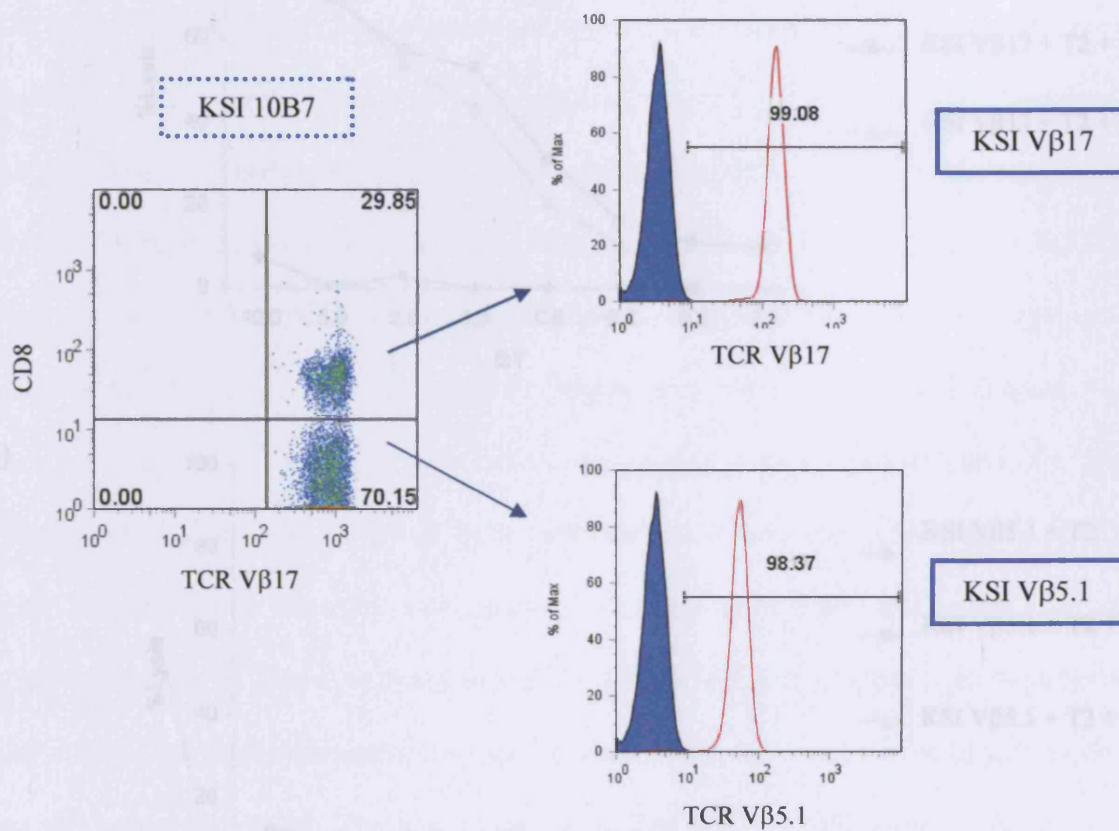


Figure 4.10 Generation of the TCRVβ5.1+ and TCR Vβ17+ T cells from KSI 10B7. KSI 10B7 was shown to consist of 70.15% TCR Vβ5.1+ CD8+ T cells and 29.85% TCR Vβ17+ CD8+ T cells (left panel). To generate a TCR Vβ5.1+ line and a TCR Vβ17+ line, 5×10^6 KSI 10B7 T cells were incubated with an anti-TCR Vβ17-PE antibody. Cells were sorted based upon positive and negative PE staining using a MoFlo cell sorter. 3.6×10^6 TCR Vβ5.1+ cells and 1×10^6 TCR Vβ17+ T cells were recovered after sorting. The cells obtained were expanded using the antigen-independent protocol. The purity of both lines was assessed by flow cytometry using an anti-TCR Vβ17-PE antibody (right panel) and histograms depict the percentage of the lines expressing TCR Vβ17. Flow cytometric analysis was done using Flowjo software.

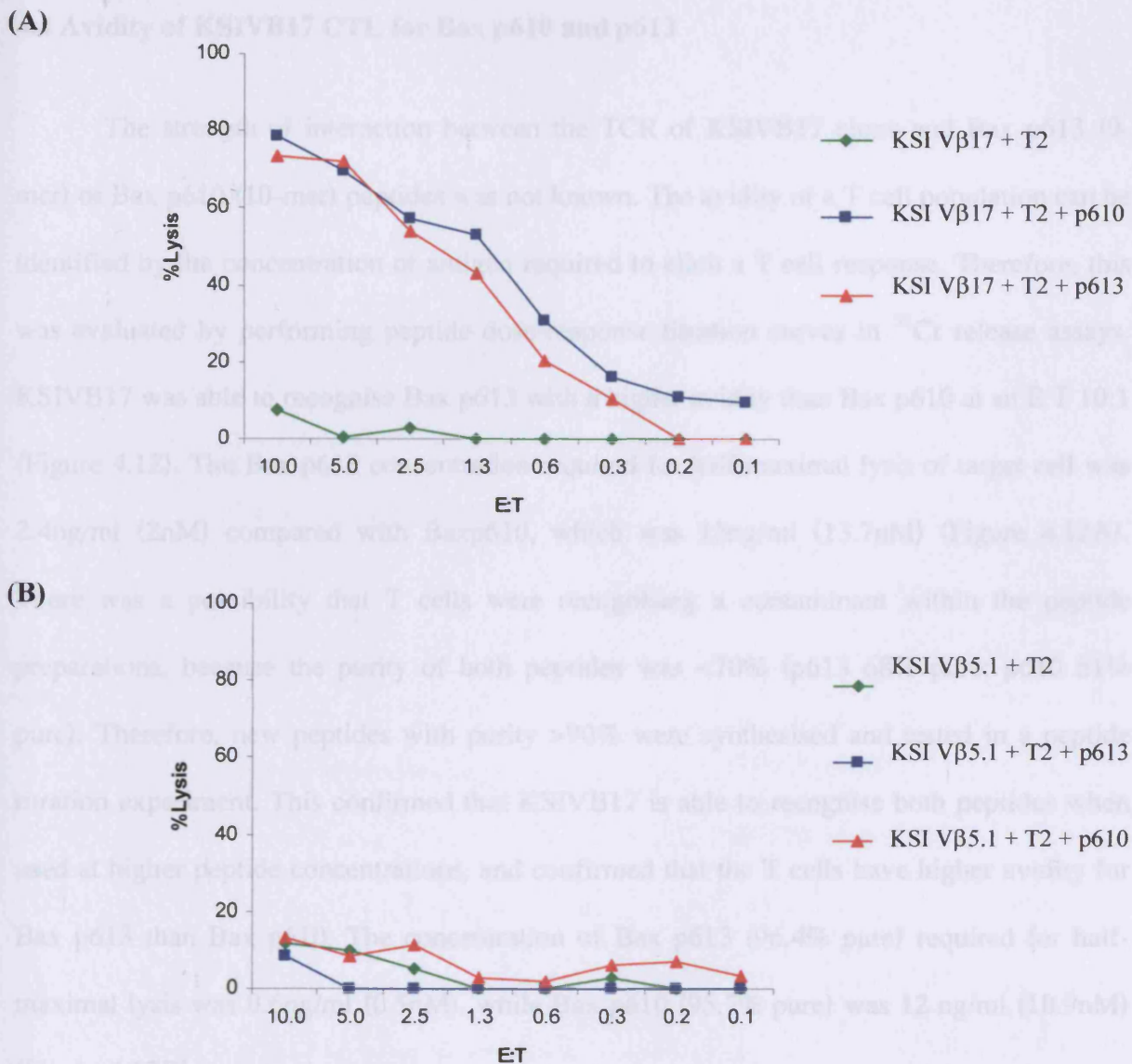
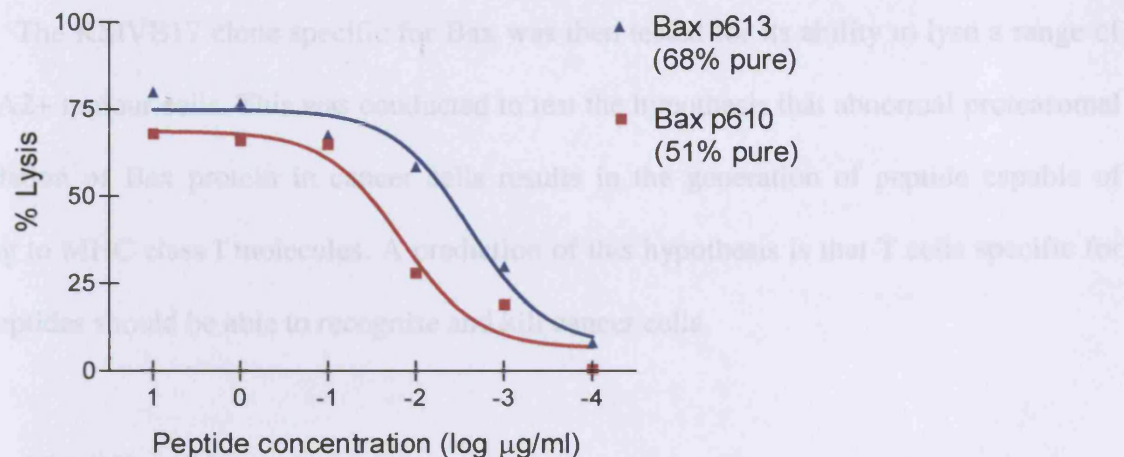


Figure 4.11 KSI V β 17+ T cells are able to lyse targets pulsed with Bax p610 or p613. KSI V β 17+ T cells (A) and KSI V β 5.1+ T cells (B) were assayed against T2 which were pulsed with Bax p610 (■), Bax p613 (▲) or unpulsed (◆). Peptide-pulsed targets were pulsed with peptides at 10 μ g/ml for 1 hour at 37°C after ^{51} Cr-labelling. Cytotoxicity was measured in a 4 hours ^{51} Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate samples. A positive result is considered > 20% lysis.

4.4 Avidity of KSIVB17 CTL for Bax p610 and p613

The strength of interaction between the TCR of KSIVB17 clone and Bax p613 (9-mer) or Bax p610 (10-mer) peptides was not known. The avidity of a T cell population can be identified by the concentration of antigen required to elicit a T cell response. Therefore, this was evaluated by performing peptide dose-response titration curves in ^{51}Cr release assays. KSIVB17 was able to recognise Bax p613 with a higher avidity than Bax p610 at an E:T 10:1 (Figure 4.12). The Bax p613 concentration required for half-maximal lysis of target cell was 2.4ng/ml (2nM) compared with Baxp610, which was 15ng/ml (13.7nM) (Figure 4.12A). There was a possibility that T cells were recognising a contaminant within the peptide preparations, because the purity of both peptides was <70% (p613 68% pure; p610 51% pure). Therefore, new peptides with purity >90% were synthesised and tested in a peptide titration experiment. This confirmed that KSIVB17 is able to recognise both peptides when used at higher peptide concentrations, and confirmed that the T cells have higher avidity for Bax p613 than Bax p610. The concentration of Bax p613 (96.4% pure) required for half-maximal lysis was 0.6ng/ml (0.5nM), while Bax p610 (95.7% pure) was 12 ng/ml (10.9nM) (Figure 4.12B).

(A)



(B)

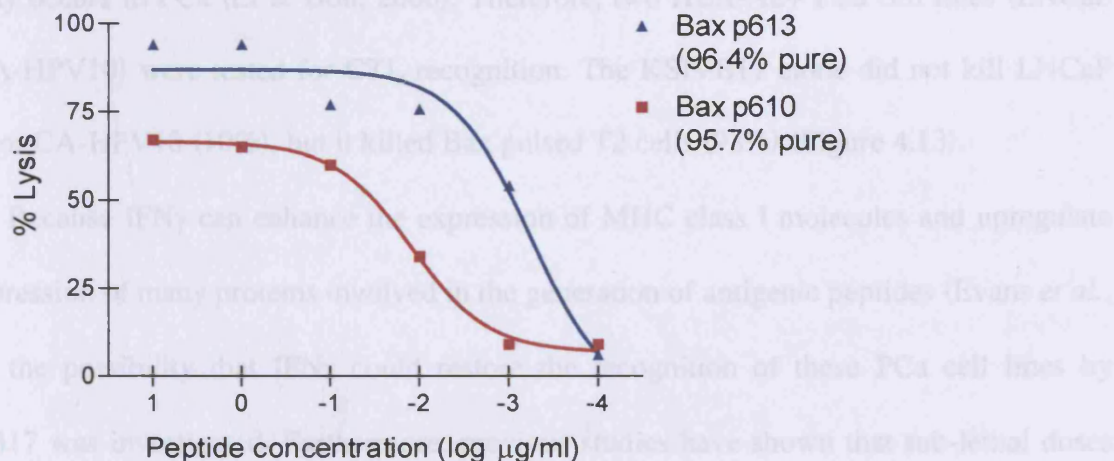


Figure 4.12 Peptide dose-response for KSIVB17 recognition of Bax p613 and Bax p610 -pulsed target cells. KSIVB17 was assayed against ^{51}Cr -labelled T2 cells pulsed with varying concentrations of Bax p613 ($\blacktriangle$) and Bax p610 ($\blacksquare$) from $10\mu\text{g/ml}$ to 0.1ng/ml for 1 hour at 37°C after ^{51}Cr -labelling. (A) Peptide titration for Bax p613 with purity of 68% and Bax p610 with purity of 51%. (B) Peptide titration for Bax p613 with purity of 96.4% and Bax p610 with purity of 95.7%. Both the results shown are at an effector:target ratio of 10:1. Results are expressed as mean of triplicate samples. The molecular weights (Mw) of Bax p613 (IMGWTLDFL) and Bax p610 (TIMGWTLDFL) were calculated from their amino acid sequences (1096 and 1196 respectively) and from this, the Molarity of each peptide concentration was determined ($10\mu\text{g/ml}$ equals $8.36\mu\text{M}$ for Bax p613 and $9.13\mu\text{M}$ for Bax p610). The EC50 value was calculated using the curve fitted to the data.

4.5 Recognition of tumour cells by KSIVB17 CTL

The KSIVB17 clone specific for Bax was then tested for its ability to lyse a range of HLA-A2+ tumour cells. This was conducted to test the hypothesis that abnormal proteasomal degradation of Bax protein in cancer cells results in the generation of peptide capable of binding to MHC class I molecules. A prediction of this hypothesis is that T cells specific for Bax peptides should be able to recognise and kill cancer cells.

4.5.1 PCa cell lines

It has been shown that abnormal Bax degradation by the ubiquitin/proteasome pathway occurs in PCa (Li & Dou, 2000). Therefore, two HLA-A2+ PCa cell lines (LNCaP and CA-HPV10) were tested for CTL recognition. The KSIVB17 clone did not kill LNCaP (16%) or CA-HPV10 (10%), but it killed Bax pulsed T2 cells (93%) (Figure 4.13).

Because IFN γ can enhance the expression of MHC class I molecules and upregulate the expression of many proteins involved in the generation of antigenic peptides (Evans *et al.*, 2001), the possibility that IFN γ could restore the recognition of these PCa cell lines by KSIVB17 was investigated. Furthermore, previous studies have shown that sub-lethal doses of irradiation can upregulate MHC class I, tumour-associated molecules and enhanced antigen-specific killing of tumour cells by CTL (Garnett *et al.*, 2004). Therefore, it was also examined whether exposure of both targets to a sub-lethal dose of irradiation (1000 rads) could sensitize these targets to Bax-specific T cells. However, no substantial increase in killing by KSIVB17 was seen when LNCaP and CA-HPV10 were IFN γ treated (1% and 19%, respectively) or irradiated (21% and 17%, respectively).

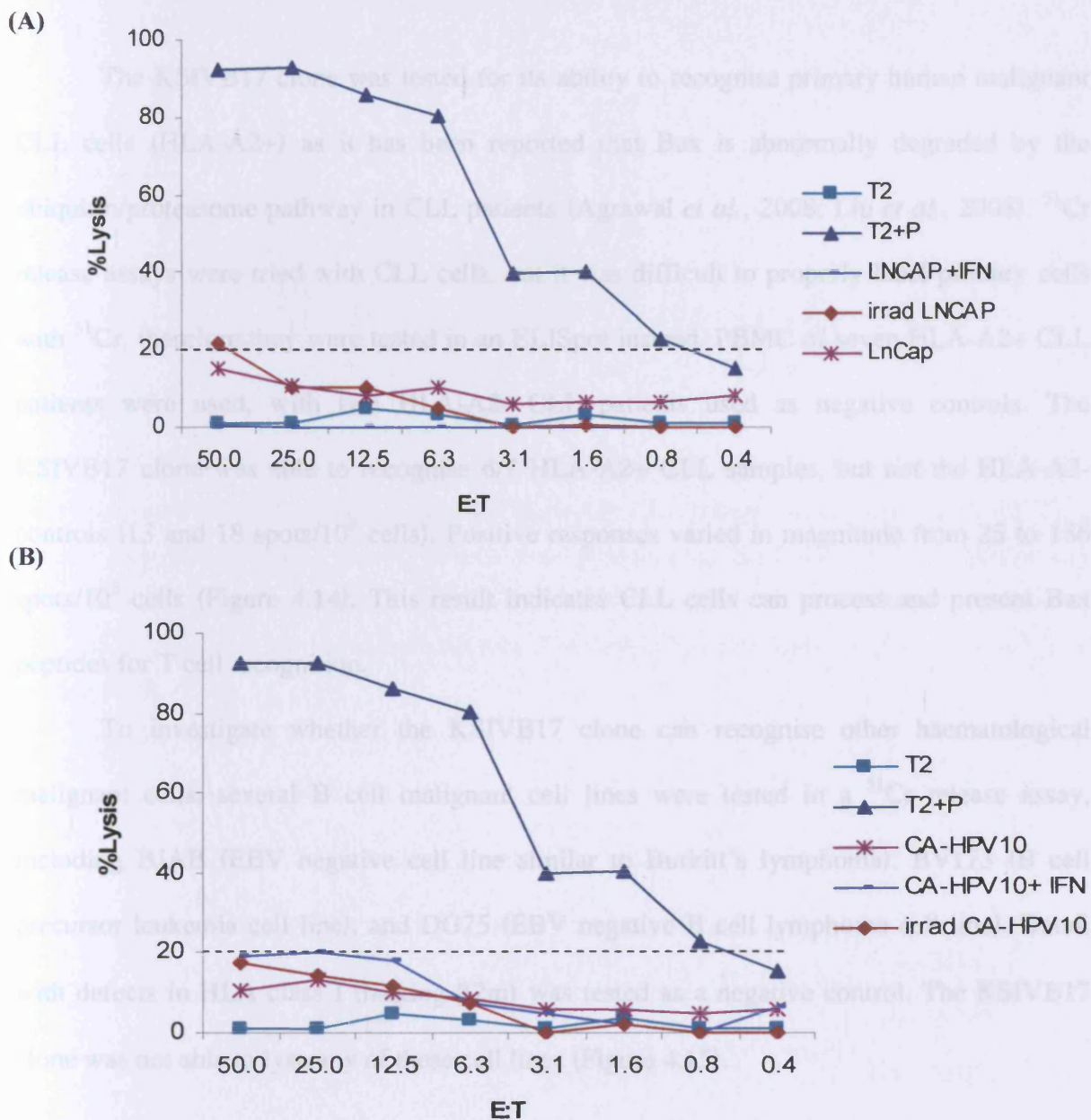


Figure 4.13 KSIVB17 clone does not lyse HLA-A2+ PCa cell lines LNCaP and CA-HPV10. KSIVB17 was assayed against ^{51}Cr -labelled LNCaP (A) and CA-HPV10 (B) that were either untreated (*), treated with 200 units/ml of IFN γ for 48 hours (-) or irradiated at 1000 rads 72 hours prior to the assay (♦). Peptide pulsed T2 (▲) and unpulsed T2 (■) were added as controls. Cytotoxicity was measured in a ^{51}Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate samples. The dashed line represents the 20% lysis cut-off for a positive result.

4.5.2 Lymphoid malignancies

The KSIVB17 clone was tested for its ability to recognise primary human malignant CLL cells (HLA-A2+) as it has been reported that Bax is abnormally degraded by the ubiquitin/proteasome pathway in CLL patients (Agrawal *et al.*, 2008; Liu *et al.*, 2008). ^{51}Cr release assays were tried with CLL cells, but it was difficult to properly label primary cells with ^{51}Cr , therefore they were tested in an ELISpot instead. PBMC of seven HLA-A2+ CLL patients were used, with two HLA-A2- CLL patients used as negative controls. The KSIVB17 clone was able to recognise 6/7 HLA-A2+ CLL samples, but not the HLA-A2- controls (13 and 18 spots/ 10^5 cells). Positive responses varied in magnitude from 25 to 186 spots/ 10^5 cells (Figure 4.14). This result indicates CLL cells can process and present Bax peptides for T cell recognition.

To investigate whether the KSIVB17 clone can recognise other haematological malignant cells, several B cell malignant cell lines were tested in a ^{51}Cr release assay, including BJAB (EBV negative cell line similar to Burkitt's lymphoma), BV173 (B cell precursor leukemia cell line), and DG75 (EBV negative B cell lymphoma cell line). Daudi with defects in HLA class I (lacking $\beta 2\text{m}$) was tested as a negative control. The KSIVB17 clone was not able to lyse any of these cell lines (Figure 4.15).

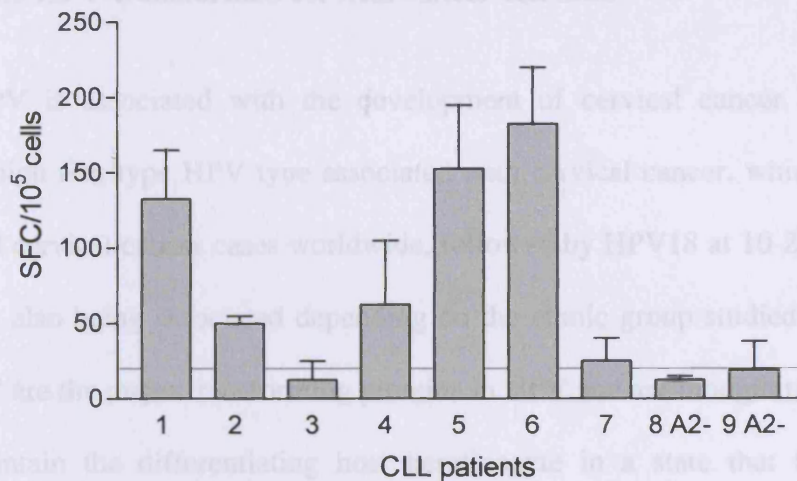


Figure 4.14 KSIVB17 clone recognises HLA-A2+ CLL cells using ELISpot assay. KSIVB17 clone was tested in an IFN γ ELISpot against 7 HLA-A2+ CLL patient samples and 2 HLA-A2- CLL samples (negative controls 8 and 9). T cells were plated out at 2×10^4 cells/well in triplicate and incubated with CLL cells at a ratio of 1:3. Results are shown as SFC/ 10^5 cells. Background responses (CLL alone) have been subtracted from the data. The dashed line represents the 20 spot cut-off for a positive result.

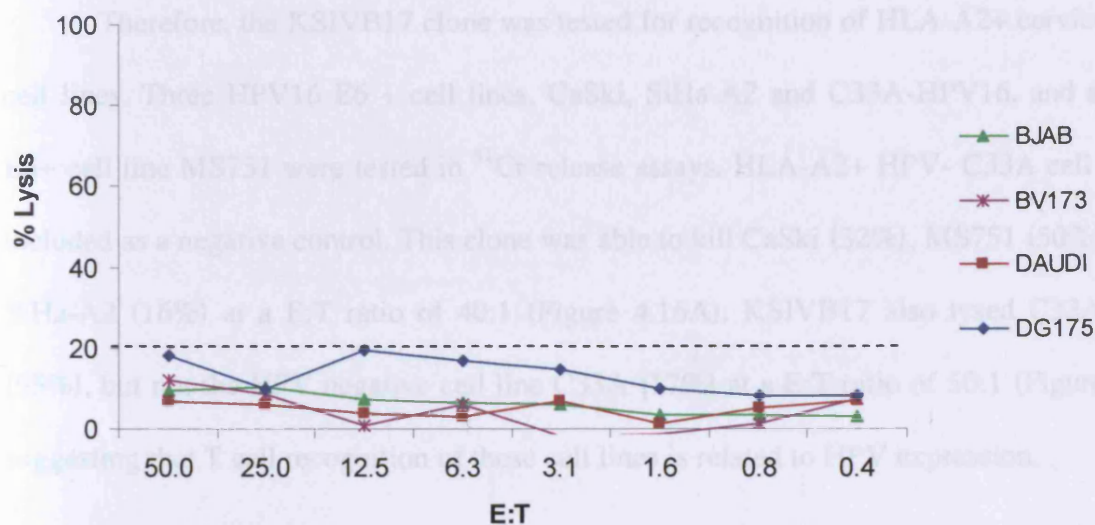


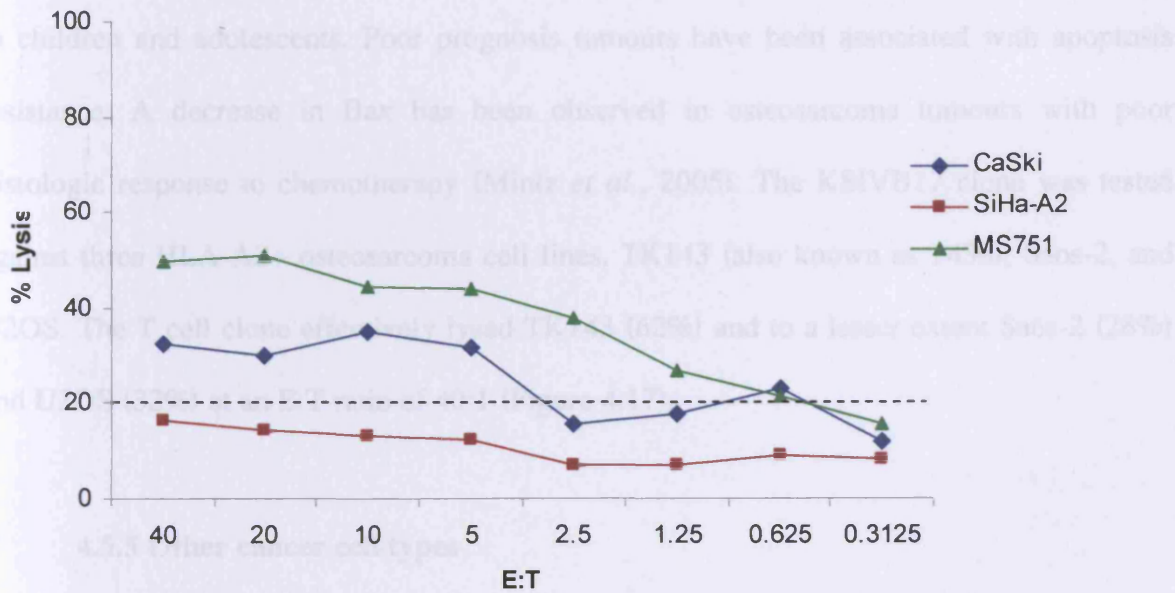
Figure 4.15 KSIVB17 clone does not lyse HLA-A2+ lymphoid malignant cell lines. KSIVB17 was assayed against ^{51}Cr -labelled BJAB ($\blacktriangle$), BV173 ($\ast$), DG175 ($\blacklozenge$), and Daudi (negative control) ($\blacksquare$). Cytotoxicity was measured in a ^{51}Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate samples. The dashed line represents the 20% lysis cut-off for a positive result.

4.5.3 HPV-transformed cervical cancer cell lines

HPV is associated with the development of cervical cancer. HPV16 is the most common high risk type HPV type associated with cervical cancer, which is detected in over 50% of all cervical cancer cases worldwide, followed by HPV18 at 10-20%, with HPV31, 45, 52 and 58 also being associated depending on the ethnic group studied (Bosch *et al.*, 1995). E6 and E7 are the major transforming proteins in HPV and are thought to modify cell cycle so as to maintain the differentiating host keratinocyte in a state that facilitates viral DNA replication and uncontrolled cell proliferation (Ruesch & Laimins, 1998). E6 protein contributes to oncogenesis in part by interacting with or inactivating proteins involved in apoptosis, such as Bak (Thomas & Banks, 1998). Both Bax mRNA and protein stability have been shown to be reduced in human differentiating keratinocytes by the expression of HPV16 E6. This study also revealed that E6 can enhance Bax degradation and inhibit Bax-induced apoptosis in stable and transiently expressing cells (Magal *et al.*, 2005).

Therefore, the KSIVB17 clone was tested for recognition of HLA-A2+ cervical cancer cell lines. Three HPV16 E6 + cell lines, CaSki, SiHa-A2 and C33A-HPV16, and a HPV45 E6+ cell line MS751 were tested in ⁵¹Cr release assays. HLA-A2+ HPV- C33A cell line was included as a negative control. This clone was able to kill CaSki (32%), MS751 (50%), but not SiHa-A2 (16%) at a E:T ratio of 40:1 (Figure 4.16A). KSIVB17 also lysed C33A-HPV16 (55%), but not the HPV negative cell line C33A (17%) at a E:T ratio of 50:1 (Figure 4.16B), suggesting that T cell recognition of these cell lines is related to HPV expression.

(A)



(B)

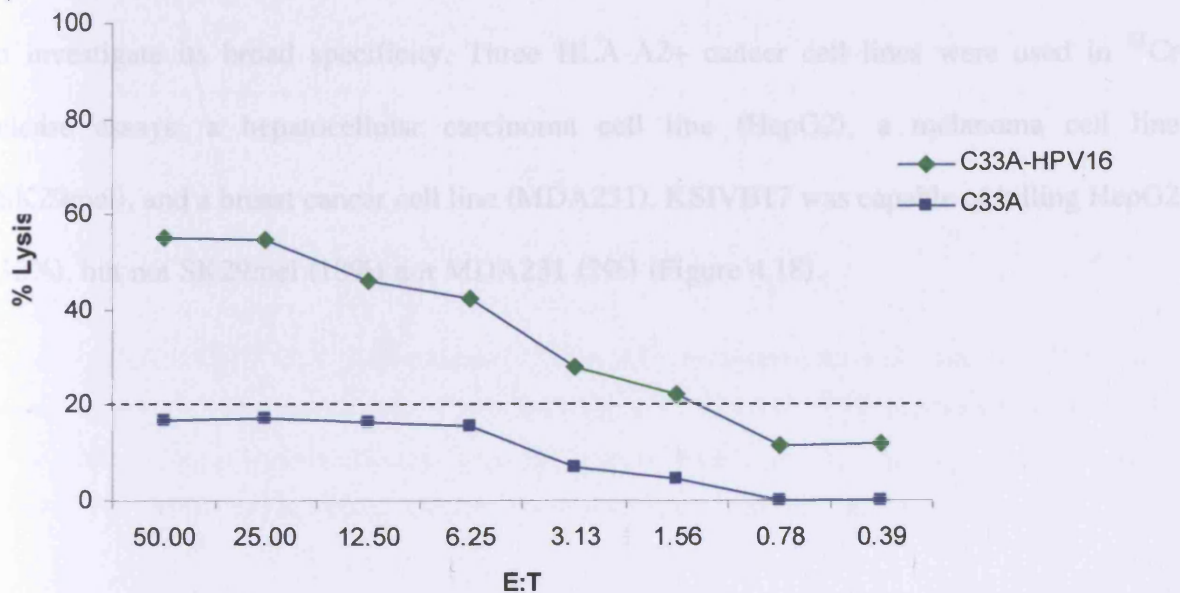


Figure 4.16 KSIVB17 CTL recognition of HPV-transformed cervical cancer cell lines. KSIVB17 was assayed against ^{51}Cr -labelled Caski (HPV16+) (◆), MS751 (HPV45+) (▲), SiHa-A2 (HPV16+) (■) (A); C33A-HPV16 (◆) and C33A (HPV-) (■) (B). Cytotoxicity was measured in a ^{51}Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate samples. The dashed line represents the 20% lysis cut-off for a positive result.

4.5.4 Osteosarcoma cell lines

Osteosarcoma is the most common type of malignant bone cancer, mainly occurring in children and adolescents. Poor prognosis tumours have been associated with apoptosis resistance. A decrease in Bax has been observed in osteosarcoma tumours with poor histologic response to chemotherapy (Mintz *et al.*, 2005). The KSIVB17 clone was tested against three HLA-A2+ osteosarcoma cell lines, TK143 (also known as 143b), Saos-2, and U2OS. The T cell clone effectively lysed TK143 (62%) and to a lesser extent Saos-2 (28%) and U2OS (32%) at an E:T ratio of 40:1 (Figure 4.17).

4.5.5 Other cancer cell types

The ability of KSIVB17 clone to interact with other cancer cell types was also tested to investigate its broad specificity. Three HLA-A2+ cancer cell lines were used in ⁵¹Cr release assays: a hepatocellular carcinoma cell line (HepG2), a melanoma cell line (SK29mel), and a breast cancer cell line (MDA231). KSIVB17 was capable of killing HepG2 (32%), but not SK29mel (18%) nor MDA231 (2%) (Figure 4.18).

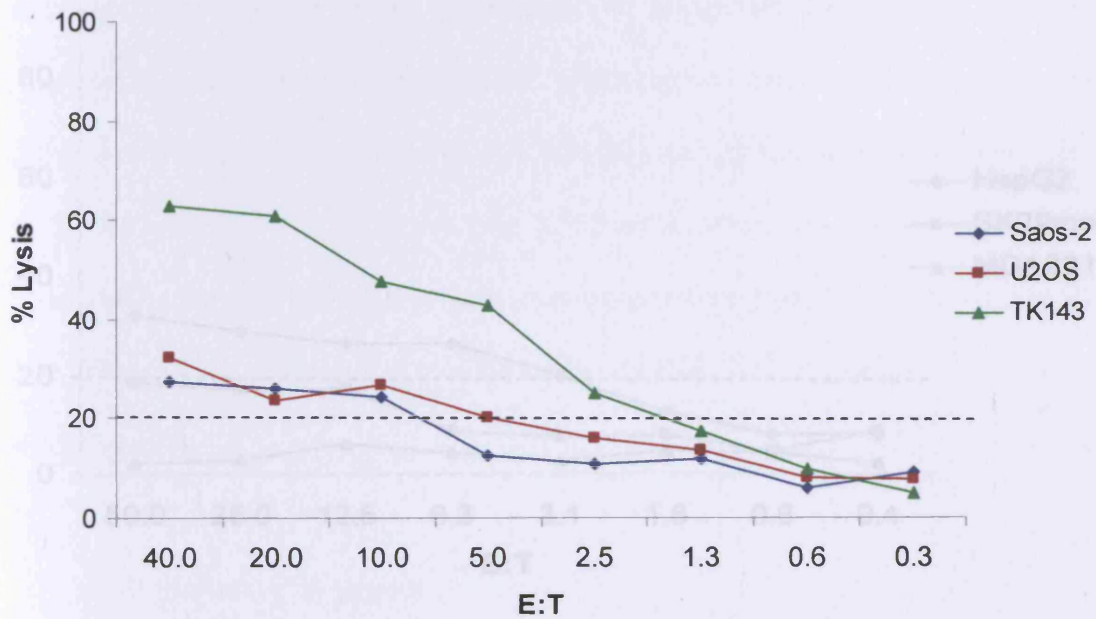
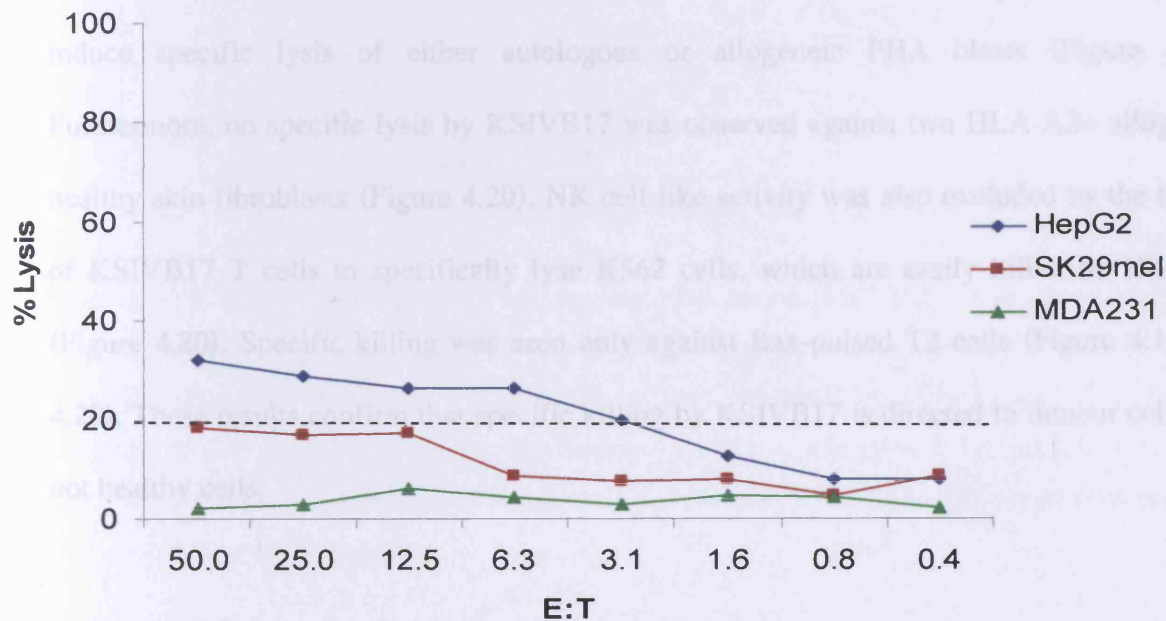


Figure 4.17 KSIVB17 CTL recognition of HLA-A2+ osteosarcoma cell lines. KSIVB17 was assayed against ^{51}Cr -labelled TK143 ($\blacktriangle$), Saos-2 ($\blacklozenge$), and U2OS ($\blacksquare$). Cytotoxicity was measured in a ^{51}Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate samples. The dashed line represents the 20% lysis cut-off for a positive result.

4.3.6 Negative controls

Since Bax protein is also expressed in normal, non-transformed cells, there is the potential that Bax peptides could be processed and presented for T cell recognition on these cells as well. Therefore, the KSIVB17 clone was tested on autologous and allogeneic healthy PHA blasts and allogeneic healthy skin fibroblasts in ^{51}Cr release assays. KSIVB17 did not



4.3.7 Summary of targets

Figure 4.18 KSIVB17 clone lyses a hepatocellular cell line but not a breast cancer or melanoma cell lines. KSIVB17 was assayed against ^{51}Cr -labelled MDA231 ($\blacktriangle$), HepG2 ($\blacklozenge$), and SK29mel ($\blacksquare$). Cytotoxicity was measured in a ^{51}Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate samples. The dashed line represents the 20% lysis cut-off for a positive result.

4.5.6 Negative controls

Since Bax protein is also expressed in normal, non-transformed cells, there is the potential that Bax peptides could be processed and presented for T cell recognition on these cells as well. Therefore, the KSIVB17 clone was tested on autologous and allogeneic healthy PHA blasts and allogeneic healthy skin fibroblasts in ^{51}Cr release assays. KSIVB17 did not induce specific lysis of either autologous or allogeneic PHA blasts (Figure 4.19). Furthermore, no specific lysis by KSIVB17 was observed against two HLA-A2+ allogeneic healthy skin fibroblasts (Figure 4.20). NK cell-like activity was also excluded by the failure of KSIVB17 T cells to specifically lyse K562 cells, which are easily killed by NK cells (Figure 4.20). Specific killing was seen only against Bax-pulsed T2 cells (Figure 4.19 and 4.20). These results confirm that specific killing by KSIVB17 is directed to tumour cells and not healthy cells.

4.5.7 Summary of targets

The KSIVB17 clone was able to recognise primary human CLL cells, kill HPV-transformed cervical cancer cell lines CaSki, MS751 and C33A-HPV16, osteosarcoma cell lines TK143, Saos-2 and U2OS, and a hepatocellular cell line HepG2. A summary of the target cells tested in ^{51}Cr release assays and their respective lysis is shown in Table 4.2.

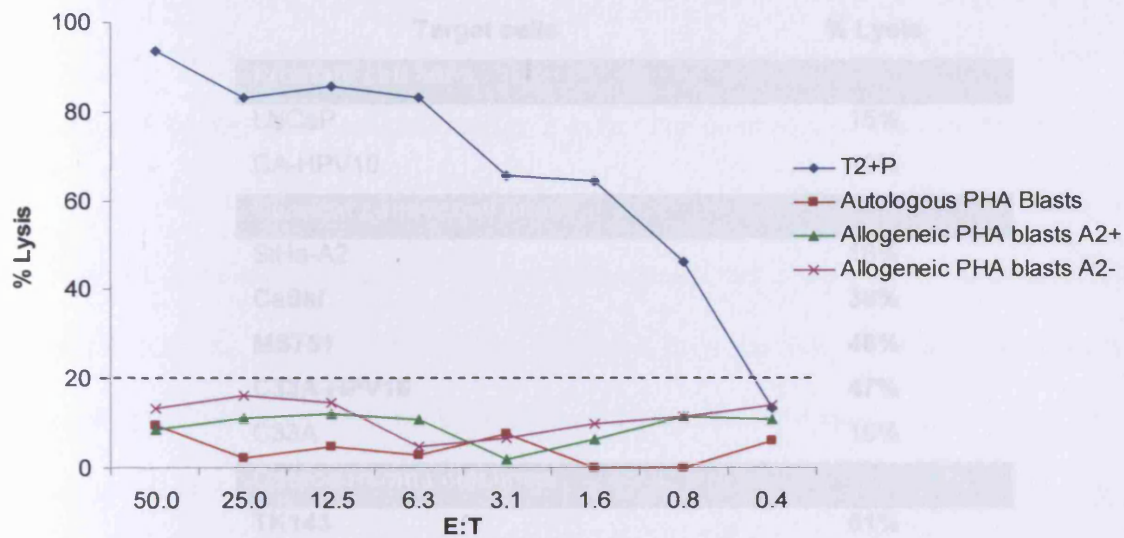


Figure 4.19 KSIVB17 clone does not lyse healthy PHA blasts. KSIVB17 was assayed against ^{51}Cr -labelled T2+Bax (◆), autologous PHA blasts (■), allogeneic HLA-A2+ PHA blasts (▲), and allogeneic HLA-A2- PHA blasts (×). PHA blasts were generated by culturing PBMC with PHA (10 $\mu\text{g}/\text{ml}$) for 3-5 days. Cytotoxicity was measured in a ^{51}Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate samples. The dashed line represents the 20% lysis cut-off for a positive result.

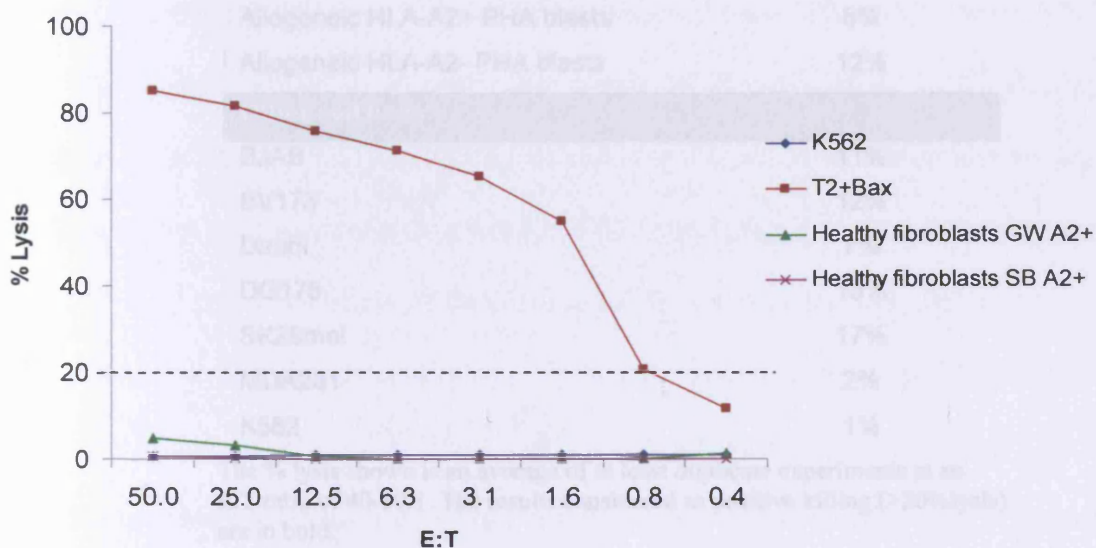


Figure 4.20 KSIVB17 clone does not lyse healthy skin fibroblasts and K562 cell line. KSIVB17 was assayed against ^{51}Cr -labelled T2+Bax (■), K562-A2 (◆), and allogeneic HLA-A2+ healthy fibroblasts GW (▲), and SB (×). Cytotoxicity was measured in a ^{51}Cr release assay at various Effector:Target (E:T) ratios. Results are expressed as mean of triplicate samples. The dashed line represents the 20% lysis cut-off for a positive result.

Table 4.2 Summary of target cells tested in ^{51}Cr release assays and their respective lysis.

Target cells	% Lysis
Prostate cell lines	
LNCaP	15%
CA-HPV10	10%
Cervical cancer cell lines	
SiHa-A2	16%
CaSki	38%
MS751	46%
C33A-HPV16	47%
C33A	16%
Osteosarcoma cell lines	
TK143	61%
Saos-2	23%
U20S	28%
Hepacellular cell line	
HepG2	22%
Healthy cells	
HLA-A2+ skin fibroblasts GW	4%
HLA-A2+ skin fibroblasts GW	1%
Autologous PHA blasts	9%
Allogeneic HLA-A2+ PHA blasts	8%
Allogeneic HLA-A2- PHA blasts	12%
Other cell lines	
BJAB	11%
BV173	12%
Daudi	7%
DG175	18%
SK29mel	17%
MDA231	2%
K562	1%

The % lysis shown is an average of at least duplicate experiments at an E:T ratio of 40-50:1. The results considered as positive killing (>20% lysis) are in bold.

4.6 Specificity of KSIVB17 clone

It was possible that the lysis of tumour cell lines by the KSIVB17 clone was not mediated by the Bax peptide-specific T cells. For example, it was possible that KSIVB17 was not clonal, and contained multiple sub-populations of TCR V β 17+ T cells. Some of these sub-populations could be Bax peptide specific but unable to kill tumour cells. In that case, the killing of the tumour cells is mediated by a separate sub-population that is tumour reactive but not Bax specific. Therefore cold target competition experiments were done to confirm that the CTLs that lyse the tumour cells are the same as those that recognise Bax peptides. Unlabeled tumour cells or T2 cells alone, pulsed with Bax p613 or with an irrelevant peptide (cold targets) were included in ^{51}Cr release assays at different ratios. Addition of unlabelled cold targets TK143 and T2 cells pulsed with Bax p613 decreased the CTL activity against labelled (hot) TK143 targets in a cold:hot target ratio-dependent manner, whereas unlabelled T2 alone or pulsed with flu M1 showed no effect on cytotoxic lysis (Figure 4.21). The lysis of hot TK143 decreased from 77% to 33% when T2+Bax p613 or TK143 were added at a cold : hot target (C:T) ratio of 20:1. In addition, it was demonstrated that the KSIVB17 CTLs do not lyse T2 pulsed with an irrelevant peptide (Flu M1) (data not shown), confirming that KSIVB17 specificity is against Bax (Figure 4.21).

Cold target inhibition of Bax-induced CTL cytotoxicity was also observed using the target MS751. Addition of unlabelled MS751 or T2 pulsed with Bax p613 decreased the CTL activity against MS751 from 36% to 13 and 10%, respectively (Figure 4.22). Collectively, these experiments confirm that killing of tumour cells is mediated by Bax peptide-specific T cells.

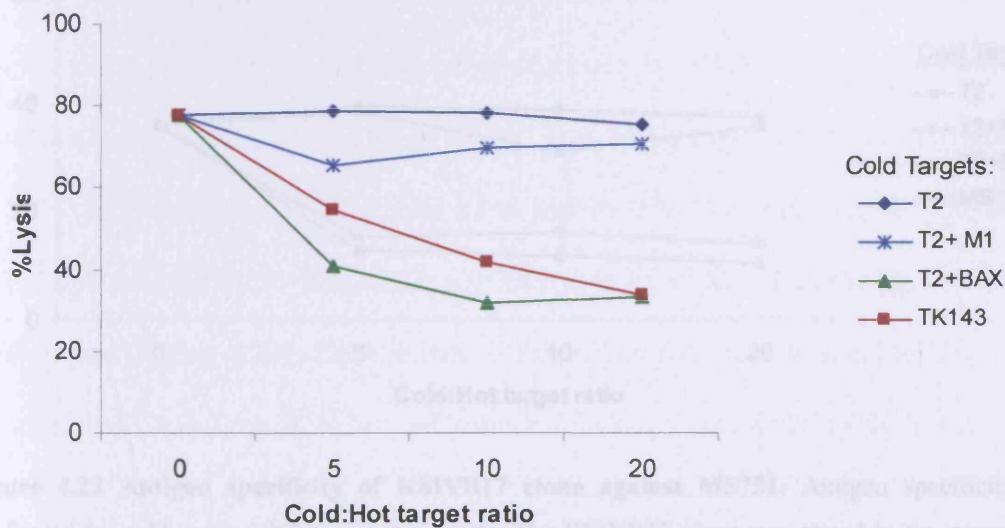


Figure 4.21 Antigen specificity of KSIVB17 clone against TK143. Antigen specificity was confirmed by cold target inhibition experiments. The KSIVB17 clone was tested in the presence of unlabelled cold targets: TK143 (■), T2 alone (◆) or pulsed with the cognate (Bax p613) (▲) or an irrelevant peptide (Flu M1) (*) at various cold:hot target ratios (5:1, 10:1 and 20:1) and incubated with ^{51}Cr labelled TK143 (hot target). The KSIVB17 clone was also tested against ^{51}Cr labelled T2+Flu M1 without any addition of cold targets. No specific killing (4%) was found against T2+ Flu M1 (data not shown). Cytotoxicity was measured in a ^{51}Cr release assay. Results shown are at an E:T ratio of 50:1 and are expressed as mean of triplicate samples.



Figure 4.22 Antigen specificity of KSIVB17 clone against MS751. Antigen specificity was confirmed by cold target inhibition experiments. The KSIVB17 clone was tested in the presence of unlabelled cold targets: MS751 (■), T2 alone (*) or pulsed with the cognate (Bax p613) (▲) or an irrelevant peptide (Flu M1) (◆) at various cold:hot target ratios (5:1, 10:1 and 20:1) and incubated with ^{51}Cr labelled MS751 (hot target). Cytotoxicity was measured in a ^{51}Cr release assay. Results shown are at an E:T ratio of 40:1 and are expressed as mean of triplicate samples.

The hypothesis being tested predicts that Bax peptides are being presented by HLA-A2 on the tumour cells. Since these tumour cells were allogeneic, it was possible that the lysis of the tumour cells was due to alloreactivity rather than HLA-A2 restricted recognition. Therefore MHC restriction of the tumour cell recognition was tested by blocking experiments using an anti- HLA-A2 antibody (Ma2.1) and two negative controls, anti-HLA-DR (L243) and anti-HLA-A23/24 antibody. T2 pulsed Bax p613 and TK143 were tested in present of MA2.1 and L243 antibodies. MS751 was tested with the HLA-A23/24 antibody as a negative control because both donor 11 and this tumour cell line are HLA-A24+. As shown in Figure 4.23, incubation of peptide-pulsed T2 or TK143 with MA2.1 resulted in the inhibition of the target cell lysis, whereas incubation with L243 had no effect. A similar result was seen with MS751. Lysis of MS751 by KSIVB17 was inhibited by Ma2.1, but not by the anti-HLA-23/24 antibody (Figure 4.24). These results confirm that Bax peptide specific CTL response is restricted to HLA-A2, and that lysis of tumour cells is also restricted to HLA-A2.

Together, the cold target competition and antibody blocking results, suggest that the KSIVB17 T cell clone is recognising Bax peptide presented by HLA-A2 on the tumour cells.

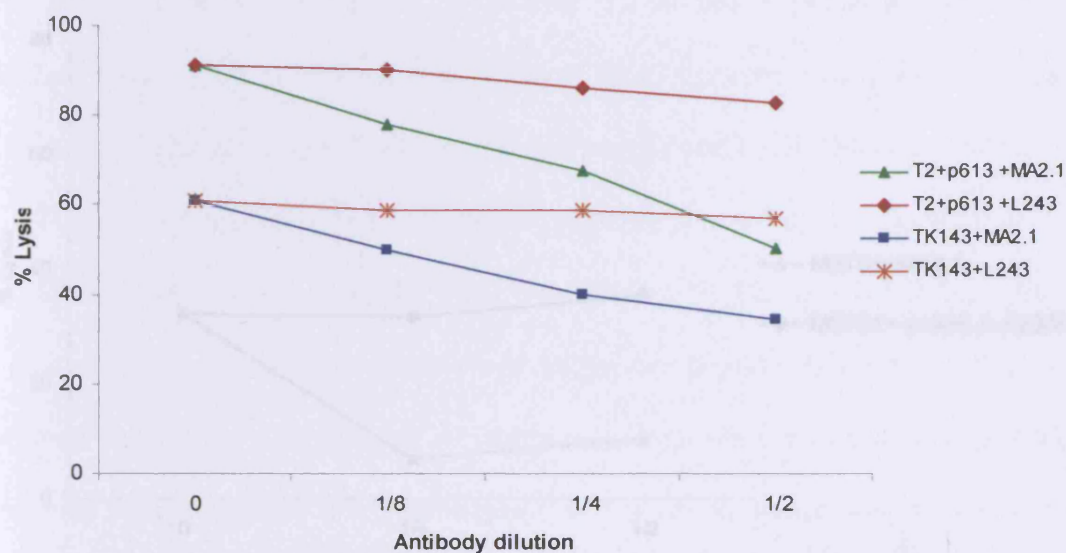


Figure 4.23 Anti-HLA-A2 antibody inhibition of TK143 and Bax-pulsed T2 lysis by KSIVB17 clone. HLA-A2 restriction was confirmed by antibody blocking experiments. This was done by incubating the ^{51}Cr labelled TK143 and T2 pulsed with p613 with or without different dilutions of HLA-specific antibodies (Ma2.1:HLA-A2 and L243:HLA-DR) for 30 minutes before adding KSIVB17 cells. Lysis of Bax p613-pulsed T2 + Ma2.1 antibody (▲), Bax p613-pulsed T2 + L243 antibody (◆), TK143 + Ma2.1 antibody (■) and TK143 + L243 antibody (*) are shown. Cytotoxicity was measured in a ^{51}Cr release assay. Results shown are at an E:T ratio of 40:1 and are expressed as mean of triplicate samples.

4.7 The effect of proteasome inhibition on MHC class I presentation of Bax peptides in KSIVB17 cells.

Another prediction of the hypothesis is that blocking proteasome function will prevent generation of Bax peptides on cancer cells. To test this, a proteasome inhibitor called bortezomib (also known as Velcade, PS-341) was used. Bortezomib is a reversible inhibitor of both standard proteasomes and immunoproteasomes (Vocals et al., 2006).

The tumour cell lines with the highest percentage of killing by the KSIVB17 were MS751, TK143 and C33A-HPV16 (see Table 4.1). T2 mixed with Bax p63 was used as a negative control as presentation of exogenous peptide should not be affected by proteasome inhibition. The lysis of all the tumour cell lines was partially inhibited by bortezomib, suggesting that proteasome plays a role in the generation of the Bax p63 epitope in these tumour cells. Lysis of T2 with exogenous peptide was not affected by the treatment with proteasome inhibitor (Figure 4.25). MS751 was then further tested in the

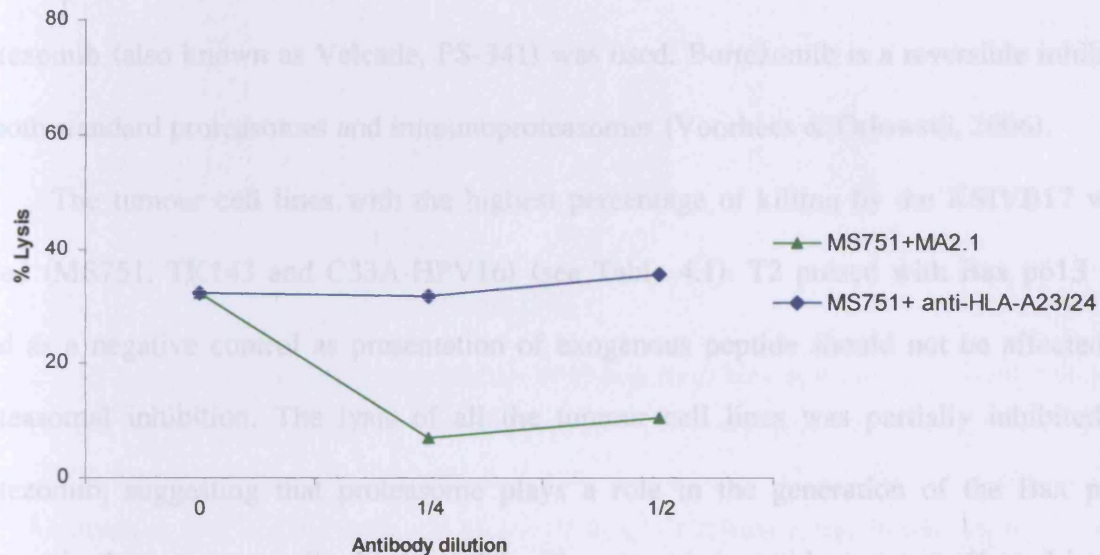


Figure 4.24 Anti-HLA-A2 antibody inhibition of MS751 lysis by KSIVB17 clone. HLA-A2 restriction was confirmed by antibody blocking experiments. This was done by incubating the ^{51}Cr labelled MS751 with or without different dilutions of HLA-specific antibodies (Ma2.1 and anti-HLA-23/24) for 30 minutes before adding KSIVB17 cells. Lysis of MS751 + Ma2.1 antibody ($\blacktriangle$) and MS751 + anti-HLA-23/24 antibody ($\blacklozenge$) are shown. Cytotoxicity was measured in a ^{51}Cr release assay. Results shown are at an E:T ratio of 40:1 and are expressed as mean of triplicate samples.

4.7 The effect of proteasome inhibition on MHC class I presentation of Bax peptides to KSIVB17 clone

Another prediction of the hypothesis is that blocking proteasome function will prevent generation of Bax peptides on cancer cells. To test this, a proteasome inhibitor called bortezomib (also known as Velcade, PS-341) was used. Bortezomib is a reversible inhibitor of both standard proteasomes and immunoproteasomes (Voorhees & Orlowski, 2006).

The tumour cell lines with the highest percentage of killing by the KSIVB17 were tested (MS751, TK143 and C33A-HPV16) (see Table 4.1). T2 pulsed with Bax p613 was used as a negative control as presentation of exogenous peptide should not be affected by proteasomal inhibition. The lysis of all the tumour cell lines was partially inhibited by bortezomib, suggesting that proteasome plays a role in the generation of the Bax p613 epitope in these tumour cells. Lysis of T2 with exogenous peptide was not affected by the treatment with proteasome inhibitor (Figure 4.25). MS751 was then further tested in the presence of different concentrations of bortezomib (Figure 4.26). Inhibition of MS751 lysis was seen with all the concentrations of the proteasome inhibitor tested, but this inhibition was reversed by adding exogenous peptide (Bax p613). This suggests that endogenous Bax peptides can be generated by the proteasome pathway in tumour cells lines.

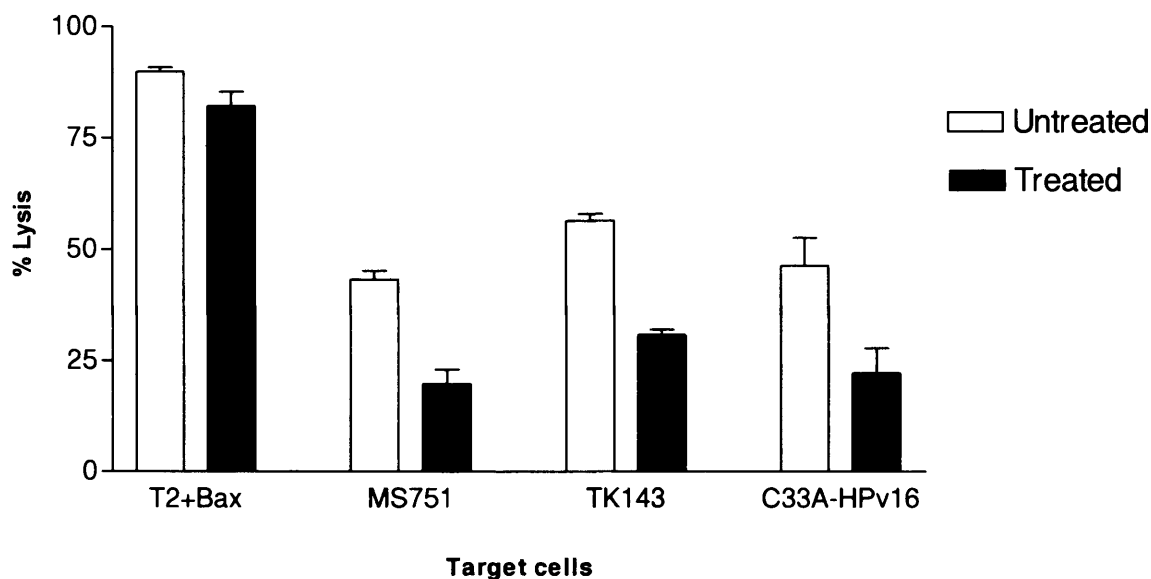


Figure 4.25 Bortezomib inhibits presentation of endogenous Bax epitopes in tumour cell lines. KSIVB17 clone was assayed against ^{51}Cr -labelled T2 pulsed with Bax p613, MS751, TK143 and C33A-HPV16 which were either untreated (white bars) or treated with $0.1\mu\text{M}$ of bortezomib (grey bars) overnight at 37°C . Cytotoxicity was measured in a ^{51}Cr release assay. Results shown are at an E:T ratio of 30:1 and are expressed as the mean of triplicate samples.

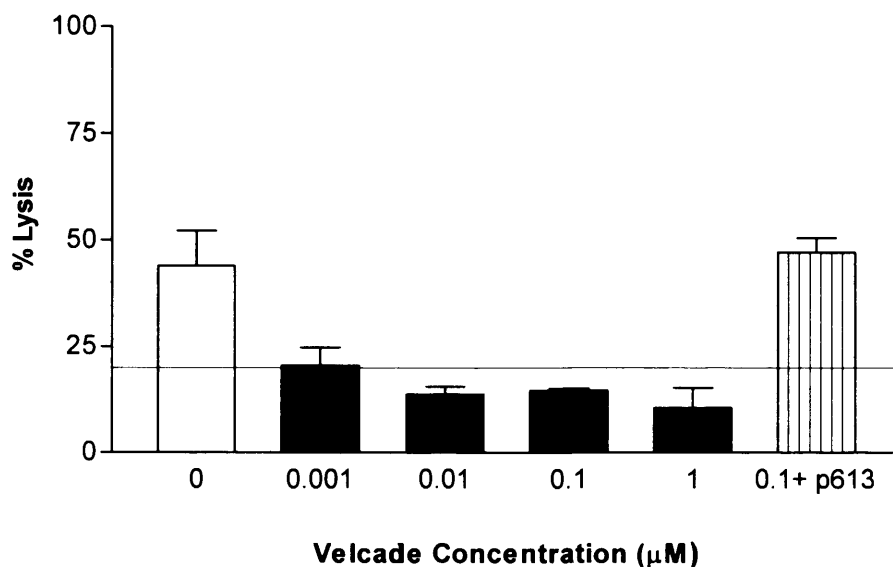


Figure 4.26 Presentation of endogenous Bax epitopes in MS751 is blocked with various concentrations of the proteasome inhibitor Bortezomib. KSIVB17 clone was assayed against ^{51}Cr -labelled MS751 untreated (white bar) or treated with various concentrations of bortezomib (μM) overnight at 37°C . As a control, MS751 was treated with $0.1\mu\text{M}$ of velcade and pulsed with Bax p613 (stripy bar). Cytotoxicity was measured in a ^{51}Cr release assay. Results shown are at an E:T ratio of 30:1 and are expressed as the mean of triplicate samples. The dashed line represents the 20% lysis cut-off for a positive result.

4.8 Discussion

In the first part of this chapter, characterisation of the T cell lines confirmed that it was possible to generate stable populations of T cells specific for Bax peptides from low frequency starting populations. It was shown that these T cell lines increased their specificity against Bax peptides after immunomagnetic enrichment. Based on the epitope mapping experiments and analysis of TCR V β usage, culturing cells under limiting dilution conditions clearly selected T cells with particular TCRs and specificity towards restricted epitopes.

Both the JSBI line and JSBI 10E6 were predominantly TCR V β 14+ and recognise Bax p3 (IMGWTLDFL) as a dominant epitope. However, the JSBI 10E6 were 98.5% TCR V β 14+ and had a higher magnitude of response against Bax p3, confirming that the culturing T cells under limiting dilution conditions was a superior approach to selectively enrich peptide-specific T cells. This also indicated that the TCR V β 14 was likely to be involved in the recognition of Bax p3 in this donor.

KSI 10B7, which was generated from a different donor (donor11), was found to be an oligoclonal T cell line, containing two main T cell populations: a V β 5.1+ and a V β 17+ population. It was able to recognise the same Bax peptide IMGWTLDFL (Bax p613) from Bax 601-23 pool, but interestingly it also reacted against a related peptide, the 10-mer TIMGWTLDFL (Bax p610) at higher peptide concentrations. In contrast, KSI line had similar percentages of TCR V β chains to the T cells from pre-sort and contained multiple antigen-specific reactivities. A way to further study the KSI line is to sort this polyclonal T cell line using individual peptides rather than entire Bax 601-23 pool and culture them under limiting dilution conditions, thus narrowing down the selection of highly specific T cells responding to individual peptides. But this would be a very labour intensive approach. Results from this donor suggested that 7-8 Bax peptides were immunogenic to T cells. Interestingly, the KSI line was able to recognise Bax p615 that is predicted to bind to HLA-B44. T2 cells were used as APC in ELIspot assays, and these express HLA-A2 and have

extremely low expression of HLA-B5. The recognition of p615 could be explained by T cells from donor 11 presenting Bax p615 to each other (HLA type of donor 11: HLA-A2, A24, B44, B60, Bw4, Bw6, Cw5, Cw20). Alternatively, this could be due to cross-reactive binding of p615 to HLA-B5+. It would be interesting to test the immunogenicity of this peptide with T cells from HLA-A2-, HLA-B44+ or HLA-B5+ individuals.

JSBI 10E6, KSI line, KSI 10B7 (and KSIVB17) were further shown to be cytotoxic against T2 cells pulsed with exogenous Bax peptides, and thus proven that these T cells have lytic efficacy towards Bax peptides. The avidity of the KSIVB17 (TCR V β 17+ clone) for both Bax p613 and p610 was determined from peptide dose-response assays. The first set of results showed that KSIVB17 had higher avidity for Bax p613 than Bax p610. As shown in the Chapter 3, Bax p613 showed a measurable binding capacity to HLA-A2 (115% increase of HLA-A2 expression) whereas Bax p610 showed only a 5% increase of HLA-A2 expression, indicating that Bax p613 is a stronger binder to HLA-A2 (see Figure 3.8). Several studies have shown that T cell clones can recognise multiple peptides (Hagerty & Allen, 1995; Loftus *et al.*, 1996). Since the sequences of two peptides are almost identical, it is possible that T cells can cross-react on the two peptides because they present a similar structure to the TCR once they are complexed to HLA-A2. The stronger response against Bax p613 may be caused by its higher affinity towards HLA-A2, resulting in more stable peptide:MHC complex. Another explanation for recognition of both peptides is that there are two TCR V β 17+ subpopulations, one recognising the Bax p610 and the other Bax p613. This possibility could be tested by sequencing of the TCR chains using PCR, and thus would confirm whether only one TCR V β 17+ population exists.

The possibility of KSIVB17 recognising a common contaminant was considered because the purity of both synthetic peptides was <70%. This possibility seems unlikely because new peptides with purity >90% were also being recognised and with higher avidity than the unpurified peptides. However, since the purity is not 100%, there is always a slight

chance that KSIVB17 could be recognising p613 only, but this is a contaminant of Bax p610. The results using pure peptides confirmed that KSIVB17 had higher avidity for Bax p613 (0.5nM for half-maximal lysis) than Bax p610 (10.9nM). This is higher than the figures obtained for T cells against other HLA-A2 associated epitopes, such as gp100 (946) peptide (1 to 10pM) (Cox *et al.*, 1994) and HPV16 E7 11-20 peptide (10pM) (Youde *et al.*, 2000). However, it is similar or lower than the concentrations required for half-maximal lysis of some other HLA-A2-associated peptide epitopes such as WT1-derived peptide WT126 (10nM) (Bellantuono *et al.*, 2002), Melan-A/Mart1 A27L analogue (0.4 to 113nM) (Palermo *et al.*, 2001), NY-ESO-1 157-165 (50nM) (Chen *et al.*, 2000) and MUC-1 derived peptides (10 to 100nM) (Brossart *et al.*, 1999). Thus, it can be said the avidity of KSIVB17 clone for Bax p610 is low and for Bax p613 is intermediate, comparable to the avidity of some other well-known tumour CTLs capable of recognizing endogenously processed tumour antigens.

The KSIVB17 clone was able to lyse targets pulsed with Bax peptides and recognise HLA-A2+ tumour cells of various types, including CLL, HPV transformed cervical, osteosarcoma, and hepatocellular carcinoma cells. Based on these results, it appears these cancer cells are effective in processing and presenting the same endogenous Bax peptides. However, these results need to be confirmed with T cell clones from other donors. Formal validation of Bax being processed and presented will require elution of naturally occurring peptides associated with MHC molecules from the tumour cells and their analysis by mass spectrometry.

Killing of tumour cells by the KSIVB17 clone was antigen-specific and HLA-A2 restricted as shown by a number of control experiments, including cold-target competition and monoclonal antibody blocking experiments. The lack of killing of the K562 cells indicated that the cytotoxic activity against tumour cells was not NK cell mediated.

Bax-specific responses mediated by KSIVB17 were seen against HLA-A2+ primary CLL cells and no HLA-A2- CLL cells using an ELISpot. These observations indicate that the

primary CLL cells can naturally process and display immunogenic peptides derived from Bax, and thus induce functional responses by Bax-specific T cells. The findings that abnormal degradation of Bax occurs in CLL cells (Agrawal *et al.*, 2008; Liu *et al.*, 2008) and Bax-specific T cell responses were detected against CLL cells seem to support the hypothesis proposed in this study. However, to further address this hypothesis, it will be required to evaluate T cell responses against Bax in CLL patients. In contrast to several solid tumours, there are only relatively few tumour-associated antigens that have been identified for CLL and thus defining new tumour antigens with a role in cancer is desirable. Bax is an attractive tumour antigen for CLL because of its reduced expression is linked to resistance to chemotherapy and apoptosis of tumour cells, thereby playing a crucial role in cancer progression. Interestingly, other lymphoid malignant cell lines were not killed by the Bax-specific T cells, indicating that the Bax-specific T cells may have a particular specificity towards CLL and not to the other lymphoid malignancies.

Although it has been shown that abnormal degradation of Bax occurs in advanced PCa patients (Li & Dou, 2000), PCa cell lines (LNCaP, HPV10) were not lysed by the Bax-specific T cells. The study carried out by Li and Dou (2000) used PCa tissue samples and not tumour cell lines. Therefore, to support the hypothesis proposed here, tumour cells from biopsy samples of PCa patients would have been ideal to test for KSIVB17 recognition, but unfortunately, no PCa samples were available for this study. Breast cancer (MDA231) and melanoma (SK29mel) were also not lysed by the KSIVB17 clone. It is possible that Bax p613 and Baxp610 epitopes are not naturally processed and presented on the cell surface of these tumour cells or they might be present but at too low a level for detection by the CTLs.

Interestingly, the KSIVB17 clone was able to lyse HPV-transformed cervical cancer cell lines, Caski (HPV16), C33A-HPV16 and MS751 (HPV45), but not a HPV negative cervical cancer cell line C33A. The Bax specific cytotoxic responses towards HPV positive cervical tumour cells suggest that these responses are related with HPV expression as the

HPV negative C33A was not killed. HPV16 E6 expression has been shown to shorten the half-life of Bax protein in differentiating keratinocytes. In addition, expression of E6 has been demonstrated to enhance Bax degradation and inhibit Bax-induced apoptosis in stable and transiently E6 expressing human cells (Magal *et al.*, 2005). The results obtained here demonstrate that some HPV transformed cervical cancer cell lines can process and present Bax epitopes associated with MHC class I molecules for KSIVB17 recognition. It is possible that this is a result of HPV E6 expression.

However, KSIVB17 did not kill SiHa-A2 cells, which is an HPV16 transformed cell line. Many of HPV16 E6 interacting proteins have been shown to be targets of E6 dependent ubiquitin-proteasomal degradation, including p53 (Scheffner & Whitaker, 2003). Previous studies demonstrated that SiHa cells have the highest steady-state levels of p53 because SiHa cells contain only one to two copies of HPV16 per cell, compared with other HPV-transformed cell lines, such as CaSki cells containing 600 copies of HPV16 (Ding *et al.*, 2007; Scheffner *et al.*, 1991). Therefore, it is possible that SiHa-A2 was not killed by KSIVB17 because the low copy number of HPV16 E6 meant that not enough Bax was being targeted for degradation and thus less Bax peptides were being generated and presented at the cell surface.

Unexpectedly, Bax-specific CTLs were also able to efficiently lyse osteosarcoma cell lines (TK143, U2OS, Saos-2) and one hepatocellular cancer cell line (HepG2). It has been reported that reduced expression of Bax is associated with poor response to treatment and shorter survival in osteosarcoma and hepatocellular carcinoma patients, respectively (Garcia *et al.*, 2002; Mintz *et al.*, 2005). This underscores the essential role of Bax in tumour development and survival. However, these studies did not demonstrate whether Bax is degraded by the proteasome. Expression can be altered in many ways, including mutations and transcription. Therefore, it will be important to test whether proteasomal degradation of Bax occurs in all the different cancer cell lines recognised by KSIVB17.

More importantly, the KSIVB17 clone did not lyse any of the healthy controls, including autologous and allogeneic PHA blasts, and allogeneic healthy skin fibroblasts. These results are in agreement with the proposed hypothesis. In healthy cells, Bax has a long half-life and predominantly localized in cytosol or loosely attached to outer mitochondrial membranes in an inactive form and may not be efficiently targeted by the proteasome. So, it is expected that there would not be substantial quantities of Bax-derived peptides for MHC class I binding. Possibly, the quantity of Bax peptides generated in normal cells is insufficient to mediate lysis by CTL. The preferential lysis of tumour cells but not normal cells by KSIVB17 might be related to the affinity of the T cells and higher presentation of Bax-derived peptides by tumour cells. Another explanation might be that normal cells present a different repertoire of T cell epitopes compared with tumour cells, and the Bax p613 and Baxp610 epitopes are not naturally processed and presented on the cell surface of normal cells. This leads to the assumption that there are differences in the processing and presentation of endogenous Bax peptides by tumour cells and normal cells. This could be confirmed by the elution of natural processed peptides complexed with MHC class I molecules from the tumour cells and normal cells, and analysis of their sequences by mass spectrometry.

Moreover, the proteasome pathway was shown to be involved in the generation of endogenous Bax peptides in tumour cells. This was demonstrated by inhibition of tumour cell lysis after treatment of tumour cells with proteasome inhibitor (bortezomib), even at relatively low concentrations. No inhibition of peptide pulsed target T2 cells was seen, ruling out any non-specific toxicity effect. Further experiments will include analysis of the effect of different doses of proteasomes inhibitor on T cell recognition of all the other tumour cells that were found to be recognised by the Bax-specific T cells.

A report carried out by Maia *et al.* (2005) has shown that Bax- δ (143a.a), an isoform resulting from alternative splicing of Bax, can act as a potential tumour antigen in acute

lymphoblastic leukemia. However, the functional significance of this isoform remains unclear (Akgul *et al.*, 2004). The epitopes identified from Bax- δ (143a.a) by that group do not correspond to the epitopes from Bax- α (192 a.a) investigated here in this study. Bax- α is the best-characterised isoform and is essential in induction apoptosis (Akgul *et al.*, 2004; Nechushtan *et al.*, 1999; Wolter *et al.*, 1997). To our knowledge, this is the first study that demonstrates Bax- α protein as a potential tumour antigen for various cancers of different origins and types.

Collectively, this study presents the first identification of antigenic peptide epitopes within the Bax protein based on its abnormal expression in cancer. The data reported herein provide evidence that Bax peptides are naturally processed by several tumour types and are presented in the context of HLA-A2 in sufficient quantities to allow recognition by Bax-specific T cells. It was demonstrated that two HLA-A2-binding epitopes (Bax p610 and Bax p613) derived from Bax can elicit epitope-specific CTL responses against tumour cells. In conclusion, these data revealed that peptides derived from the ubiquitously expressed Bax protein are immunogenic to human CD8⁺ T cells and support the concept that Bax could serve as a potential tumour antigen for immunotherapy of several human cancers.

Overall, the data presented in this chapter supports the hypothesis, but to strengthen these findings it would be necessary to show whether increased proteasomal degradation of Bax occurs in cancer cells. This will be addressed in the next chapter.

Chapter 5

Analysis of Bax expression in cancer cells

In the last chapter, it was revealed that Bax peptides can be naturally processed and presented by several tumour types for recognition by Bax-specific T cells. Here, it will be investigated whether T cell reactivity correlates with Bax expression and its degradation in cancer cells. Based on the hypothesis, there should be an increased proteasomal degradation of Bax in cancer cells, but not normal cells, resulting in these being recognised by Bax-specific T cells. Therefore, Bax expression will be analysed in PCa cell lines, CLL patient samples, cervical cancer cell lines, osteosarcoma and normal (non-cancerous) cells by western blotting. In order to assess the expression of Bax protein and its degradation by proteasomes, the cells were treated with proteasome inhibitors.

In this study, two proteasome inhibitors were used, clasto-lactacystin and bortezomib. At the beginning of this project, the cells (PCa, CLL and healthy donors) were tested with clasto-lactacystin, but this proteasome inhibitor was changed later to bortezomib. Clasto-lactacystin is a specific, but metabolically unstable proteasome inhibitor, and thus is only used for studying proteasomal processes *in vitro*. While, Bortezomib is a specific proteasome inhibitor and the first proteasome inhibitor approved for human applications, including treatment of multiple myeloma and continues to be studied in various haematological malignancies and solid tumours (Voorhees & Orlowski, 2006).

5.1 Bax expression in human PCa cell lines

Four PCa cell lines (see Table 2.1) were used to analyse steady-state Bax expression by western blotting. LNCaP is a metastatic androgen sensitive PCa cell line, CA-HPV10 is a less aggressive non-metastatic, androgen sensitive PCa cell line, and PC3 is androgen resistant, and derived from aggressive PCa. DU145 (androgen resistant, metastatic cell line) was used as a negative control as it is a Bax null cell line. Analysis of these PCa cell lines demonstrated that there was a differential expression of Bax levels as detected by western blotting (Figure 5.1). Both CA-HPV10 and LNCaP showed increased full-length Bax protein (21 kda, Bax α) levels, compared to PC3. Lower levels of Bax expression in PC3 were revealed by western blotting. Proteasome inhibition slightly increased Bax expression in advanced PCa cell line PC-3 and CA-HPV10. However, no increase was seen in LNCaP after treatment. As expected, no Bax expression was detected in DU145 (negative control).

5.2 Bax expression in CLL patients

Bax expression and its degradation were determined in fresh peripheral blood samples from 19 CLL patients, using the proteasome inhibitor clasto-lactacystin (2 μ M and 10 μ M). Figure 5.2 shows representative immunoblots of ten CLL patients. A slight upregulation of the full length Bax protein (21 kda) was detected in 11/19 CLL patients, after treatment with clasto-lactacystin. However, accumulation of a lower molecular weight form of Bax (18 kda) was clearly seen in most CLL patients (18/19) after clasto-lactacystin treatment. The degree of accumulation varied among these samples. Only one CLL patient (CLL 6) did not show detectable levels of this product after proteasome inhibition. The molecular weight of this lower molecular form of Bax corresponds to a truncated version of Bax (p18 Bax) that is formed during apoptosis by calpain cleavage of the full-length form (p21 Bax) (Wood *et al.*, 1998). The results suggest that active proteasomal degradation of Bax p18 cleavage product is occurring in most CLL patients.

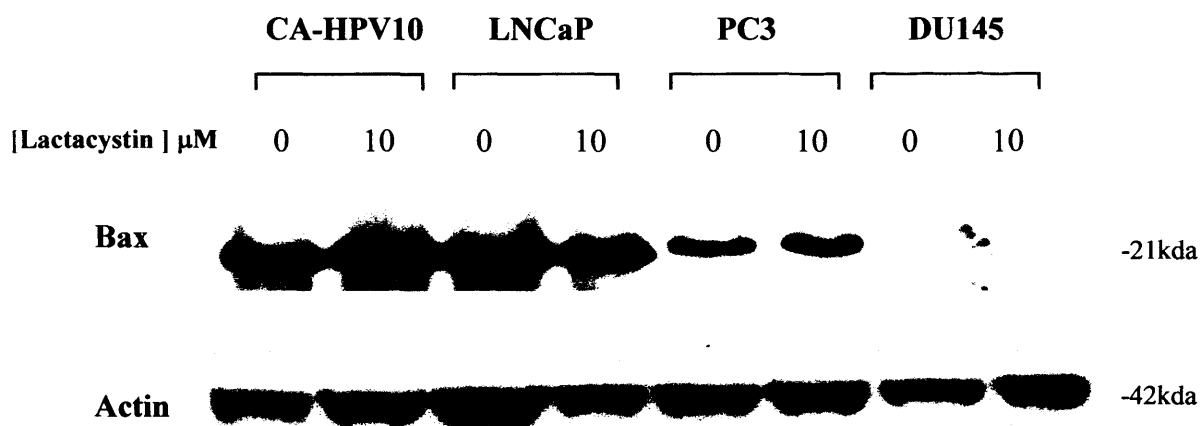


Figure 5.1 Expression of Bax in PCa cell lines in the presence or absence of the proteasome inhibitor, clasto-lactacystin. PCa cells (CA-HPV10, LNCaP, PC3, and DU145) were treated with or without the proteasome inhibitor clasto-lactacystin (10 μ M) overnight, followed by preparation of whole cell lysates. These were then analysed by western blotting for the expression of Bax. The blots were incubated with specific antibodies to Bax (21 kda) at 1/1000 dilution and Actin (42 kda; loading control) at 1/10000 dilution.

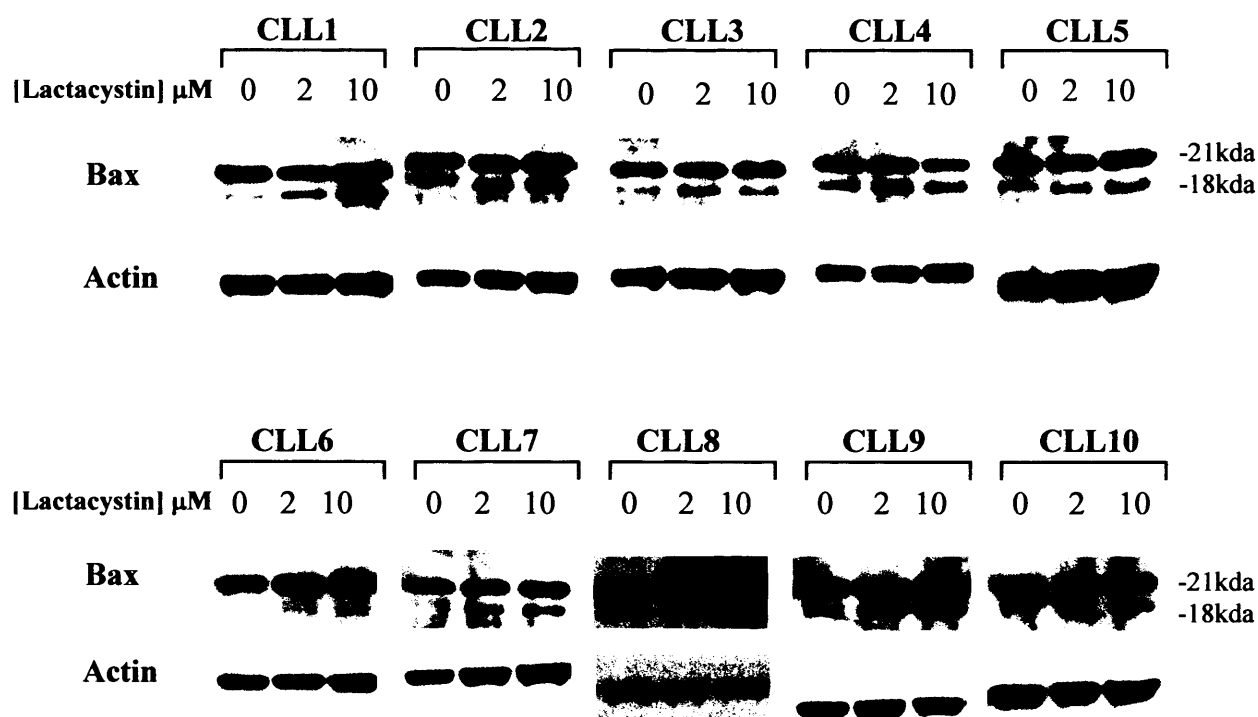


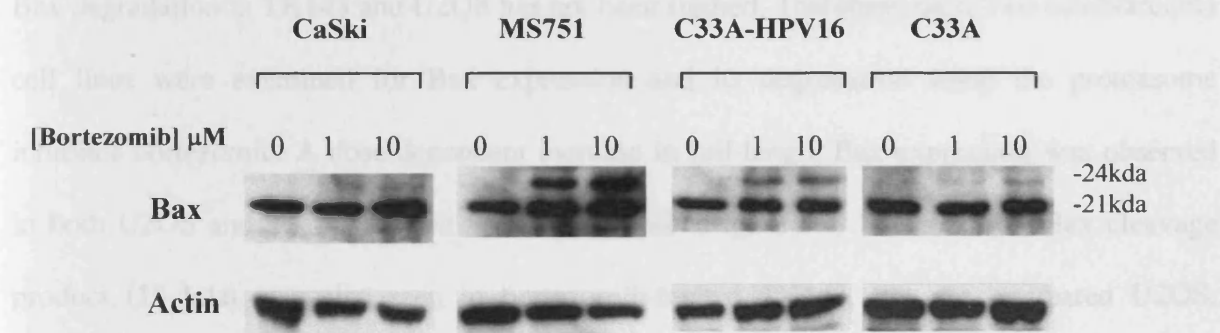
Figure 5.2 Representative immunoblots showing expression of Bax in CLL cells in the presence or absence of the proteasome inhibitor, clasto-lactacystin. CLL cells were treated with or without clasto-lactacystin (2μM and 10 μM) overnight, followed by preparation of whole cell lysates. These were then analysed by western blotting for the expression of Bax. The blots were incubated with specific antibodies to Bax (21kda and 18kda) at 1/1000 dilution and Actin (42 kda; loading control) at 1/10000 dilution.

5.3 Bax expression in cervical cancer cell lines

It has previously been reported that transfection of HPV16 E6 into human fibroblasts resulted in reduced Bax mRNA and protein expression. The reduction in protein appeared to be related to increased degradation of Bax (Magal *et al.*, 2005). However, Bax expression in cervical cancer cell lines was not studied. Therefore, four cervical cancer cell lines CaSki (HPV16 metastatic cell line), MS751 (HPV45 metastatic cell line), C33A-HPV16 and C33A (HPV negative cell line) were examined for their Bax expression and degradation by the proteasome, using the proteasome inhibitor bortezomib (Figure 5.3). Dose-dependent accumulation of Bax (21kda, Bax α) was seen in CaSki and MS751 treated with bortezomib. Interestingly, an additional protein of approximately 24kda was detected after proteasome inhibition particularly in MS751, and C33A-HPV16. It has been recently demonstrated that this protein corresponds to the isoform Bax β (Fu *et al.*, 2009). As for C33A, no significant increase of Bax α and only a slight accumulation of Bax β were seen after treatment. These results indicate that both Bax isoforms are degraded by the proteasome in HPV-transformed cell lines. No cleavage to Bax p18 form was observed in any of the cervical cancer cell lines.

5.4 Bax expression in osteosarcoma cell lines

Poor prognosis of patients has been associated with apoptosis resistance in osteosarcoma. A decrease in Bax has been observed in osteosarcoma tumours with poor histologic response to chemotherapy [Matsuda et al., 2005]. Bax degradation by the proteasome has been shown in one of the osteosarcoma cell lines, U2OS [Kang et al., 2005]. However, Bax degradation by 15 and 420S proteasomes in other osteosarcoma cell lines were not examined.



Overall, this data show that Bax is degraded by the proteasome in these osteosarcoma cell lines.

Figure 5.3 Expression of Bax in cervical cancer cell lines in the presence or absence of the proteasome inhibitor, bortezomib. Cervical cancer cell lines (CaSki, MS751, C33A-HPV16 and C33A) were treated with or without bortezomib (1 μ M and 10 μ M) overnight, followed by preparation of whole cell lysates. These were then analysed by western blotting for the expression of Bax. The blots were incubated with specific antibodies to Bax (24kda and 21kda) at 1/1000 dilution and Actin (42 kda; loading control) at 1/1000 dilution.

Western blots for Bax expression and potential degradation by the proteasome (Figure 5.3).

Full-length Bax (21kda) expression did not seem to significantly increase with inhibition of

the proteasome activity in all the donors. HD7 was the only donor where a slight increase of

the full-length Bax protein after proteasome inhibition. Low levels of Bax cleavage product

(24kda) were detected without treatment in PBMC of five donors HD1, HD2, HD6, HD7 and

HD9) and the PBMC donor. However, no accumulation of the cleavage product was seen in the

majority of donors when treated with the proteasome inhibitor, with exception of PBMC from

HD1, which showed a modest increase from a very low initial expression. Overall, this data

indicates that Bax protein expression in these cells is usually not regulated by the proteasome

pathway.

5.4 Bax expression in osteosarcoma cell lines

Poor prognosis of patients has been associated with apoptosis resistance in osteosarcoma. A decrease in Bax has been observed in osteosarcoma tumours with poor histologic response to chemotherapy (Mintz *et al.*, 2005). Bax degradation by the proteasome has been shown in one of the osteosarcoma cell lines, Saos-2 (Chang *et al.*, 1998). However, Bax degradation in TK143 and U2OS has not been studied. Therefore, these two osteosarcoma cell lines were examined for Bax expression and its degradation using the proteasome inhibitor bortezomib. A dose-dependent increase in full-length Bax expression was observed in both U2OS and TK143 treated with bortezomib (Figure 5.4). Detection of Bax cleavage product (18 kda) was also seen in bortezomib-treated TK143, but not in treated U2OS. Overall, this data show that Bax is degraded by the proteasome in these osteosarcoma cell lines.

5.5 Bax expression in normal (non-cancerous) cells

PBMC from nine healthy donors and PHA blasts generated from one healthy donor were examined for Bax expression and potential degradation by the proteasome (Figure 5.5). Full-length Bax (21kda) expression did not seem to significantly increase with inhibition of the proteasome activity in all the donors. HD7 was the only one to show a slight increase of the full-length Bax protein after proteasome inhibition. Low levels of Bax cleavage product (p18) were detected without treatment in PBMC of five donors (HD4, HD5, HD6, HD7 and HD9) and the PHA blasts. However, no accumulation of the cleavage product was seen in the majority of donors when treated with the proteasome inhibitor, with exception of PBMC from HD1, which showed a modest increase from a very low initial expression. Overall, this data indicates that Bax protein expression in these cells is usually not regulated by the proteasome pathway.

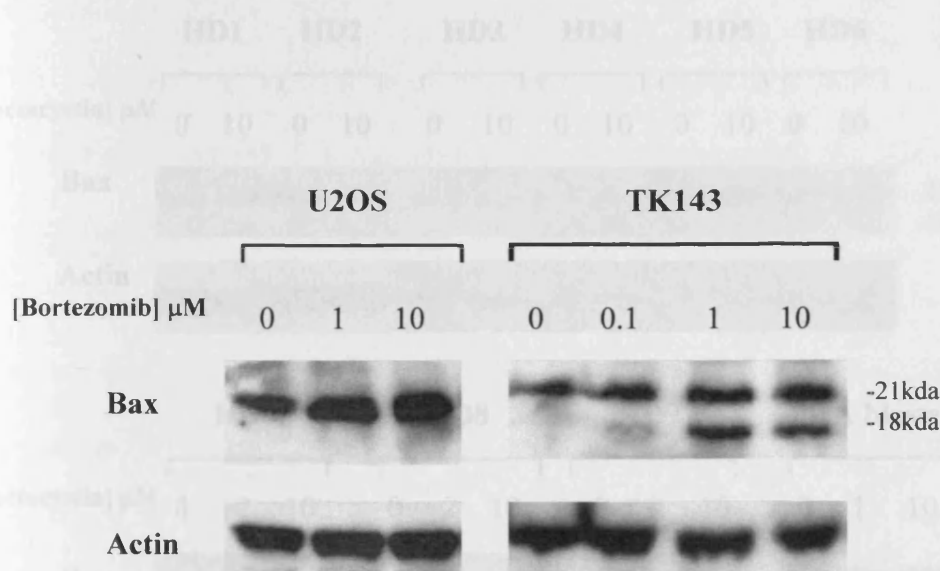


Figure 5.4 Expression of Bax in osteosarcoma cell lines in the presence or absence of the proteasome inhibitor, bortezomib. Osteosarcoma cell lines (U2OS and TK143) were treated with or without bortezomib (1 μM and 10 μM for U2OS; 0.1 μM , 1 μM and 10 μM for TK143) overnight, followed by preparation of whole cell lysates. These were then analysed by western blotting for the expression of Bax. The blots were incubated with specific antibodies to Bax (21kda and 18kda) at 1/1000 dilution and Actin (42 kda; loading control) at 1/10000 dilution.

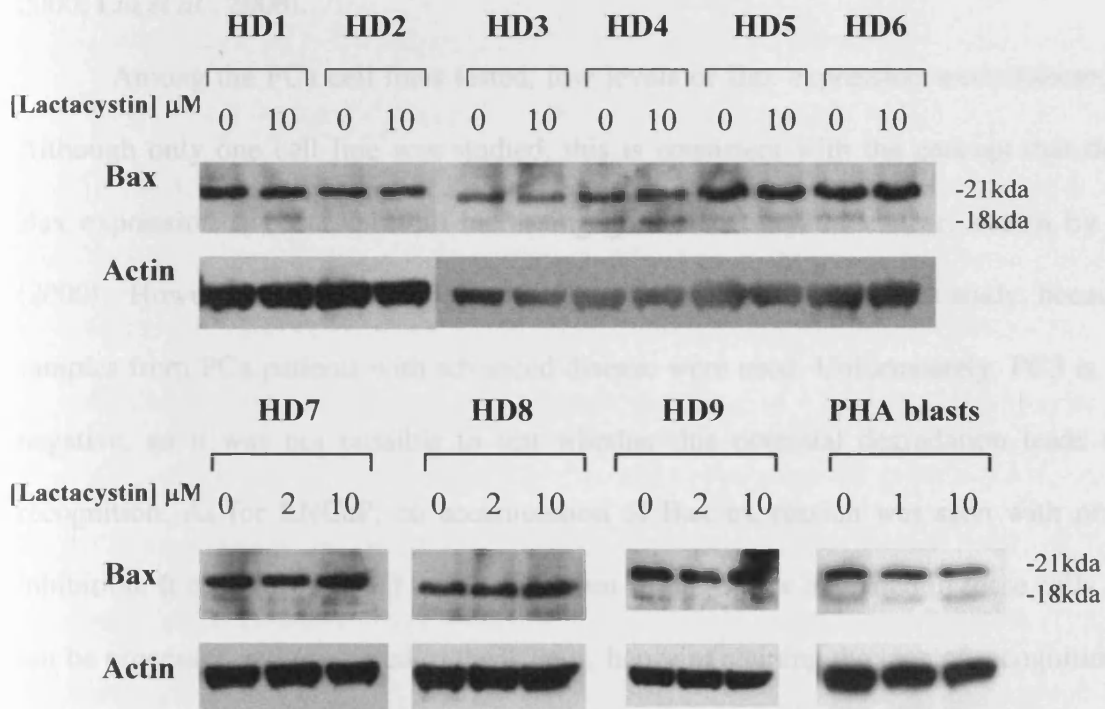


Figure 5.5 Expression of Bax in normal cells in the presence or absence of the proteasome inhibitor, clasto-lactacystin. Fresh PBMC from nine healthy donors were treated with or without clasto-lactacystin (2 μM and 10 μM) overnight. PHA blasts from a healthy donor were generated by culturing PBMC with PHA (10 $\mu\text{g}/\text{ml}$) for 3 days and treated with or without clasto-lactacystin (1 μM and 10 μM) overnight, followed by preparation of whole cell lysates. These were then analysed by western blotting for the expression of Bax. The blots were incubated with specific antibodies to Bax (21kda and 18kda) at 1/1000 dilution and Actin (42 kda; loading control) at 1/10000 dilution. HD = healthy donor.

5.6 Discussion

In this chapter, Bax degradation by the ubiquitin-proteasome pathway was examined in different cell types. Bax is normally ubiquitously expressed but decreased expression has been observed in various cancer cells. It has been reported that Bax is short-lived in cancer cells and is abnormally degraded by the ubiquitin-proteasome system (Agrawal *et al.*, 2008; Li & Dou, 2000; Liu *et al.*, 2008).

Among the PCa cell lines tested, low levels of Bax expression were detected in PC3. Although only one cell line was studied, this is consistent with the concept that decreasing Bax expression is observed with increasing grade (severity) of cancer, shown by Li *et al.* (2000). However, it is difficult to compare the present results with this study, because tissue samples from PCa patients with advanced disease were used. Unfortunately, PC3 is HLA-A2 negative, so it was not possible to test whether this potential degradation leads to T cell recognition. As for LNCaP, no accumulation of Bax expression was seen with proteasome inhibition. It can be suggested that Bax protein would not be degraded in these cells so would not be processed and presented to the T cells, hence explaining the lack of recognition by Bax specific T cells.

By contrast in CLL patients, the western blot data shows that proteasomal degradation of Bax is enhanced in most CLL cells. These findings are in accordance with previous studies (Agrawal *et al.*, 2008; Liu *et al.*, 2008). Bax is short-lived in malignant B cells and increased Bax degradation activity has been associated with poor prognosis in CLL and resistance to chemotherapy in lymphoid malignancies (Agrawal *et al.*, 2008; Liu *et al.*, 2008; Pepper *et al.*, 1997; Pepper *et al.*, 1998). This Bax degradation activity was seen in CLL cells but not in normal lymphocytes. Correlation between increased apoptosis and accumulation of Bax following treatment with proteasome inhibitors indicates that increased proteasomal degradation of Bax contributes to resistance to apoptosis in cancer cells. The mechanism of Bax degradation is still not clear, but it has been recently suggested that Bax degradation

occurs when Bax is translocated and undergoes conformational changes at the mitochondrial level, probably before its stable mitochondrial membrane insertion. The exposure of Bax hydrophobic domains during its conformational changes triggers ubiquitin recognition and proteasomal degradation (Yu *et al.*, 2008).

A Bax cleavage product (p18) became detectable after treatment, with very low levels of p18 being rescued by proteasome inhibition. It has been previously reported that cleavage of full-length Bax (p21) during apoptosis, to the p18 form may enhance its cell death function (Cao *et al.*, 2003; Wood & Newcomb, 2000). Although cleavage to p18 Bax has been shown not to be necessary for Bax to initiate apoptosis, this cleavage product can accelerate apoptosis and displays a more potent ability to disrupt mitochondrial membrane integrity and promote cell death than p21 Bax (Cao *et al.*, 2003; Wood & Newcomb, 2000). The selective degradation of the cleavage product by proteasome seen in CLL cells may thus be a way to silence this potent form of Bax, delaying or avoiding the apoptosis process. Conversely, some of the tumour cell lines tested (PCa, cervical cancer and U2OS cell lines) did not show detectable levels of the cleavage product with or without proteasome inhibition. This suggests that cleavage of Bax may not be a major mechanism to induce apoptosis in these cell lines.

Bax degradation by the proteasome was also detected in cervical cancer cell lines. Interestingly, increased levels of an additional band (24kda), corresponding to the isoform Bax β were found in MS751 and C33A-HPV16 after proteasomal inhibition, indicates that this isoform is also degraded by the proteasome. This is in agreement with recent findings showing that this isoform is tightly regulated by the ubiquitin proteasome pathway. This study also reported that Bax β is highly potent in inducing cytochrome c release from mitochondria and it can associate with Bax α (21kda) to promote its change to active conformation (Fu *et al.*, 2009)

It is possible that the degradation of Bax in cervical carcinoma cell lines is a consequence of HPV gene expression. Expression of E6 oncoprotein has been reported to

induce Bax degradation and inhibit Bax-induced apoptosis (Magal *et al.*, 2005). Furthermore, HPV E6 can target many cellular proteins including proteins involved in apoptosis, such as Bak, for ubiquitin-proteasomal degradation (Scheffner & Whitaker, 2003). The results in this thesis also support this concept. There was an accumulation of Bax α /Bax β in HPV E6+ cells after treatment with bortezomib, whereas only a slight increase of Bax β with the highest dose of bortezomib was seen in HPV negative C33A. It would be of interest to directly the effect of HPV E6 by transfecting it into cell lines that do not show any proteasomal degradation of Bax.

Osteosarcoma cells TK143 and U2OS also showed degradation of Bax by the proteasome. However, differential expression of Bax was detected between cell lines. TK143, but not U2OS, showed detectable levels of cleavage product after treatment with proteasome inhibitor. Although Saos-2 was not tested in the present study, it has previously been reported by others that Bax is degraded by the ubiquitin-proteasome pathway in these cells (Chang *et al.*, 1998).

Important evidence to support the concept of differential degradation of Bax between tumour cells and normal cells, was that there was no substantial accumulation of Bax in non-transformed cells (PBMC and PHA blasts) after treatment with proteasome inhibitor. This is consistent with previous findings, demonstrating that malignant B cells, but not normal lymphocytes, have a defect in Bax stability (Agrawal *et al.*, 2008). This group found an increased Bax degradation at the mitochondrial level in malignant cells, but not in normal peripheral blood lymphocytes, indicating that Bax in healthy cells is stable and has a long half-life. It was also suggested that ubiquitin recognizes the active form of Bax (Agrawal *et al.*, 2008; Liu *et al.*, 2008). A recent study proposed that the exposure of Bax hydrophobic domains during conformation change and translocation to the mitochondria triggers ubiquitin recognition and proteasome degradation in cancer cells (Yu *et al.*, 2008). In healthy cells, Bax is stable and predominantly localized in cytosol or loosely attached to outer mitochondrial membranes in an inactive state (Nechushtan *et al.*, 1999; Wolter *et al.*, 1997). Therefore, Bax

in healthy tissues does not appear to be efficiently targeted for proteasomal degradation. This is consistent with the hypothesis and explains why healthy cells are not recognized by Bax-specific T cells.

In this study, Bax stability was not tested and only a measurement of Bax protein level at a single time point was done using whole cell lysates. It will be useful to use cytosol and mitochondrial fractions, instead of whole cell lysates, as it will give information about the location of Bax in the different cancer cells and normal cells. Future experiments include the investigation of the stability of Bax and the rate of degradation using cycloheximide in these tumour cells compared with healthy cells (and other tumour cells). Cycloheximide inhibits protein synthesis and allows the measurement of degradation only. Therefore, this will provide information about the intrinsic ubiquitin/proteasome activity of these tumour cells and dynamic changes in Bax protein levels over time. It will also be essential to examine both Bax stability and T cell recognition for fresh tumour tissue compared to cell lines of solid cancers. Investigation of Bax degradation in other non-cancerous cells such as skin fibroblasts, will also be important.

In conclusion, Bax was found to be ubiquitously expressed as expected, but differential degradation by proteasomes was revealed between cell types. It was shown here that Bax degradation by proteasome occurs in cancer cells, but not in the healthy cells tested. This correlates with the T cell recognition data and supports the hypothesis. Overall, the results from this chapter and chapter 4 support the concept of Bax as a tumour antigen for several human cancers.

Chapter 6

Generation of T cell Responses in CLL patients

The previous chapters have shown that Bax peptides were immunogenic in healthy donors, and could generate T cells able to recognise tumour cells. For Bax to be useful in immunotherapy, it is required that T cell responses can be generated in cancer patients. In this chapter, the general immune responsiveness in CLL patients will be evaluated. In order to know if it is possible to generate T cell responses in CLL patients, memory responses were tested against a mixture of recall antigens and primary T cell responses against MART-1 peptide. MART-1 was used as a model antigen for naïve T cell responses in a HLA-A2 restricted system. Naïve and memory T cell responses were characterized using samples from both healthy donors as a control, and CLL patients. Ultimately, T cell responses against the candidate tumour antigen Bax were investigated in CLL patients, particularly because Bax-specific T cells from a healthy donor were able to recognise CLL cells, indicating that Bax can be processed and presented by CLL cells.

6.1 T cell responses against PPP

The capacity of T cells from CLL patients to respond against a mixture of recall antigens (PPP) was tested as a general indication of immune competence. PPP contained both CD8+ and CD4+ T cells epitopes from common recall antigens. The source of these antigens included EBV, CMV, influenza A and tetanus. This protocol has been used as a positive control for memory T cell responses in testing candidate therapeutic vaccines (Smith *et al.*, 2005).

PBMC from thirteen CLL patients and eleven healthy donors were used to assess memory T cell responses against PPP *in vitro*. T cell responses against PPP were detected in 9/13 CLL patients, with a range of responses between 27 and 298 spots /10⁵ cells (mean = 88 spots $\pm$ 24) (Figure 6.1). All the patients were capable of responding to mitogens (PHA, PMA, Concanavalin A, ionomycin), which were used as a positive control in ELISpot (between 150-300 spots/10⁵ cells) (data not shown). Unlike the CLL patients, memory T cell responses against PPP were detected in all eleven healthy donors, with responses ranging from 29 to 384 spots /10⁵ cells (mean = 177 spots $\pm$ 37.3). It was also evident that responses in healthy donors were significant higher than in CLL patients ($p = 0.0127$) (summarized in Figure 6.2). Overall, this data shows that fewer CLL patients were able to respond to recall antigens and T cell responses were weaker when compared to healthy donors.

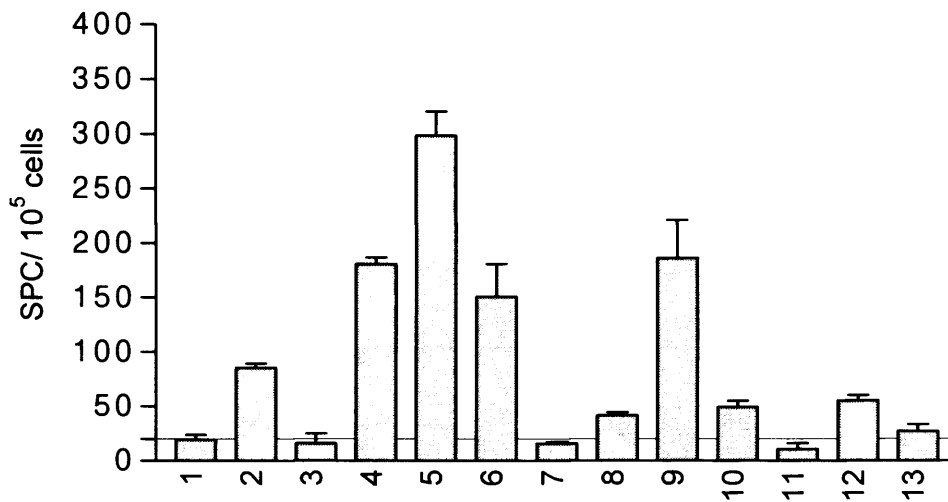


Figure 6.1 Memory T cell responses against recall antigens in CLL patients. PBMC from CLL patients were cultured with a mixture of peptides from influenza, EBV, CMV and tetanus toxoid (PPP). After 7 days, T cells were harvested and IFN γ -secreting T cells reactive against PPP were measured using an ELISpot assay. The dashed line represents the 20 spot cut-off for a positive response and the standard deviation is also shown. Background responses (+PBMC without peptide) have been subtracted from data.

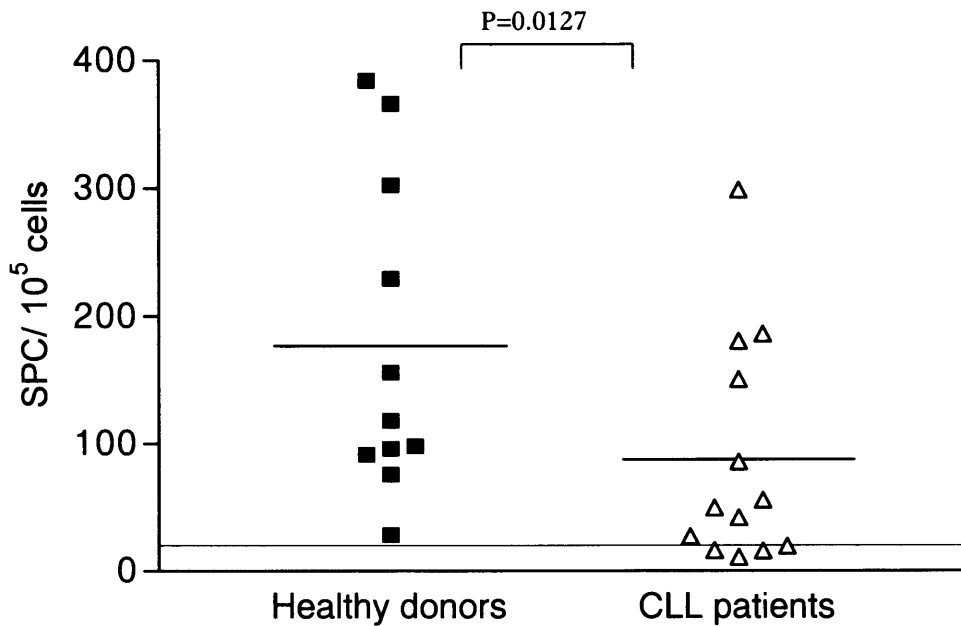


Figure 6.2 Memory T cell responses against recall antigens in CLL patients and healthy donors. PBMC from either CLL patients or healthy donors were culture with PPP. After 7 days, T cells were harvested and IFN γ -secreting T cells reactive against PPP were measured using an ELISpot assay. The dashed line represents the 20 spot cut-off for a positive response and the solid horizontal lines represent the mean. Background responses (+PBMC without peptide) have been subtracted from data. Statistical analysis (Mann Whitney test) was done using GraphPad Prism software.

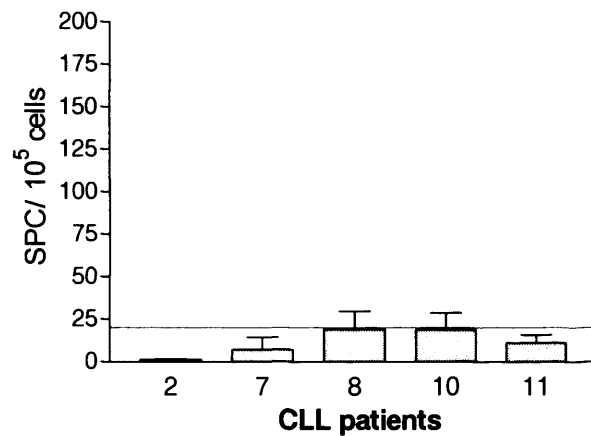
6.2 T cell responses against MART-1

MART-1 antigen is highly immunogenic and it has been shown that this antigen is capable of eliciting robust primary T cell responses in a large proportion of healthy HLA-A2+ donors (Pittet *et al.*, 1999). Therefore, MART-1 was used to evaluate whether CLL patients were capable of mounting naïve T cell responses. Five CLL patients in this study were HLA-A2+ and so tested in parallel for responses against MART-1 peptide (Patients 2, 7, 8, 10 and 11). However, none of the HLA-A2+ patients generated positive MART-1 T cell responses (Figure 6.3A). Interestingly, CLL patients 7 and 11 were also not able to mount memory responses against recall antigens, whilst in patients 2, 8 and 10, it was possible to detect a weak PPP response (41-88 spots/ 10^5 cells) (Figure 6.1). PBMC from seven HLA-A2+ healthy donors were also stimulated with MART-1 peptide. Primary T cell responses could be detected in 4/7 healthy donors, with responses ranging from 89 to 168 spots/ 10^5 cells (Figure 6.3B).

Since in CLL patients, 80-95% of PBMC are CD19+ B cells and only 1-15% are CD3+ T cells, it was also evaluated whether the low frequency of T cells in the PBMC was having an impact on the detection of T cell responses. CD8+ T cells were enriched (using immunomagnetic beads) from five HLA-A2+ CLL patients and healthy donors, and stimulated with autologous PBMC and MART-1 peptide for up to 3 weeks to determine if MART-1 T cell responses could be detected. However, a weak positive response against MART-1 peptide was detected in only one of the patients (34 spots/ 10^5) (Figure 6.4A), suggesting that other factors, besides the frequency of CD3+T cells influenced the priming of MART-1 T cells. In contrast, it was possible to detect T cell responses against MART-1 in 4/5 healthy donors (Figure 6.4B).

Overall, this data suggests that priming of naïve CD8+ T cell responses is impaired in CLL patients, compared to healthy donors.

(A)



(B)

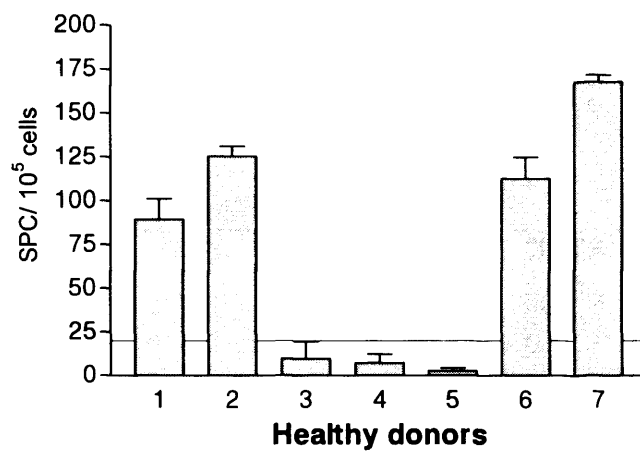
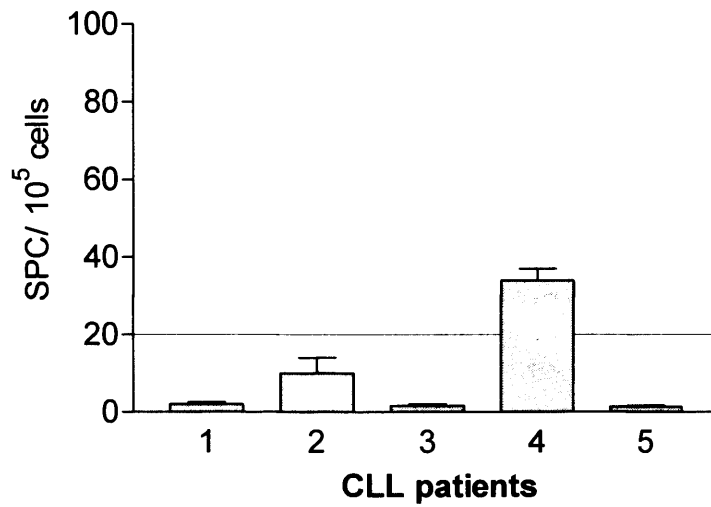


Figure 6.3 Naive T cell responses against MART-1 peptide in CLL patients (A) and healthy donors (B). PBMC from either CLL patients or healthy donors were cultured with MART-1 peptide (ELAGIGILTV) for 7 days. T cells were harvested and IFN γ -secreting T cells reactive against MART-1 were measured using an ELISpot assay. All subjects were typed as HLA-A2. The dashed line represents the 20 spot cut-off for a positive response. Background responses (+PBMC without peptide) have been subtracted from data.

(A)



(B)

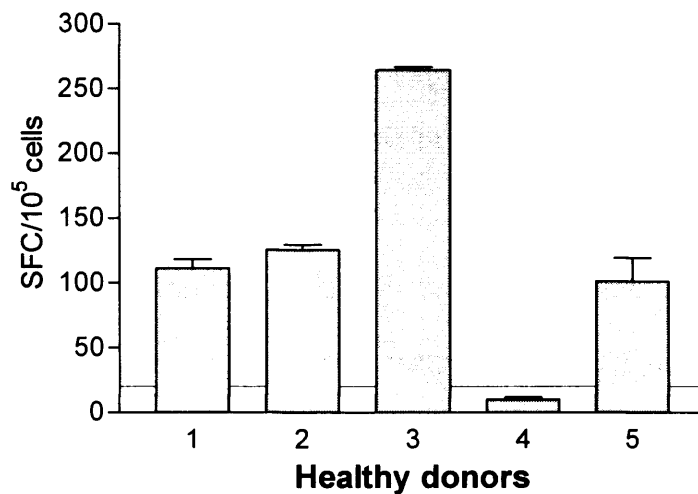


Figure 6.4 MART-1 T cell responses after 2-3 weeks of stimulation in CLL patients (A) and healthy donors (B). CD8⁺ T cells were purified using immunomagnetic beads and cultured with irradiated autologous PBMC and MART-1. Cultures were restimulated with MART-1 weekly. T cells were harvested after 2 to 3 weeks stimulation and IFN γ -secreting T cells reactive against MART-1 were measured using ELISpot assay. All subjects were typed as HLA-A2. The dashed line represents the 20 spot cut-off for a positive response. Background responses (+PBMC without peptide) have been subtracted from data.

6.3 T cell responses against Bax peptides

Although it was previously revealed that CLL cells can be recognised by Bax-specific T cells from a healthy donor (section 4.5.2), it was not known whether the candidate tumour antigen Bax is effective in generating T cell responses in CLL patients. Therefore, T cell responses against Bax peptides were investigated in CLL (summarised in table 6.1). PBMC from two HLA-A2+ patients (CLL1 and CLL2) were stimulated with Bax pool 1-15 for up to 4 weeks, but Bax specific T cell responses were not detected. Taking into account that 80-95% of CLL cells are B cells, CD8+ T cells from one HLA-A2+ patient (CLL3) were purified by flow sorting and cultured with irradiated autologous CLL cells in the presence of Bax pool 1-15 for one week. A positive T cell response against Bax peptides was detected after being stimulated for one week and expanded using the antigen-independent protocol (110 spots/ 10^5 cells). However, Bax specific T cells from this patient were difficult to grow and no further characterisation of these T cells was done.

CD8+ T cell enrichment using flow sorting was time consuming (~ 4 hours to purify only 1×10^6 cells), expensive and a low number of T cells was recovered after stimulation (2×10^5). Therefore, CD8+ T cells from additional four HLA-A2+ CLL patients (CLL4, CLL5, CLL6 and CLL7) were enriched using immunomagnetic beads (MACs), as previously done for healthy donors. Cells were harvested after 1-3 weeks and tested in an ELISpot assay, depending on the numbers of CD8+ T cells after each stimulation. A weak response against Bax peptides was detected in one of these patients (21 spots/ 10^5 cells) after 1 week of stimulation with Bax pool. No additional stimulations with Bax pool were possible for this patient due to limited numbers of T cells.

Overall, these results indicate Bax peptide specific T cells can be detected occasionally in CLL patients but that it is difficult to generate robust T cell responses from the blood using current protocols.

Table 6.1 Summary of T cell responses against Bax in CLL patients

CD8+ T cell Purification		Bax peptide pool stimulation	ELISpot (/10 ⁵ cells)
CLL1	No	PBMC+ irradiated autologous PBMC	4 weeks: < 20 spots
CLL2	No	PBMC+ irradiated autologous PBMC	4 weeks: < 20 spots
CLL3	Flow sorting	T cells + irradiated autologous PBMC	1 week: 110 spots
CLL4	MACs sorting	T cells + irradiated autologous PBMC	2, 3 weeks: < 20 spots
CLL5	MACs sorting	T cells + irradiated autologous PBMC	1 week: 22 spots
CLL6	MACs sorting	T cells + irradiated autologous PBMC	2, 3 weeks: < 20 spots
CLL7	MACs sorting	T cells + irradiated autologous PBMC	3 weeks : < 20 spots

Background responses (+PBMC without peptide) have been subtracted from data.

6.4 Discussion

The data from this *in vitro* study suggests that naïve and, to a certain extent, memory T cell responses are compromised in CLL patients. It was particularly difficult to detect naïve responses to MART-1 in HLA-A2+ CLL patients even when CD8+ T cells were purified. However, in 9/13 CLL patients tested in this study, T cell responses were detected against recall antigens without purification of T cells, indicating that functional memory T cells can be found in the peripheral blood of most patients. The frequency of responses against recall antigens was significant lower than in healthy donors, but it must be taken into consideration the immunosuppressive effect of leukaemic B cells, which were predominant in the cultures tested against PPP, accounting for 80-95% of PBMC in culture. Furthermore, the age of healthy donors tested was lower (30-60 years) compared to the age of the CLL cohort (>45 years) and thus might have a slight influence on the detection of higher magnitude of responses in the healthy cohort. One major limitation of using CD8+ T cells from CLL patients is the starting numbers of T cells. Lower CD8+ T cell numbers were obtained from CLL

patients since only 3 mls of blood samples were collected from patients compared with 50-100 mls of blood from healthy donors. Furthermore, only 1-15% CD3+ T cells are found in the peripheral blood of CLL patients, resulting in just 2×10^5 to 1.5×10^6 CD8+ T cells being recovered from each patient. Since repeat blood sample could not be obtained from the CLL patients, it was not possible to culture T cells for more than 3 weeks.

It is interesting to see that it is possible to detect memory T cell responses, as it implies that CLL cells, despite their reduced antigen presenting ability, can still be competent APCs in presenting recall antigens *in vitro*. This is in accordance with the concept that memory T cells are less dependent on co-stimulation for their activation compared with naïve T cells (Croft *et al.*, 1994). It has been reported that CLL patients have increased numbers of CMV-reactive T cells and that the T cell population is altered towards a cytotoxic phenotype in an effort to control persistent viral infections, including CMV (Mackus *et al.*, 2003). Since PPP contained CMV peptides, it is possible that most of detectable T cell responses are against CMV. However, mapping PPP specific T cell responses to individual peptides, in conjunction with assessment of CMV serology would be needed to prove this.

It is well known that CLL cells express low levels of the adhesion and co-stimulatory molecules essential for functional APCs, therefore it is not completely unexpected that CLL cells failed to prime naïve T cell responses against MART-1. There are other immune defects in CLL that can contribute to the failure in inducing activation and differentiation of tumour-specific T cells. These include abnormal maturation and function of DCs (Orsini *et al.*, 2003), secretion of inhibitory cytokines and expression of inhibitory molecules such as CD200 by B cells, that actively suppress T cell activation, expansion and effector function (Kretz-Rommel *et al.*, 2007; Pallasch *et al.*, 2009). CLL cells can also manipulate the gene expression profile of T cells through cell-cell contact, inducing abnormal expression of genes involved in CD4+ T cell differentiation, and impaired production of cytolytic granzyme granules in CD8+ T cells

(Gorgun *et al.*, 2005). Furthermore, impairment of immune synapse formation between CLL cells and T cells, which in turn can prevent T cell activation, has also been demonstrated in CLL patients (Ramsay *et al.*, 2008). The T cells themselves can also have abnormalities, which include inverted ratio of CD4:CD8 (Platsoucas *et al.*, 1982; Totterman *et al.*, 1989), reduced expression of CD28, ζ chain of TCR complex, and CD40L, and reduced IL-2 (IL-2R) that collectively suggest impairment of ability to initiate an immune response, and maintenance and expansion of T cell responses to antigens (Rossmann *et al.*, 2003). All of these defects are likely to contribute to the failure to generate a primary T cell response in CLL patients.

Only two CLL patients (CLL3 and CLL5) were able to respond to the candidate tumour antigen Bax. Although in CLL5 only a weak response was detected, it was possible to detect a positive response in CLL3 after only one week stimulation with Bax pool. This might imply that the Bax specific T cell responses in these patients are memory responses. However, without further characterisation of these T cells, this cannot be confirmed. Therefore, it will be of great interest to generate a tetramer for Bax p610 and/or p613 as this will allow examination of Bax specific T cells directly *ex vivo* and allow assessment of memory markers. The Bax-specific T cell responses were not robust responses, but this was not surprising, since even T cell responses against a highly immunogenic tumour antigen like MART-1 are difficult to detect. Similar to MART-1, most of the patients were not able to respond to Bax, suggesting that the immunosuppressive effects of CLL cells and T cell abnormalities may be preventing the activation of T cells. Alternatively, this might simply reflect that these patients do not host a spontaneous response to Bax peptides or that responses in these patients may have been present but below the detection limit of the ELISpot. Due to reduced number of CD8⁺ T cells obtained from CLL patients, it was not possible to restimulate T cells with Bax pool for more than 3 weeks (unlike healthy donors). Further restimulations may be necessary to generate a Bax-specific T cell response in CLL patients.

Despite the fact that several defects are found in both T cells and B cells of CLL patients, a few tumour antigens have been identified in CLL patients, such as CD23 (Bund *et al.*, 2007), CD229 (Bund *et al.*, 2006), survivin (Schmidt *et al.*, 2003), MDM2 (Mayr *et al.*, 2006), hTERT (Kokhaei *et al.*, 2007) and fibromodulin (Mayr *et al.*, 2005).

Some of these studies were able to demonstrate specific T cell responses using native CLL cells as APC after several weeks of stimulation but in some cases CLL cells had to be stimulated prior to co-incubation with T cells using CD40L-expressing feeders (Bund *et al.*, 2006; Bund *et al.*, 2007; Mayr *et al.*, 2006). The low immunogenicity of CLL cells is due at least in part to their poor antigen-presenting competence, making it extremely difficult to initiate an effective immune response. CD40L stimulation has been shown to be effective in enhancing their antigen presenting capacity by increasing the expression of adhesion and co-stimulatory molecules. In several studies, this resulted in autologous T cell activation and expansion within 3 to 4 weeks of stimulation with CD40L-stimulated CLL cells, and most importantly allowed detection of peptide-specific T cell responses (Bund *et al.*, 2006; Bund *et al.*, 2007; Mayr *et al.*, 2006).

Taken together, the findings of these studies imply that stimulation or pre-activation of CLL cells is required to allow antigen presentation to T cells. To induce effective Bax (or MART-1) specific T cell responses in CLL patients, it will likely be necessary to enhance antigen presentation, possibly to overcome tolerance on both the antigen presenting and effector mechanisms. Importantly, HLA-A2+ CLL cells were recognised by the Bax-specific T cells from a healthy donors, indicating that CLL cells are processing and presenting Bax peptides and thus it is possible that there are T cells specific for Bax in the circulation. Therefore, additional experiments must be attempted with improved stimulation protocols before excluding the possibility of Bax as a potential tumour antigen for CLL.

Different *in vitro* strategies could be developed to repair T cell function and overcome T cell tolerance. First, CLL cells need to be stimulated with CD40L prior to incubation with T

cells. Stimulations including native CLL cells or CD40L activated CLL cells co-cultured with T cells in presence of Bax peptides should be tried for more than 3 weeks *in vitro* culture. As culture with CD40L-activated CLL cells allows T cell expansion with several weeks of stimulation, limited numbers of T cells might no longer be a major problem. An additional strategy can also be applied by using an antibody to block CD200, which is an immune suppressive molecule over-expressed on CLL cells, that is capable of inhibiting T cell proliferation, antigen-specific T cell responses and promoting induction of Tregs. It has been recently shown that expansion of antigen-specific cytotoxic T cells and suppression of Tregs can be enhanced by culturing T cells with CD40L-stimulated APCs and an anti-CD200 antibody (Pallasch *et al.*, 2009). Therefore, this approach should also be considered to assess Bax-specific T cell responses in CLL patients. The use of an immunomodulatory drug called lenalidomide to improve antigen presentation by CLL cells is also an attractive strategy to investigate Bax specific T cell responses in CLL patients. Lenalidomide has been recently shown to enhance immune synapse formation between T cells and CLL cells (Ramsay *et al.*, 2008). Because of its T cell stimulatory effect, this could result in improved effector functions against CLL cells.

Applying these *in vitro* approaches will most likely provide decisive information whether Bax protein could be a tumour antigen in CLL, but will also help to optimise protocols for priming and maintaining functional T cells against other candidate tumour antigens in CLL patients.

Chapter 7

Immunophenotyping of CLL patients

CLL was studied as a model system for generation of human tumour specific T cell responses. The general cell-mediated immunity of patients was investigated in Chapter 6. These *in vitro* studies suggested that both naïve and memory T cell responses are compromised in CLL patients. It is known that high numbers of systemic tumour cells in CLL can suppress T cell responses, but the mechanism(s) behind this suppression is still not clear. Recently, the development of multi-colour flow cytometry has allowed more detailed functional and phenotypic characterisation of T cell subsets in human disease. It has provided valuable information about aberrant phenotypes in diseases, such as a skewed maturation of CD8⁺ T cells in HIV-patients (Champagne *et al.*, 2001; Hoji *et al.*, 2007) and increased Tregs in cancer patients (Wolf *et al.*, 2003). The main aim of this chapter is to use this technology to investigate the possibility that such skewed T cells subsets could also be identified in CLL patients (n=51). A smaller number of age-matched healthy donors (n=10) were analysed to provide direct comparators for the lymphocyte subsets under investigation. Eight-colour flow cytometry was used for detailed phenotypic analysis of peripheral blood lymphocyte populations, in particularly T cells (see table 2.6 in Materials &Methods).

7.1 Frequencies of peripheral blood lymphocyte populations

The first investigation of this study was to assess the frequency of peripheral blood lymphocyte populations. The percentages of B cells (CD19+CD5+), T cells (CD3+ CD4+ and CD8+), innate NK (CD56+ CD16+), $\gamma\delta$ T cells (CD3+ TCR $\gamma\delta$ +) (Figure 7.1) and Tregs (CD4+ CD25^{high} Foxp3) (Figure 7.2) were analysed in both CLL patients and healthy donors. However, the absolute numbers of these populations were not evaluated in this study. Consistent with previous studies, a significant increase was seen in the percentage of CD19+CD5+ B cells in patients (83.4%) compared to healthy donors (8.9%) ($P<0.0001$), along with a significant decrease of percentage of CD3+ T cells (9.6% vs 62.2%, $P<0.0001$) and NK cells (CD56+CD16+) (2.3% vs 12.6%, $P<0.0001$). An increase in the frequency of CD8+ T cells among CD3+ T cells was observed in CLL patients (40.3% vs 28.5%, $P=0.056$ not significant), while the percentage of CD4+ T cells among the CD3+ T cells was significantly reduced (45.6% vs 64.4%, $P=0.0008$). An expansion in the CD8+ T cell subset relative to CD4+ T cells was seen in 29/51 patients (57%) leading to a lower CD4:CD8 ratio compared to healthy donors (mean ratio 0.77 vs 2.26, $P<0.0001$). No significant difference was seen in the $\gamma\delta$ T cells among the CD3+ T cells between CLL patient and healthy donors (4.67% vs 2.23%, $P=0.05$). Furthermore, a significant increase of Tregs was observed among CD4+ T cells in CLL patients compared to healthy donors (4.1% vs 1.8% , $P=0.0096$) (Figure 7.2).

Overall, these results showed that, besides the increase in malignant B cells in CLL patients, the frequency of NK cells and CD3+ T cells is significant reduced, and there is a tendency for an increase in frequency of CD8+ T cells and Tregs, and reduced frequencies of CD4+ T cells, compared with healthy donors.

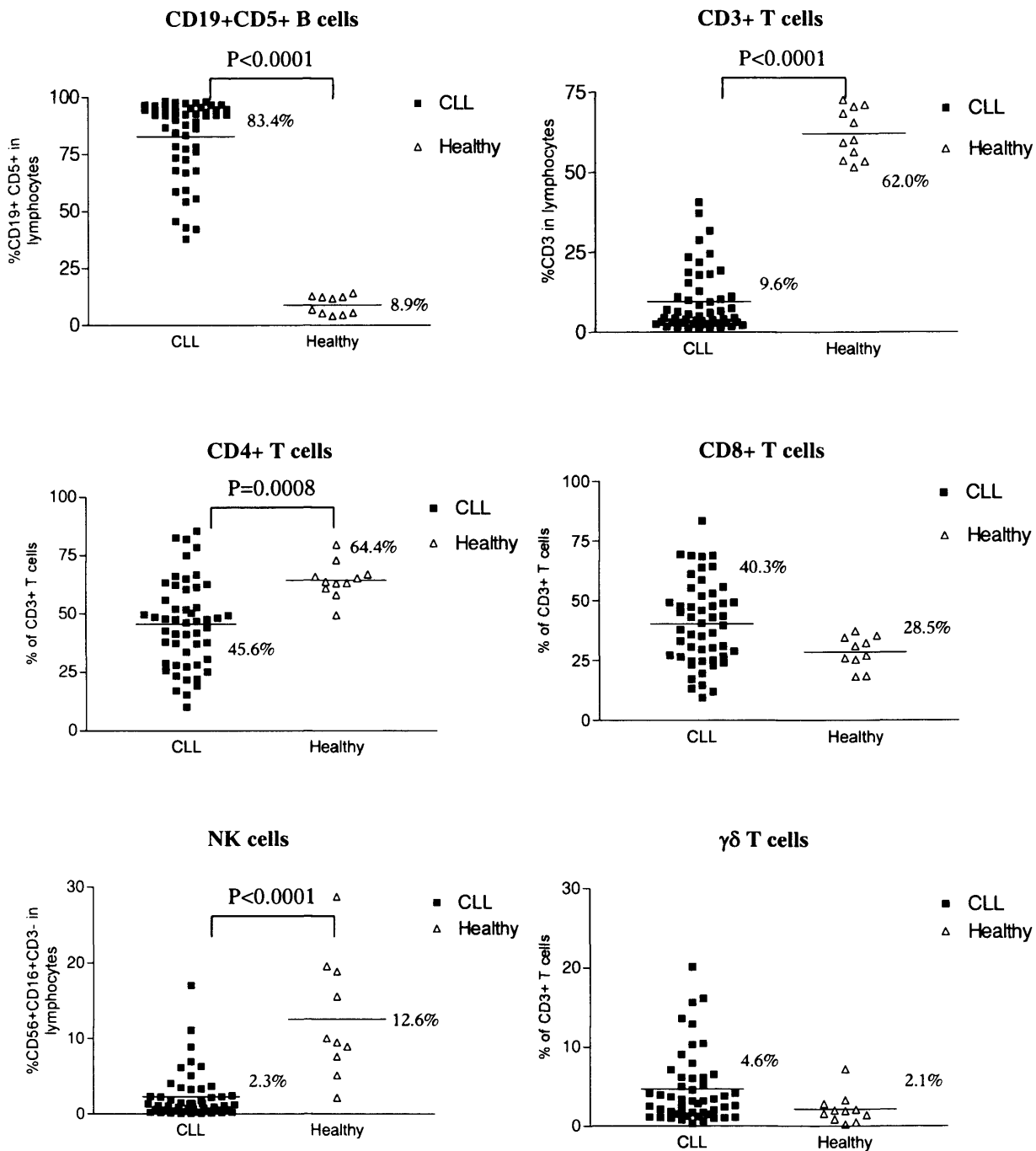
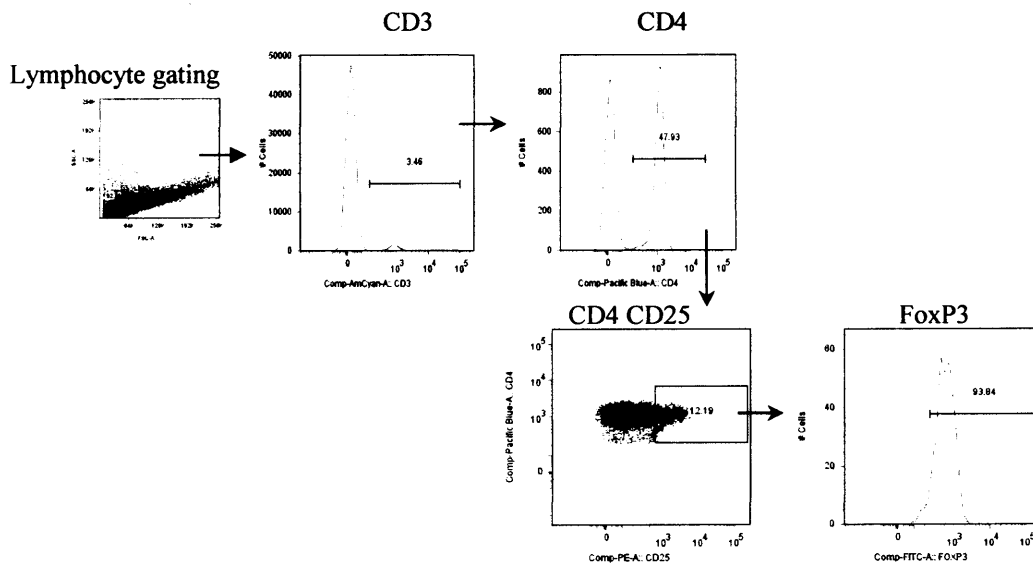


Figure 7.1 Frequency of the peripheral blood lymphocyte populations in CLL patients (n=51) and healthy donors (n=10). The number of positive cells was determined by flow cytometry (FACS CantoII). Lymphocytes were gated based on their forward and side scatter profile. % B cells (CD19+CD5+), CD3+ T cells, NK (CD56+CD16%) are shown within lymphocyte gate. % CD4+ T cells, CD8+ T cells and $\gamma\delta$ T cells (TCR $\gamma\delta$ +) are shown within the combined lymphocyte/CD3 gate. The mean within each subgroup is shown. Flow cytometric and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism software, respectively.

(A)



(B)

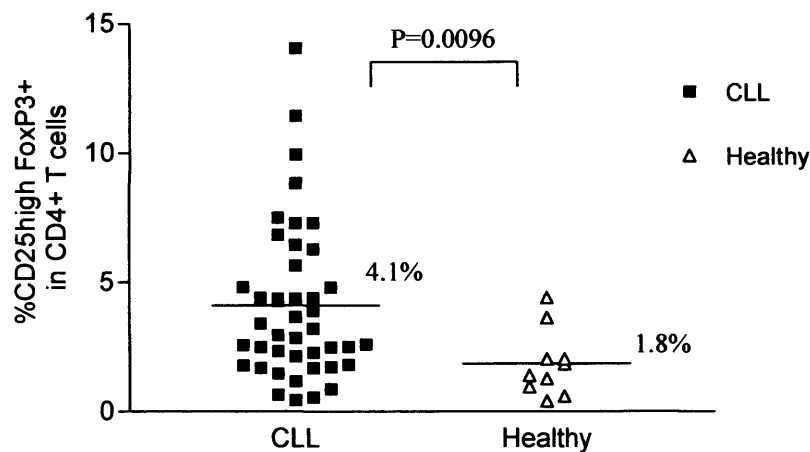


Figure 7.2 Frequency of Tregs in CLL patients (n =43) and healthy donors (n =10). The number of positive cells was determined by flow cytometry (FACS CantoII). (A) Gating strategy used to determine CD4⁺ CD25^{high} FoxP3⁺. (B) % CD4⁺ CD25^{high} FoxP3⁺ shown within lymphocyte/CD3/CD4 gate. The mean within each subgroup is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively.

7.2 Expression of immunosuppressing molecules CD200, CD200R and PD-1

Several studies have shown that immunosuppressing molecules, such as CD200 and PD-1, are overexpressed in haematological malignancies, including CLL (McWhirter *et al.*, 2006; Xerri *et al.*, 2008). Therefore, the expression of PD-1, CD200 and its receptor CD200R were investigated in order to see whether there is an altered expression on the different lymphocyte populations of CLL patients when compared with age-matched healthy donors. The immunosuppressive CD200 has been shown to play a part in regulating immune responses (Kretz-Rommel *et al.*, 2007; McWhirter *et al.*, 2006; Pallasch *et al.*, 2009). CD200 is normally expressed on DCs, B cells and T cells among others. CD200 exerts its effect by binding to its cognate receptor CD200R, which is expressed on cells specifically derived from myeloid lineage, T cells and B cells (Rijkers *et al.*, 2008). PD-1 is a lymphoid receptor that negatively regulates immune responses. PD-1 is induced in T cells, B cells and myeloid cells. Interaction of PD-1 with its ligands PD-L1 or PD-L2 on tumour cells may play a role in tumour escape from immune responses (Okazaki & Honjo, 2006; Xerri *et al.*, 2008).

CD200 expression was examined on B cells, NK, CD4+ and CD8+ T cells. Both its receptor (CD200R) and PD-1 expression was examined on B cells and CD4+ and CD8+ T cells. A representative gating strategy to determine the frequency of lymphocyte populations and expression of immunosuppressing molecules is shown in Figure 7.3.

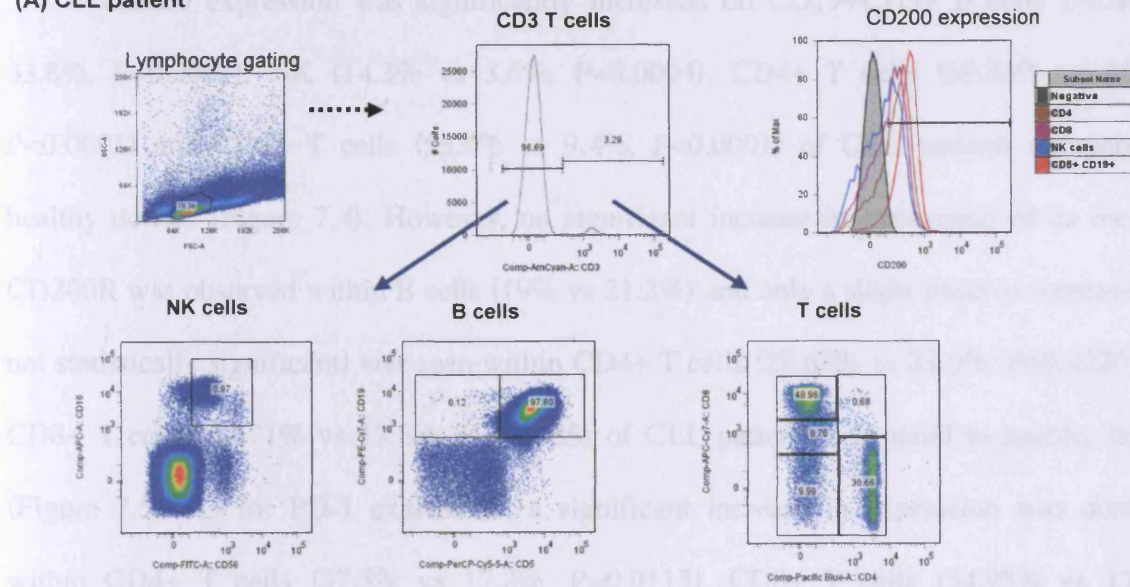
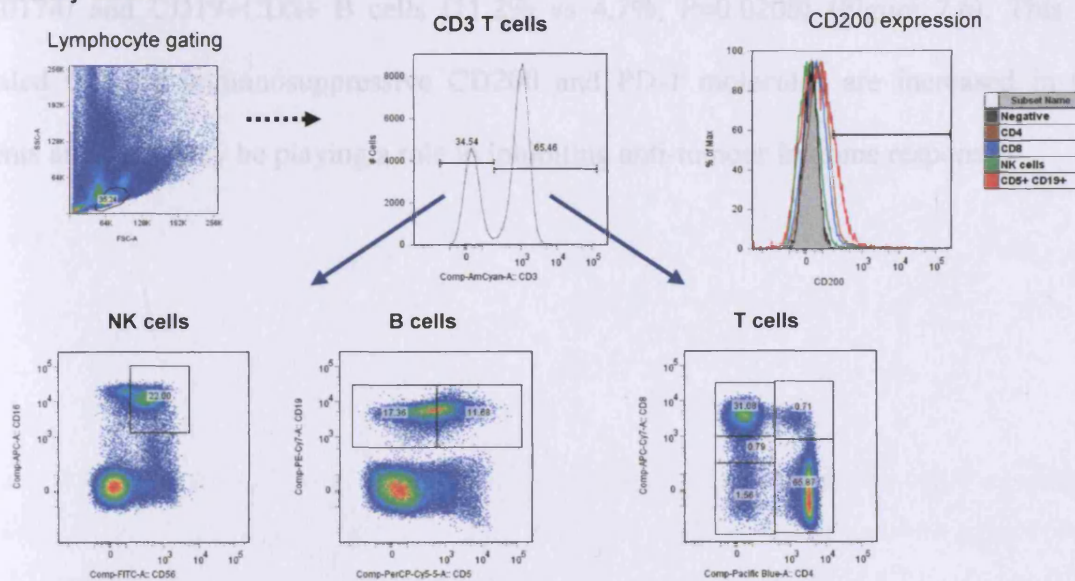
(A) CLL patient**(B) Healthy donor**

Figure 7.3 Representative results of a CLL patient (A) and healthy donor (B). The gating strategy used to determine lymphocyte populations NK (CD56+CD16+), B cells (CD19+CD5+) T cells (CD3+ CD4+ or CD8+) and expression of immunosuppressing molecules (in this case CD200) within these subsets is shown. The number of positive cells was determined by flow cytometry (FACS Cantoll) and flow cytometric analysis was carried out using FlowJo software.

CD200 expression was significantly increased on CD19+CD5+ B cells (96.4% vs 33.8%, $P<0.0001$), NK (14.3% vs 3.6%, $P=0.0004$), CD4+ T cells (69.83% vs 16.7%, $P<0.0001$) and CD8+ T cells (55.4% vs 9.4%, $P<0.0001$) of CLL patients compared to healthy donors (Figure 7.4). However, no significant increase in expression of its receptor CD200R was observed within B cells (19% vs 21.2%) and only a slight trend of increase (but not statistically significant) was seen within CD4+ T cells (29.60% vs 23.5%, $P=0.4220$) and CD8+ T cells (23.71% vs 17.5%, $P=0.2625$) of CLL patients compared to healthy donors (Figure 7.5). As for PD-1 expression, a significant increase in expression was observed within CD4+ T cells (37.5% vs 17.2%, $P=0.0113$), CD8+ T cells (34.95% vs 17.1%, $P=0.0174$) and CD19+CD5+ B cells (11.2% vs 4.7%, $P=0.0208$) (Figure 7.6). This data revealed that the immunosuppressive CD200 and PD-1 molecules are increased in CLL patients and these may be playing a role in inhibiting anti-tumour immune responses.

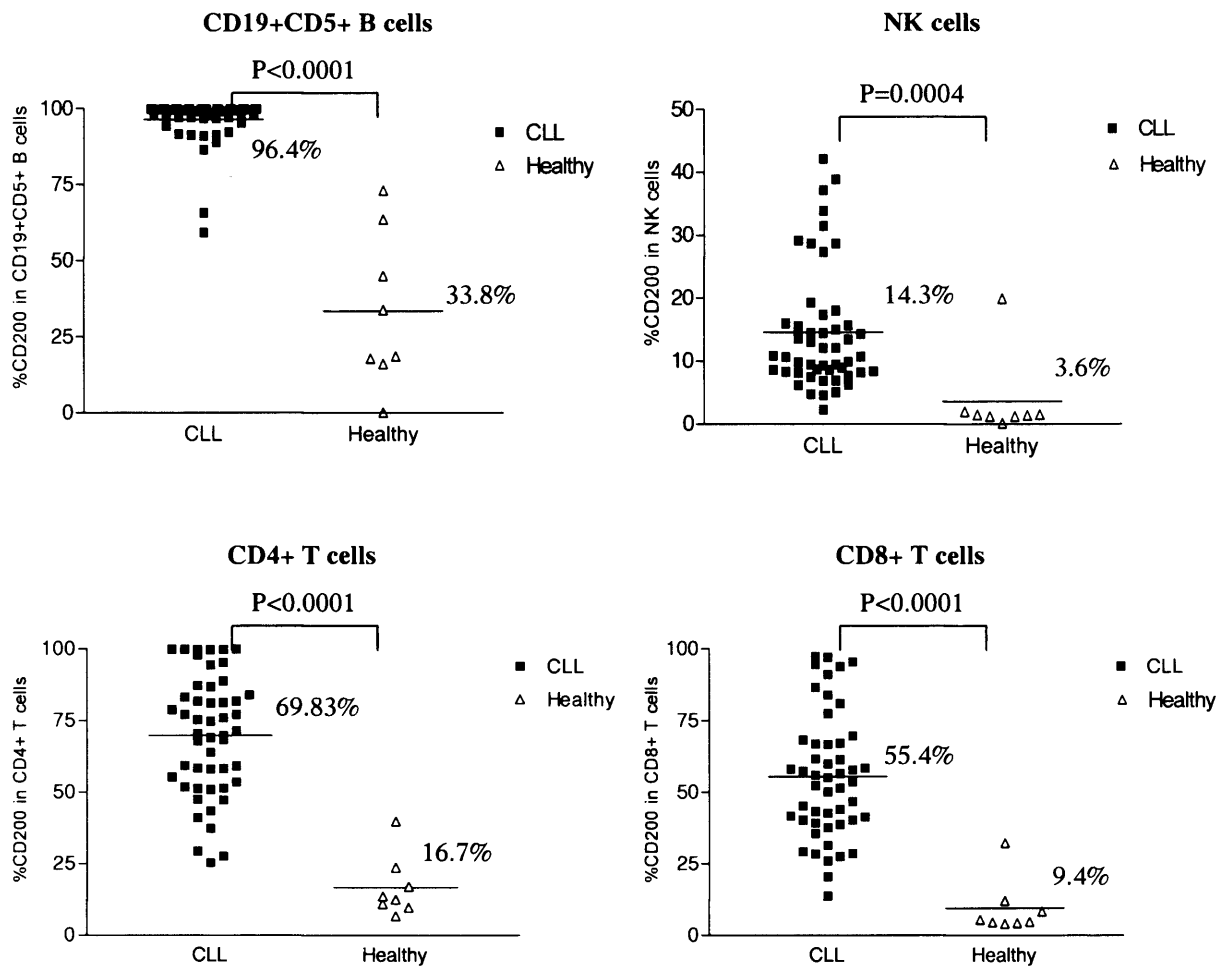


Figure 7.4 CD200 expression in CLL patients (n =50) and healthy donors (n =8). The number of positive cells was determined by flow cytometry (FACS CantoII). Lymphocytes were gated based on their forward and side scatter profile. CD200 expression was analysed within CD19+CD5+ B cells, NK (CD56+CD16+), CD4+ and CD8+ T cells. The mean within each subgroup is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively.

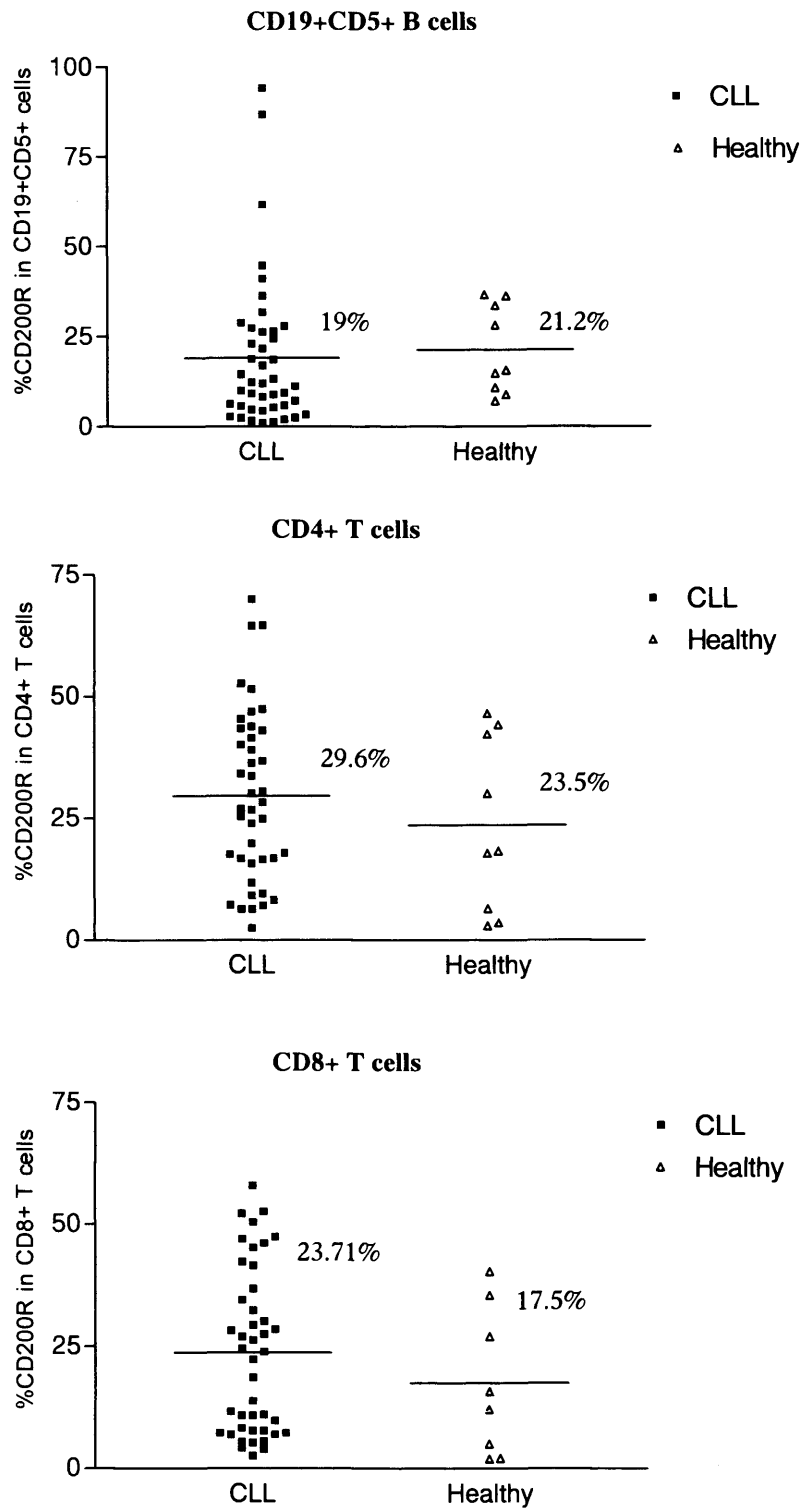


Figure 7.5 CD200R expression in CLL patients (n=42-43) and healthy donors (n=8-9). The number of positive cells was determined by flow cytometry (FACS CantoII). Lymphocytes were gated based on their forward and side scatter profile. CD200R expression was analysed within CD19+CD5+ B cells, CD4+ and CD8+ T cells. The mean within each subgroup is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively.

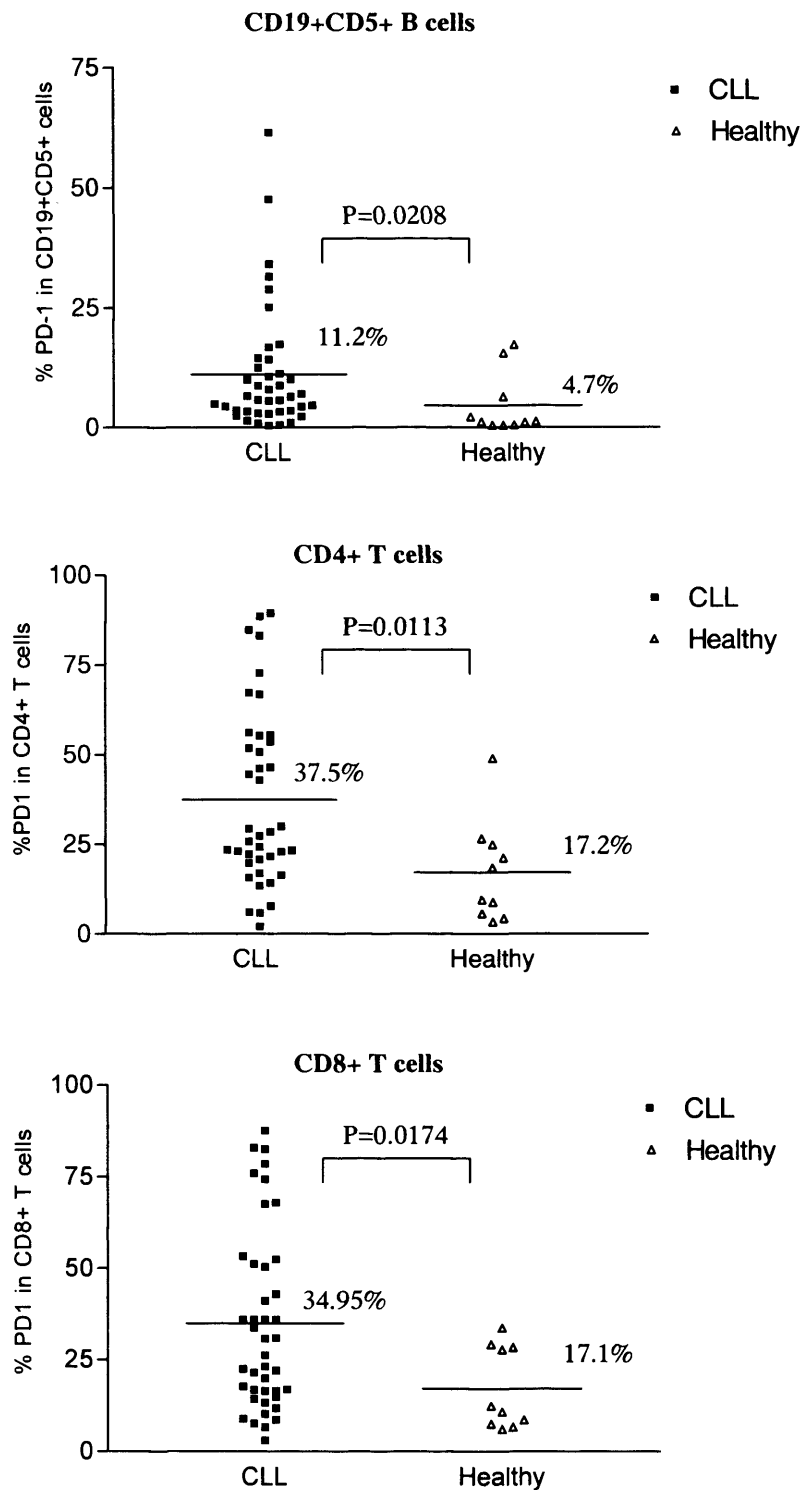


Figure 7.6 PD-1 expression in CLL patients (n=42) and healthy donors (n=10). The number of positive cells was determined by flow cytometry (FACS CantoII). Lymphocytes were gated based on their forward and side scatter profile. PD-1 expression was analysed within CD19+CD5+ B cells, CD4+ and CD8+ T cells. The mean within each subgroup is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively.

7.3 CD3+ T cells

A comprehensive analysis of CD4+ and CD8+ memory T cells in CLL patients compared with that in healthy donors was undertaken to give further insights as to why it was difficult to generate T cell responses in CLL patients (Chapter 6). It has been reported that a skewed maturation of T cells occurs with ageing and disease, including chronic viral infection, such as HIV (Champagne *et al.*, 2001; Hoji *et al.*, 2007; Koch *et al.*, 2008). In particular, a progressive downregulation of CD28 and upregulation of CD57, which are associated with replicative senescence (proliferative exhaustion), have been observed with ageing and disease, including CLL (Brenchley *et al.*, 2003; Scheuring *et al.*, 2002; Van den Hove *et al.*, 1998a).

Therefore, co-expression of CD28, CD45RO and CD57 was analysed in CD4+ and CD8+ T cell compartments to elucidate whether this phenotype is more pronounced in patients with CLL compared to age-matched healthy donors. It was also evaluated if CD4+ and CD8+ memory T cell subsets differ between CLL patients and age-matched controls. The T cell subsets were defined by dividing both CD4 and CD8 compartment on the basis CD45RO and CCR7 into naïve (CCR7+CD45RO-), central memory (CCR7+CD45RO+), effector memory (CCR7-CD45RO+) and terminally differentiated effector cells (CCR7-CD45RO+) (see Figure 1.3). A representative result from a CLL patient sample showing the detailed gating strategies using CD3, CD4, CD8, CD45RO, CCR7, CD28 and CD57 is shown in Figure 7.7.

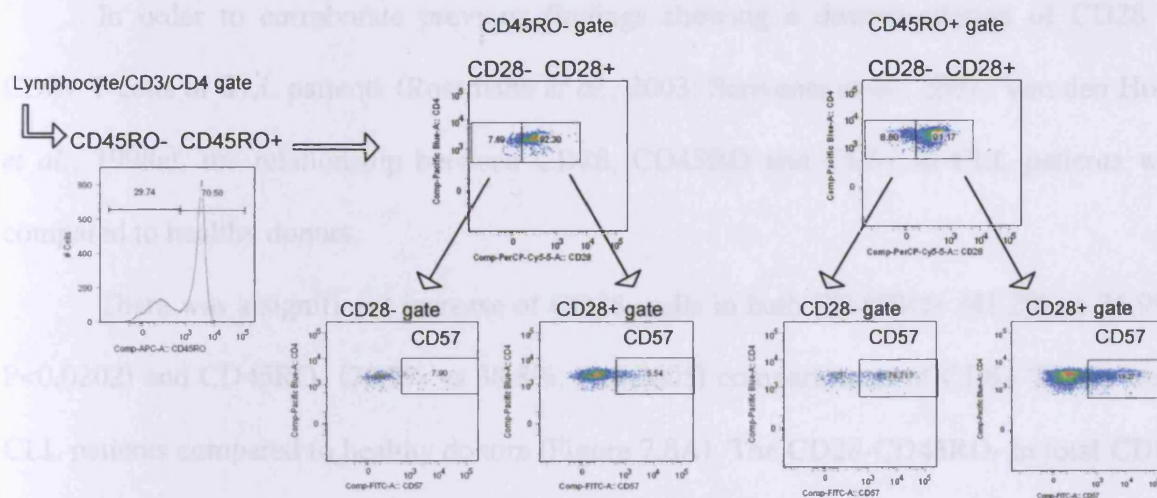
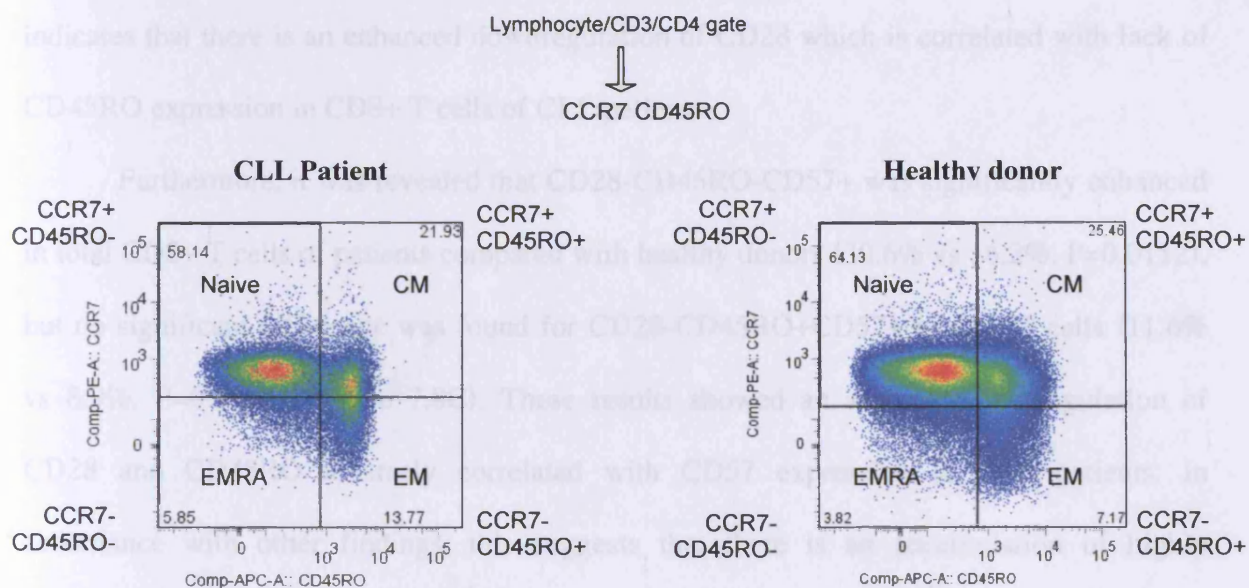
(A) Gating for CD45RO/CD28/CD57 expression**(B) Gating to define subsets**

Figure 7.7 Representative results of T cell analysis. (A) Gating strategy for CD45RO $\pm$ CD28 $\pm$ CD57 $\pm$ in CD4 $^{+}$ T cells. (B) Definition of T cell subsets Naive (CCR7 $^{+}$ CD45RO $^{-}$), Central Memory (CM) (CCR7 $^{+}$ CD45RO $^{+}$), Effector Memory (EM) (CCR7 $^{-}$ CD45RO $^{+}$), and Effector (EMRA) (CCR7 $^{-}$ CD45RO $^{-}$). Representative results of the T cell subsets are shown in a CLL patient and healthy donor. The number of positive cells was determined by flow cytometry (FACS Cantoll) and flow cytometric analysis was carried out using FlowJo software.

7.3.1 CD8+ T cells

In order to corroborate previous findings showing a downregulation of CD28 in CD8+ T cells of CLL patients (Rossmann *et al.*, 2003; Scrivener *et al.*, 2001; Van den Hove *et al.*, 1998a), the relationship between CD28, CD45RO and CD57 in CLL patients was compared to healthy donors.

There was a significant increase of CD28- cells in both CD45RO+ (41.2% vs 26.9%, $P < 0.0202$) and CD45RO- (70.7% vs 38.8%, $P < 0.0005$) compartments of CD8+ T cells from CLL patients compared to healthy donors (Figure 7.8A). The CD28-CD45RO- in total CD8+ T cells of patients was significantly enhanced compared to healthy donors (44.3% vs 23.5%, $P < 0.0038$), but no significant difference of CD28-CD45RO+ in total T cells was found between patients and healthy donors (15.6% vs 10.6%, $P = 0.1807$) (Figure 7.8B). This indicates that there is an enhanced downregulation of CD28 which is correlated with lack of CD45RO expression in CD8+ T cells of CLL patients.

Furthermore, it was revealed that CD28-CD45RO-CD57+ was significantly enhanced in total CD8+ T cells of patients compared with healthy donors (30.6% vs 16.2%, $P = 0.0112$), but no significant difference was found for CD28-CD45RO+CD57+ in total T cells (11.6% vs 8.9%, $P = 0.2991$) (Figure 7.8C). These results showed an apparent downregulation of CD28 and CD45RO inversely correlated with CD57 expression in CLL patients. In accordance with other findings, this suggests that there is an accumulation of highly differentiated T cells, supporting the concept of systemic activation of the T cell compartment (Rossmann *et al.*, 2003; Scrivener *et al.*, 2001; Serrano *et al.*, 1997; Van den Hove *et al.*, 1998a; Van den Hove *et al.*, 1998b).

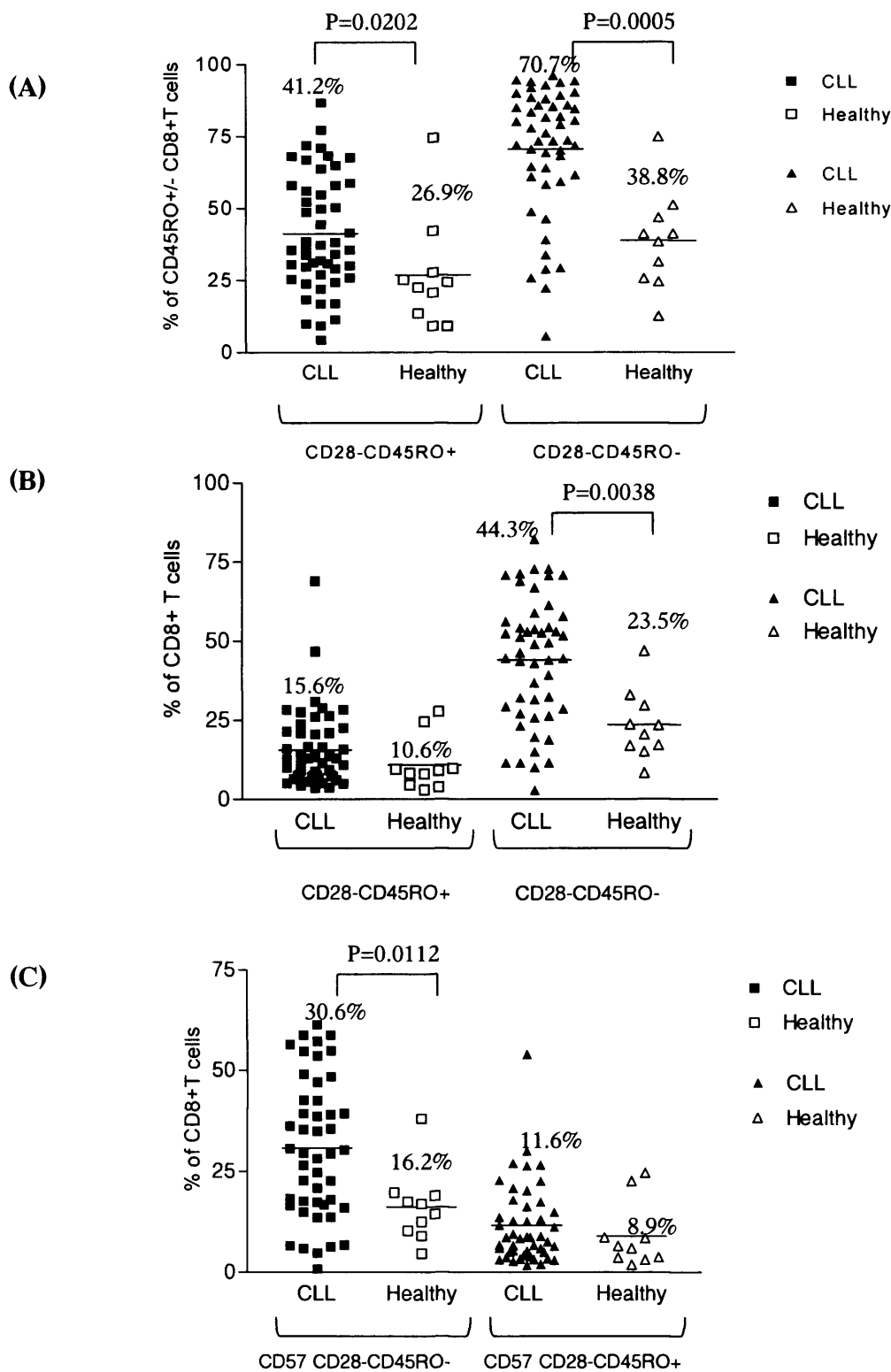


Figure 7.8 Expression of CD28, CD45RO and CD57 in CD8+ T cells of CLL patients (n=49) and healthy donors (n=10). The number of positive cells was determined by flow cytometry (FACS Cantoll). Lymphocytes were gated based on their forward and side scatter profile. T cells were gated on CD3+ CD8+. **(A)** % CD28- within CD45RO+ and CD45RO- CD8+ T cells. **(B)** % CD28-CD45RO- of CD8+T cells. **(C)** % CD28- CD45RO+ CD57+ and % CD28- CD45RO- CD57+ of CD8+ T cells. The mean within each subgroup is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively. CLL patients (filled symbols) and healthy donors (open symbols).

CD8⁺ T cell subsets were evaluated in CLL patients and compared with age-matched controls (Figure 7.9). The subsets were defined by the model which subdivides the CD8⁺ T cell compartment on the basis of CD45RO and CCR7. A significant decrease in the proportion of naïve CD8⁺ T cells in CLL patients was observed when compared to healthy donors 11.58% vs 25.6, $P=0.0083$). This is consistent with an increase in proportions of EM (32.5%) and EMRA (48%). However, no significant difference between CLL patients and healthy donors (EM: 34.1%, $P=0.6509$, and EMRA:35.5%, $P=0.0809$) was observed. The percentage of CM was low but not significantly different between patients and controls (6.79% vs 5.89%, $P=0.4911$).

The accumulation of CD28⁻ and CD57⁺ cells were further shown to be in the EM and EMRA subsets. There was a significant increased of CD28⁻ EMRA (39.3% vs 22%, $P=0.0186$) and CD57⁺ EMRA (32.8% vs 18.9, $P=0.0220$) in total CD8⁺ T cells of patients as compared to healthy donors (Figure 10). These results indicated that there is an accumulation of exhausted CD8⁺ T cells, supporting the concept of a chronic overstimulation of T cells in CLL patients.

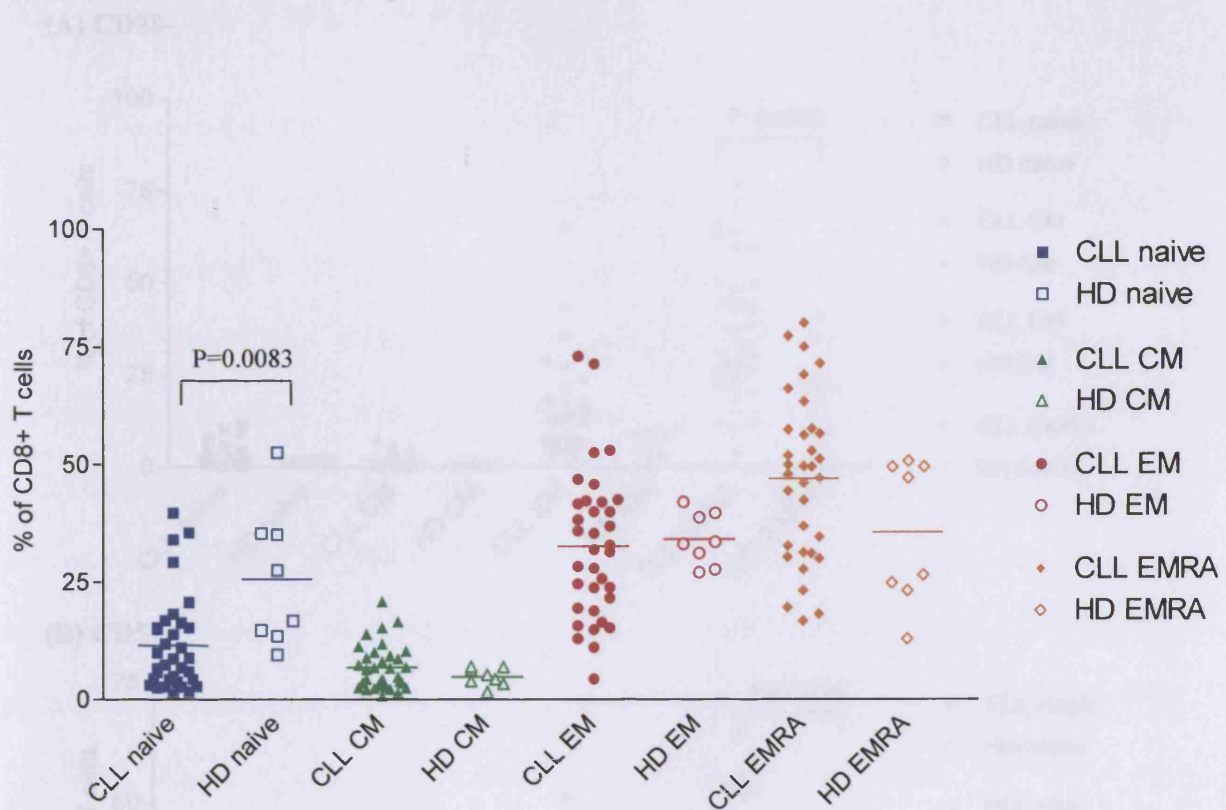
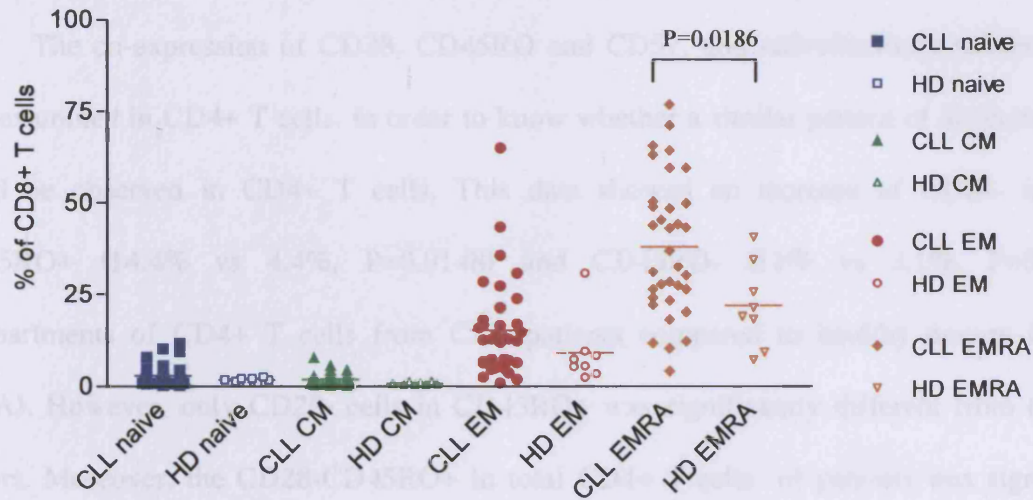


Figure 7.9 CD8+ T cell subsets of CLL patients (n=34) and healthy donors (n=8). The number of positive cells was determined by flow cytometry (FACS Cantoll). Lymphocytes were gated based on their forward and side scatter profile. T cells were gated on CD3+ CD8+. T cells subsets were defined based on the expression of CCR7 and CD45RO. Naive (CCR7+CD45RO-), Central Memory (CM) (CCR7+CD45RO+), Effector Memory (EM) (CCR7-CD45RO+), and Effector (EMRA) (CCR7-CD45RO-). Heterogeneity inside each subset was defined by co-expression of co-stimulatory molecules CD27 and CD28 was not included. The mean within each subgroup is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively. CLL patients (filled symbols) and healthy donors =HD (open symbols).

(A) CD28-



(B) CD57+

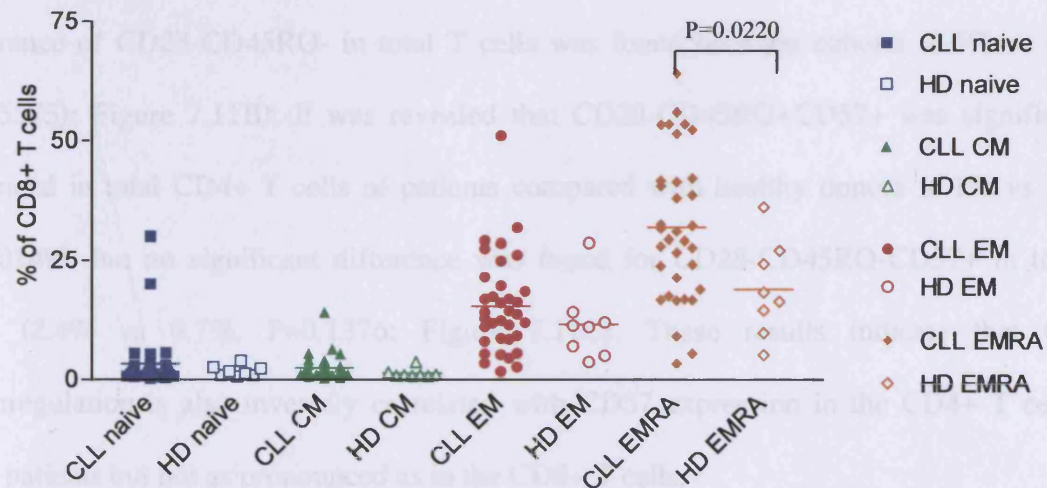


Figure 7.10 Percentage of subsets that are CD28- and CD57+ in total CD8+ T cells of CLL patients (n=34) and healthy donors (n=8). The number of positive cells was determined by flow cytometry (FACS Cantoll). Lymphocytes were gated based on their forward and side scatter profile. T cells were gated on CD3+ CD8+. T cells subsets were defined based on the expression of CCR7 and CD45RO. Naive (CCR7+CD45RO-), Central Memory (CM) (CCR7+CD45RO+), Effector Memory (EM) (CCR7-CD45RO+), and Effector (EMRA) (CCR7-CD45RO-). These subsets were further gated into CD28- or CD57+ cells. The percentage is of CD8+ total cells. The mean within each subgroup is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively. CLL patients (filled symbols) and healthy donors =HD (open symbols).

7.3.2 CD4+ T cells

The co-expression of CD28, CD45RO and CD57, and naïve/memory subsets were also examined in CD4+ T cells, in order to know whether a similar pattern of differentiation could be observed in CD4+ T cells. This data showed an increase of CD28- in both CD45RO+ (14.4% vs 4.4%, $P=0.0148$) and CD45RO- (11% vs 3.1%, $P=0.1130$) compartments of CD4+ T cells from CLL patients compared to healthy donors (Figure 7.11A). However, only CD28- cells in CD45RO+ was significantly different from healthy donors. Moreover, the CD28-CD45RO+ in total CD4+ T cells of patients was significantly enhanced compared to healthy donors (8.2% vs 1.9%, $P=0.0072$), but no significant difference of CD28-CD45RO- in total T cells was found between cohorts (2.6% vs 0.7%, $P=0.5575$); Figure 7.11B). It was revealed that CD28-CD45RO+CD57+ was significantly enhanced in total CD4+ T cells of patients compared with healthy donors (6.1% vs 1.4%, $P=0.0149$), but no significant difference was found for CD28-CD45RO-CD57+ in total T cells (2.4% vs 0.7%, $P=0.1376$; Figure 7.11C). These results indicate that CD28 downregulation is also inversely correlated with CD57 expression in the CD4+ T cells of CLL patients but not as pronounced as in the CD8+ T cells.

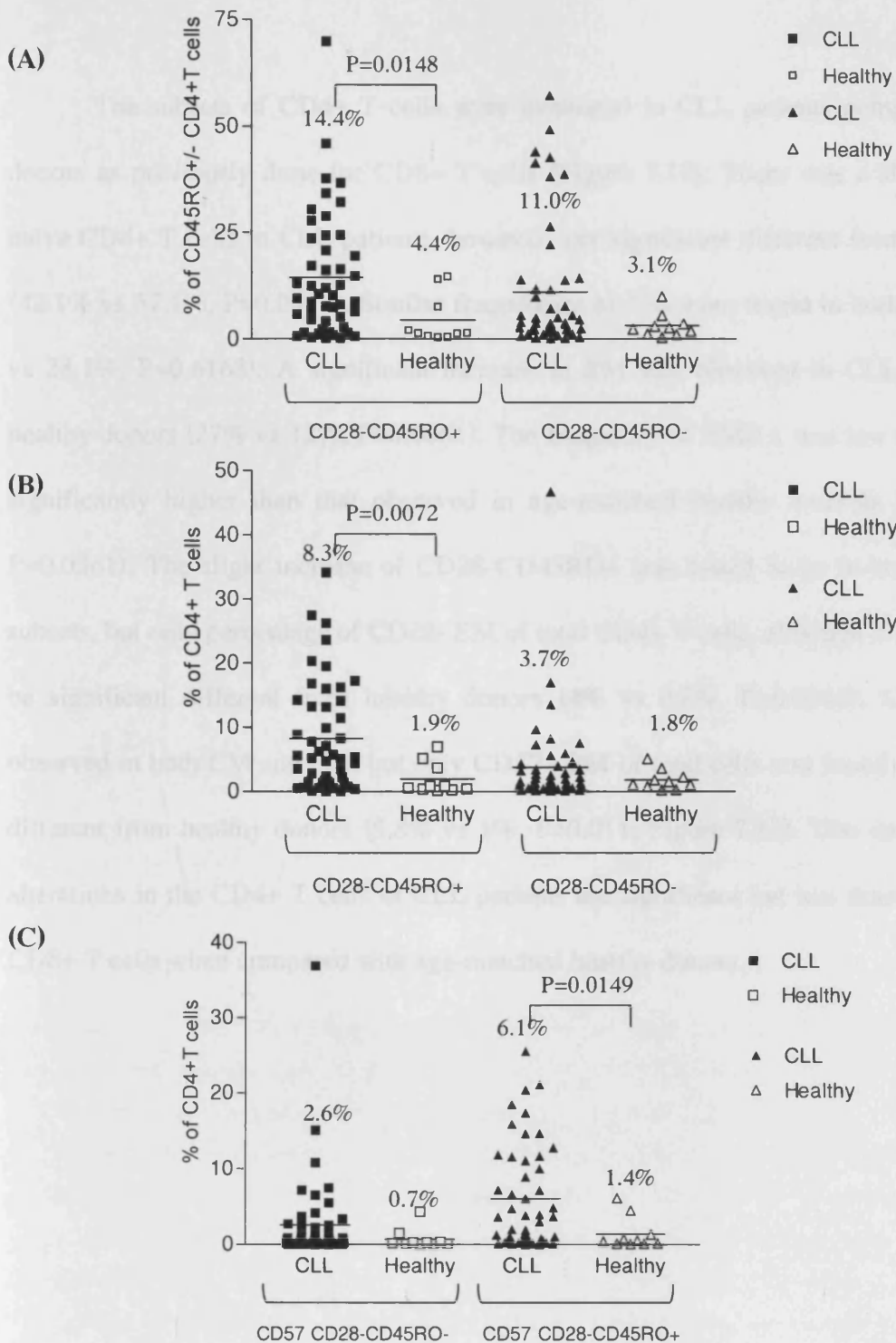


Figure 7.11 Expression of CD28, CD45RO and CD57 in CD4+ T cells of CLL patients (n=49) and healthy donors (n=10). The number of positive cells was determined by flow cytometry (FACS CantoII). Lymphocytes were gated based on their forward and side scatter profile. T cells were gated on CD3+ CD4+. (A) % CD28- within CD45RO+ and CD45RO- CD4+ T cells. (B) % CD28-CD45RO- of CD4+ T cells. (C) % CD28- CD45RO+ CD57+ and % CD28- CD45RO- CD57+ of CD4+ T cells. The mean within each subgroup is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively. CLL patients (filled symbols) and healthy donors (open symbols).

The subsets of CD4⁺ T cells were evaluated in CLL patients compared to healthy donors as previously done for CD8⁺ T cells (Figure 7.12). There was a slight decrease in naïve CD4⁺ T cells in CLL patients, however, not significant different from healthy donors (42.1% vs 57.1%, $P=0.0518$). Similar frequencies of CM were found in both cohorts (25.9% vs 28.1%, $P=0.6168$). A significant increase in EM was observed in CLL compared with healthy donors (27% vs 12%, $P=0.0061$). The frequency of EMRA was low in CLL cells but significantly higher than that observed in age-matched healthy controls (4.8% vs 2.7%, $P=0.0361$). The slight increase of CD28-CD45RO⁺ was found to be in both CM and EM subsets, but only percentage of CD28- EM of total CD4⁺ T cells, although low, was shown to be significant different from healthy donors (4% vs 0.8%, $P=0.0045$). CD57⁺ was also observed in both CM and EM, but only CD57⁺ EM of total cells was found to be significant different from healthy donors (5.8% vs 1%, $P=0.011$; Figure 7.13). This data indicates that alterations in the CD4⁺ T cells of CLL patients are significant but less dramatic than in the CD8⁺ T cells when compared with age-matched healthy donors.

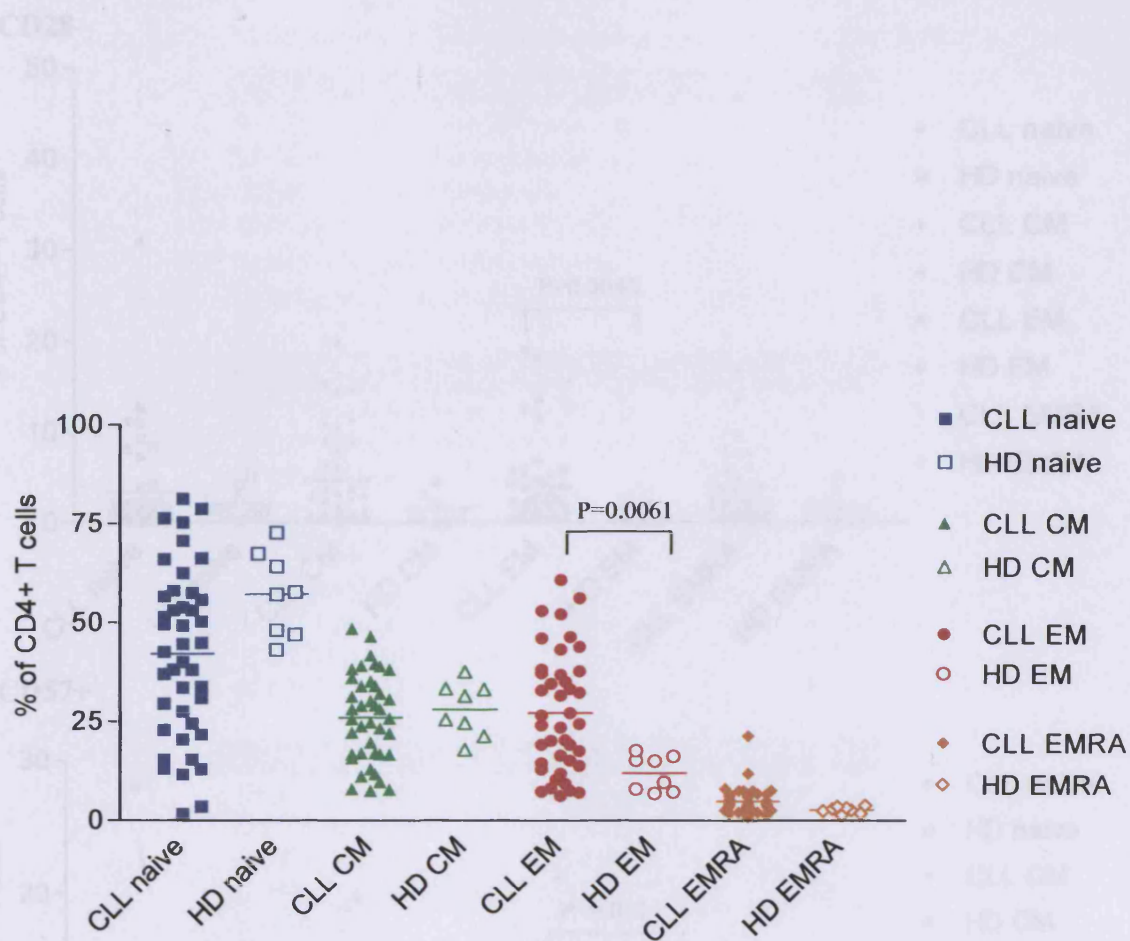


Figure 7.12 CD4+ T cell subsets of CLL patients (n=42) and healthy donors (n=8). The number of positive cells was determined by flow cytometry (FACS Cantoll). Lymphocytes were gated based on their forward and side scatter profile. T cells were gated on CD3+ CD4+ cells. T cells subsets were defined based on the expression of CCR7 and CD45RO. Naive (CCR7+CD45RO-), Central Memory (CM) (CCR7+CD45RO+), Effector Memory (EM) (CCR7-CD45RO+), and Effector (EMRA) (CCR7-CD45RO-). Heterogeneity inside each subset was defined by co-expression of costimulatory molecules CD27 and CD28 was not included. The mean within each subgroup is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively. CLL patients (filled symbols) and healthy donors =HD (open symbols).

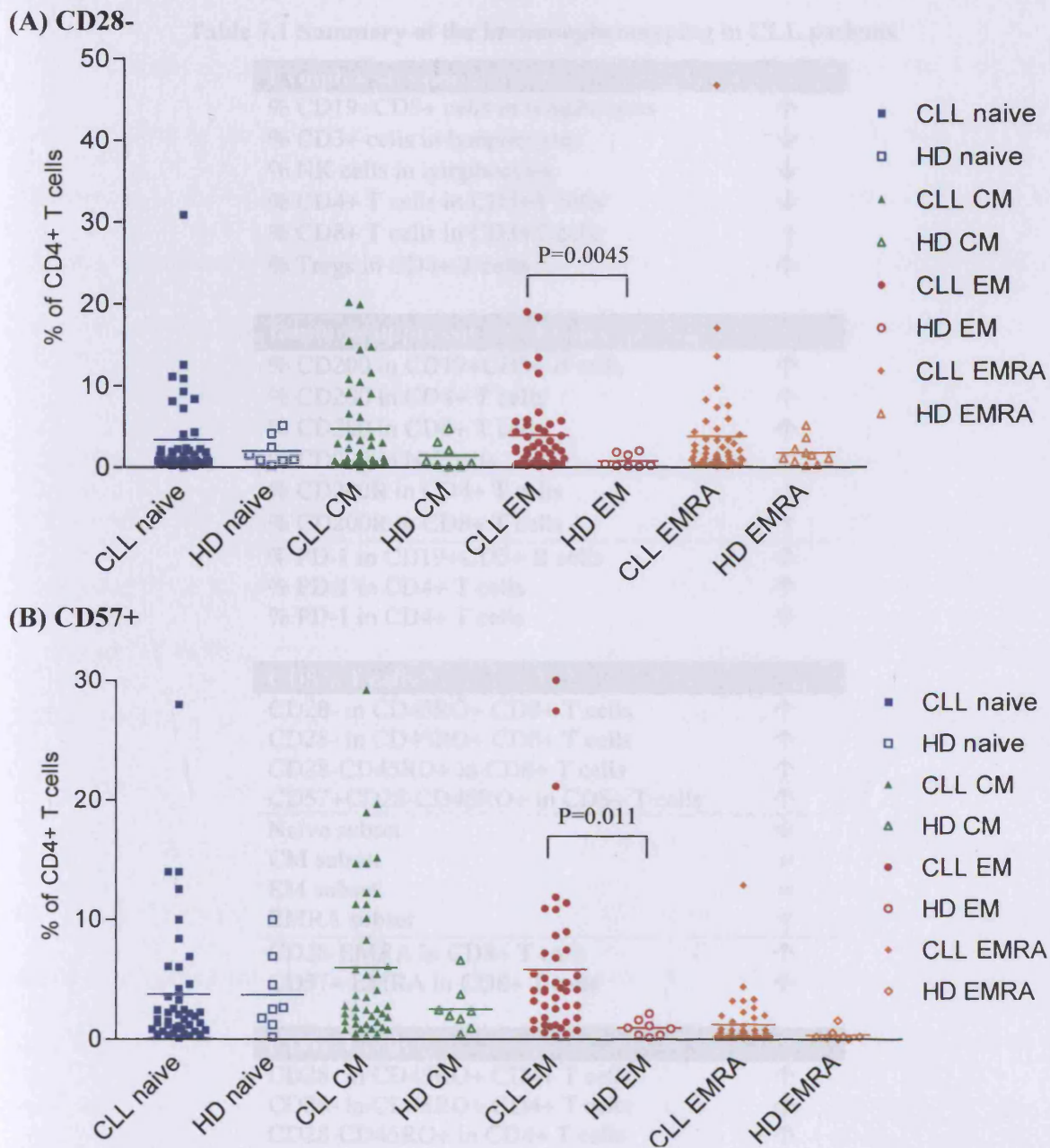


Figure 7.13 Percentage of subsets that are CD28- and CD57+ in total CD4+ T cells of CLL patients (n=42) and healthy donors (n=8). The number of positive cells was determined by flow cytometry (FACS Cantoll). Lymphocytes were gated based on their forward and side scatter profile. T cells were gated on CD3+ CD4+. T cells subsets were defined based on the expression of CCR7 and CD45RO. Naive (CCR7+CD45RO-), Central Memory (CM) (CCR7+CD45RO+), Effector Memory (EM) (CCR7-CD45RO+), and Effector (EMRA) (CCR7-CD45RO-). These subsets were further gated into CD28- or CD57+ cells. The percentage is of CD8+ total cells. The mean within each subgroup is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively. CLL patients (filled symbols) and healthy donors =HD (open symbols).

Table 7.1 Summary of the immunophenotyping in CLL patients

Lymphocytes populations	
% CD19+CD5+ cells in lymphocytes	↑
% CD3+ cells in lymphocytes	↓
% NK cells in lymphocytes	↓
% CD4+ T cells in CD3+ T cells	↓
% CD8+ T cells in CD3+ T cells	↑
% Tregs in CD4+ T cells	↑
Immunosuppressive molecules	
% CD200 in CD19+CD5+ B cells	↑
% CD200 in CD4+ T cells	↑
% CD200 in CD8+ T cells	↑
% CD200 in NK cells	↑
% CD200R in CD4+ T cells	↑
% CD200R in CD8+ T cells	↑
% PD-1 in CD19+CD5+ B cells	↑
% PD-1 in CD4+ T cells	↑
% PD-1 in CD8+ T cells	↑
CD8 + T cells	
CD28- in CD45RO+ CD8+ T cells	↑
CD28- in CD45RO+ CD8+ T cells	↑
CD28-CD45RO+ in CD8+ T cells	↑
CD57+CD28-CD45RO+ in CD8+ T cells	↑
Naïve subset	↓
CM subset	=
EM subset	=
EMRA subset	↑
CD28-EMRA in CD8+ T cells	↑
CD57+ EMRA in CD8+ T cells	↑
CD4+ T cells	
CD28- in CD45RO+ CD4+ T cells	↑
CD28- in CD45RO+ CD4+ T cells	↑
CD28-CD45RO+ in CD4+ T cells	↑
CD57+CD28-CD45RO+ in CD4+ T cells	↑
Naïve subset	↓
CM subset	=
EM subset	↑
EMRA subset	↑
CD28-EM in CD4+ T cells	↑
CD57+ EM in CD4+ T cells	↑
↓↑ significantly different from healthy controls	
↓↑ trend but not significantly different from healthy controls	

7.4 Discussion

The results presented in this chapter provide further evidence that may help in the understanding of the failure of T cells to mount effective responses in CLL patients. Several differences in the frequencies of immune cell subsets and phenotypes were detected in CLL patients compared with age-matched healthy donors. Some of these could explain the immune dysfunctions in CLL that prevent the generation of an anti-tumour response.

CLL patients have an increased frequency of leukaemia CD19+CD5+ B cells, accompanied by a decrease in frequency of T cells, and NK cells. Although the frequency of T cells and NK cells in the peripheral blood is decreased due to the accumulation of malignant B cells, previous studies have shown that the absolute number of circulating T cells and NK cells is usually elevated and associated with the clinical stage (Dianzani *et al.*, 1994; Goolsby *et al.*, 2000; Totterman *et al.*, 1989; Vuillier *et al.*, 1988). Elevated numbers of circulating T cells with a reduced CD4:CD8 ratio has been frequently reported in CLL, particularly in the more advanced stages of the disease (Goolsby *et al.*, 2000; Platsoucas *et al.*, 1982; Totterman *et al.*, 1989; Vuillier *et al.*, 1988). Indeed, it was found in this study that more than half of the patients had a reduced/inverted CD4:CD8 ratio. These findings imply that these lymphocyte populations are expanding in response to malignant cell growth and may be contributing to the growth potential and survival of leukaemia cells. Despite the associated expansion of both neoplastic B cells and T cells, CLL patients have an abnormal T cell function and there may be several mechanisms that contribute to immune dysfunction.

In this study, a significant increase of Tregs was found in CLL patients compared with healthy donors. This is in accordance with previous reports that show that the frequency of Tregs was higher in patients and increased with progression of the disease (Beyer *et al.*, 2005; Giannopoulos *et al.*, 2008). These groups also showed that Treg cells were functionally capable of abrogating T cell responses against tumour and viral antigens (Giannopoulos *et al.*, 2008), and the inhibitory function of Tregs was reduced in patients treated with

fludarabine (Beyer *et al.*, 2005). In this context, the increased frequency of Tregs could be inhibiting effective anti-tumour immunity. It will be interesting to correlate the present findings with prognosis of the patients as this may give further information about the relationship between immunosuppression and clinical prognosis. However, it seems likely that the study would require more patients in order to provide definitive results.

In accordance with previous findings (McWhirter *et al.*, 2006), CD200 was shown to be upregulated on B cells of all CLL patients. Interestingly, CD200 was also found to be significantly increased on CD4⁺ and CD8⁺ T cells but was reduced on NK cells when compared to healthy donors. However, only a slight increase of expression of its receptor CD200R was shown on T cells of CLL patients and this was not significantly different from healthy donors. It has been reported that memory T cells express higher amounts of CD200R than naïve or effector T cells (Rijkers *et al.*, 2008). It will be interesting to examine expression of CD200R in the memory T cell subsets of CLL patients. Due to the limited availability of fluorochromes for CD200R, this antibody could not be used in the same panel as the T cell subsets markers and thus this analysis was only carried on total CD8⁺ and CD4⁺ T cells. As this receptor is also found on DCs and macrophages, it will be interesting to see if its expression is upregulated on these myeloid cells in CLL patients. Given that it was shown here that T cells also have an increased expression of CD200, it can be envisaged that not only expression of CD200 on B cells but also on T cells may be contributing to the immunosuppressive interactions with DCs/macrophages, and CD200 overexpression may negatively affect immune responses through multiple different mechanisms.

The CD200 overexpression in CLL cells has been shown to inhibit T cell proliferation, suppress anti-tumour responses, and enhance Tregs (Kretz-Rommel *et al.*, 2007; Pallasch *et al.*, 2009). Blockade of CD200 has been shown to be efficient in augmenting antigen-specific T cells and deregulating the presence of Tregs (Pallasch *et al.*, 2009). The overexpression of CD200 may explain the failure to detect T cell responses

against Bax peptides and MART-1 peptide. Therefore, it would be interesting to evaluate if the blockade of CD200 can improve T cell responses against MART-1 and more importantly against Bax peptides.

The immunosuppressive PD-1 molecule was also found to have increased expression on T cells and B cells of CLL patients compared with healthy donors. This is in accordance with a recent report showing that PD-1 is expressed on CLL cells (Xerri *et al.*, 2008). Higher percentages of PD-1 were observed in both CD4+ T cells and CD8+ T cells compared with B cells. This increased expression may be preventing the T cell activation and induction of an effective naive immune response. Further evaluation of PD1/PD-1 ligands on different populations, including also Tregs, may provide further elucidation about the interactions of T cells expressing PD-1 with malignant B cells and if PD-1/PD-Ls have a role in the survival of malignant B cells. A recent study showed that the blockade of PD-1 can augment expansion and functionality of CTLs specific for melanoma antigens (Wong *et al.*, 2007). Thereby, it would be interesting to evaluate the effect of PD-1 blockade on T cell responses in CLL patients.

The analysis of CD4+ and CD8+ T cell repertoire revealed additional abnormalities that corroborate with previous findings. The results presented here showed that CD28 is downregulated particularly in CD8+ T cells of CLL patients and can be associated with increase of CD57+ T cells. The proportion of CD57+ CD28- among CD4+ T cells was less dramatic yet significantly increased in the CD45RO compartment of CLL patients, indicating that they are antigen experienced. Reduced expression of CD28 and increased expression of CD57 in T cells of CLL patients has been reported by other groups (Rossmann *et al.*, 2003; Scrivener *et al.*, 2001; Serrano *et al.*, 1997; Van den Hove *et al.*, 1998a; Van den Hove *et al.*, 1998b). Increased oligoclonal T cell expansions, predominantly expressing CD57+ are commonly found in CLL patients, and prevail in higher percentages in CLL patients with advanced disease stages than in early stages (Serrano *et al.*, 1997) .

The present results support the concept of an ongoing activation of T cells *in vivo*. Downregulation of CD28 and expression of CD57 have been associated with replicative senescence, and can lead to inability of T cells to proliferate and enhanced tendency to apoptosis (Brenchley *et al.*, 2003; Scheuring *et al.*, 2002; Van den Hove *et al.*, 1998a). Lack of CD28 and upregulation of CD57 can occur in response to repeated/chronic antigenic stimulation and has been associated with ageing. Several lines of evidence suggest that the alteration in the T cell subsets in both patients and healthy elderly can be associated with chronic antigenic stimulation by viral infections, such as CMV (Mackus *et al.*, 2003; Pita-Lopez *et al.*, 2009).

The analysis of CD8⁺ T cell subsets revealed a relative shift towards differentiated T cells. A decreased proportion of naïve CD8⁺ T cells and an increased proportion of EM and EMRA were observed in CLL patients. The relative expansion of CD8⁺ EMRA and EM T cell subsets has been previously reported by another group, but only 11 CLL patients were analysed (Schreeder *et al.*, 2008). This study is in accordance with those findings and extends these observations to a cohort of 34 patients. However, no significant difference of the increase of EM and EMRA was found between CLL patients and healthy donors. Analysis of a larger cohort of age-matched healthy donors will probably be necessary for significant results. The differences in the CD4⁺ T cell subsets were less pronounced than in the CD8⁺ T cells. A slight decrease of the naïve subset (but not significant), and a significant increase of the EM were found in CLL patients as compared with the healthy donors. These results are consistent with a recent study, which assessed CD4⁺ T cells subsets in patients with mutated (n=71) and unmutated (n= 42) IgV_H genes. This study showed a significant increase in relative numbers of CM and EM T cells which was associated with progressive disease (Tinhofer *et al.*, 2009).

The present observations confirmed previous reports and in addition demonstrated that increased CD28⁻ or CD57⁺ cells in total CD8⁺ T cells were found particularly in the EM

and EMRA subsets. As for CD4⁺ T cells, the CD28⁻ or CD57⁺ cells were found to be in the CM, EM and EMRA subsets, but only CD28⁻ EM / CD57⁺ EM were significantly different from age-matched controls. These observations indicate that several alterations in the T cell subsets in CLL patients may contribute to the impaired regulation of malignant B cells.

Initiation of a T cell response requires priming in the presence of CD28 co-stimulation. The reduced CD28 expression may cause an impaired capability of T cells to interact with DCs and CLL cells, consequently leading to impaired interaction and prevention of T cell activation. Furthermore, these findings are consistent with functional studies demonstrating a defective immune synapse between T cells and CLL (Ramsay *et al.*, 2008), and with the observations that CLL can manipulate the gene expression profile of T cells through cell-cell contact, such as abnormal expression of genes involved in CD4⁺ T cell differentiation and impaired production of cytolytic granzyme granules in CD8⁺T cells (Gorgun *et al.*, 2005). It appears that malignant B cells exert suppressive effects upon systemic T cells to prevent generation or amplification of an anti-tumour response. Taking these investigations into account, it is not perhaps surprising that priming of naïve T cell responses even against the highly immunogenic tumour antigen MART-1 is compromised in CLL patient samples. These results suggest that T cells in CLL may be incapable of initiating, continuing or completing an immune response to the leukaemia B cells and other antigens and thus may be involved directly in sustaining the tumour.

This data implies that peripheral CD8⁺ and CD4⁺ T cell repertoires are skewed towards an effector memory/effector phenotype in CLL patients. The expansion of these subsets supports the concept that the generation of these cells is driven by repeated/chronic stimulation. The expansion of EM and EMRA might be related with the fact that CMV infection can drive cells to chronic stimulation and exhaustion. Nevertheless, serological analysis needs to be performed in these patients to confirm whether they are CMV positive and the presence of CMV has an impact in the changes of T cell subsets observed in these

patients. Intuitively, large numbers of effector T cell subsets appear to be beneficial because of their ability to mount immediate protection. However, these cells may not be suitable for long-term protection as there is a reduction in the diversity and functional integrity of the T cell subsets. The increase of CD57⁺CD28⁻ effector T cells indicates that a large proportion of the effector cells are highly differentiated in a state of replicative senescence and thus have the least proliferative potential amongst the T cell subsets. This contributes to a decrease competence to effectively respond to re-infection or maintain memory for tumour antigens expressed by relapsing tumour cells. Moreover, decreased diversity of the naïve T cell repertoire can affect the capacity of these patients to resist infections to which they had not previously encountered, or to respond to the appearance of new tumour antigens. This atypical differentiation could contribute to the inability to respond appropriately to persistent infections or persistent exposure to tumour antigens, and thus to the failure of cellular immune control of CLL cells.

In line with other reports, the findings of this pilot study indicate there are prominent dysregulations in both CD8⁺ and CD4⁺ T cells that are almost certainly involved in the unresponsiveness of T cells to tumour antigens, explaining the failure to prime T cell responses against Bax and MART-1. Although some of the frequencies were not significantly different between CLL patients and healthy donors, it must be taking into consideration that only 8-10 healthy donors were included in this analysis due to the limited availability of age-matched controls; most people in the department are under 50 years of age, with an average of 30 years, whereas age of CLL cohort was between 45 to 90 years. Further experiments including larger numbers of age-matched controls will be done to confirm these differences. Furthermore, additional differences may potentially be found between patients with different disease stages and thus correlation between this analysis with clinical data of these patients is going to be performed in the near future.

In order to gain further insight into the emergence of T cell abnormalities and expression of immunosuppressive molecules in CLL, this study will be extended to 100 patients and other parameters, such as mean fluorescence intensity of certain markers, frequencies of populations within lymphocytes gate, CD3+ gate and inside each subgate, will be included. Both CD4+ and CD8+ T cell subsets will be further characterised using the heterogeneity analysis (CD57, CD27 and CD28). Altered numbers and abnormal functions of T cells have extensively been reported in CLL patients, but differences within the specific naïve and memory T cell subsets has not been studied in great detail. In conjunction with these markers, it will also be of great interest to study T cell function, such as cytokines and cytotoxic granule secretion. This study will provide useful data to discriminate T cell changes in CLL patients, namely by showing that these are caused entirely by altered frequencies of the naïve/memory subsets or by altered heterogeneity within each of the subset. The data generated will be analysed in conjunction with a large pre-existing patient database to find cell phenotypes that may correlate with clinical outcome. This will most likely allow grouping patients with different distributions of naïve and memory T cell subsets and/or with other immune alterations described here.

Immunophenotyping by flow cytometry is a valuable tool in the evaluation of immunological status, especially in haematological malignancies that involve alterations in lymphocyte populations. This study has defined phenotypes that may correlate with disease progression/response to treatment and may have implications in the development of strategies to overcome T cell tolerance for immunotherapy of CLL using Bax or other tumour antigens. Different strategies could be carried out *in vitro* to overcome T cell tolerance. These include improving antigen presentation by CLL cells, antigen-specific T cell activity by blocking immunosuppressing molecules, such as CD200 and PD-1 and/or using immunomodulating drugs. Studies are underway to address these issues.

Chapter 8

Final Discussion

The results presented in this study have provided evidence that supports a novel approach of immune targeting Bax protein based on its unique expression pattern in cancer. This is a novel concept because definition of tumour antigens usually relies on steady-state over-expression of proteins in cancer cells. Here, it was proposed that a self-protein with low or unstable expression can be a candidate tumour antigen. This was based on the hypothesis that its abnormal proteasomal degradation in cancer cells will result in enhanced generation of Bax peptides that can bind to HLA class I molecules and be displayed at the surface of cancer cells for CD8⁺ T cell recognition. Thus, Bax-specific T cells should be able to recognise and kill tumour cells, but not healthy cells.

This research defined Bax as a new potential tumour antigen for immunotherapy. Chapter 3 showed for the first time that Bax peptides can be immunogenic to human CD8⁺ T cells. Chapter 4 demonstrated that Bax-specific T cell lines/clones can be generated from low frequency peptide-specific T cells, and proved the concept that Bax-specific T cells can kill tumour cells in HLA-A2 restricted manner. Bax specific T cells were capable of recognising tumour cells from several types of cancer, revealing a unique pattern of specificity. Chapter 5 provided further evidence to support the hypothesis, by showing that Bax is targeted for proteasomal degradation in selective tumours, but not in healthy cells. A correlation between T cell reactivity and Bax degradation was observed. Chapter 6 showed that it is difficult to generate T cell responses against Bax and other tumour antigens in CLL patients, indicating that both naïve and memory T cells are compromised in CLL patients. Chapter 7 demonstrated that the unresponsiveness of T cells in CLL patients could be related to various immune abnormalities, such as increased expression of immunosuppressing molecules, Tregs and skewed T cell differentiation.

Using a reverse immunology approach, this study revealed that T cell precursors for Bax-derived peptides are present in the peripheral blood of healthy donors. The first major implication from this study is the identification of four potential immunogenic Bax-derived peptides (p610, p612, p613 and p614). Furthermore, implementation of a technology of selecting T cells with low frequency based on their IFN γ secretion resulted in the generation of purified Bax specific T cell lines and clones from two donors. Characterisation of Bax-specific T cell lines/clones unveiled Bax p610 and p613 peptides as the most promising epitopes. These HLA-A2-binding epitopes were able to elicit Bax-specific CTL responses *in vitro* against tumour cells expressing endogenous Bax peptides, but not against non-transformed cells (PHA blasts and skin fibroblasts).

A Bax-specific T cell clone was able to recognise HLA-A2+ tumour cells of various types, including CLL, HPV transformed cervical, osteosarcoma and hepatocellular carcinoma cells. This implies that similar Bax peptides are expressed and complexed with MHC class I molecules on different cancer cell types. Killing of tumour cells by the T cell clone was antigen-specific and HLA-A2 restricted. Moreover, it was established that the proteasome pathway was involved in the generation of endogenous Bax peptides in tumour cells as lysis by Bax-specific T cells was reduced in tumour cells treated with a proteasome inhibitor.

T cell reactivity against the tumour cells appeared to be correlated with Bax expression in these cells. The hypothesis was strengthened by the observation of increased Bax degradation by the proteasome in selective cancer cells, as detected by western blotting. Cancer cells that showed increased degradation of Bax were recognised by the Bax-specific T cells, whereas the normal cells and cancer cells with no substantial increased degradation were not recognised by the T cells. Together, these findings provide key evidence that support the central hypothesis. To further prove the hypothesis, Bax stability needs to be evaluated in the different cells tested as this will confirm that rapid/high degradation rates of Bax occurs in the tumour cells and probably is involved in the generation of T cell epitopes in the tumour

cells. However, increased Bax degradation is not a guarantee for recognition of tumours by Bax-specific CTLs as other tumour cells may be presenting different Bax epitopes (other than the Bax p610 and p613). Additionally, it would be of interest to use mitochondrial and cytosolic fractions to evaluate the location of Bax in these cancer cells compared with normal cells.

A way to directly validate this hypothesis will be by elution of natural processed peptides complexed with MHC class I molecules from the tumour cells tested in this study and analyse their sequences by mass spectrometry. This will prove that the Bax protein is indeed endogenously processed and Bax-derived peptides associated with MHC class I molecules are present in these tumour cells. This is currently being evaluated as one of the next steps to explore Bax protein as a tumour antigen.

The results in this thesis support the concept that decreased stability of the Bax protein is contributing to T cell recognition. This is not an unusual concept, since several studies have shown that CTL recognition of endogenously processed antigens can be governed by instability or susceptibility to degradation of proteins, rather than high levels of steady-state expression (Castilleja *et al.*, 2001; Gileadi *et al.*, 1999; Tobery & Siliciano, 1997; Vierboom *et al.*, 2000). In particular, CTL recognition of the ubiquitously expressed tumour suppressor self-protein p53 was found towards tumour cells with enhanced proteasomal degradation of p53 (Sirianni *et al.*, 2004; Vierboom *et al.*, 2000). Importantly, these findings corroborate with the concept that enhanced protein degradation by the ubiquitin-proteasome pathway can lead to the induction of vigorous CTL responses *in vitro* and *in vivo*.

To fully exploit the potential usefulness of Bax epitopes for immunotherapy, it is necessary to screen for T cell responses against Bax in cancer patients. T cell responses against Bax were evaluated to a limited extent in CLL patients in this thesis. CLL is a good

model system for this study because autologous primary tumour cells can be used as targets for evaluation of T cell recognition, whereas for other types of cancer, no tumour biopsies could be obtained and only cancer cell lines were used. Detection of T cell responses was limited and only two patients were capable of mounting a response against Bax peptides. In a way, this was expected as even the MART-1 antigen, which is a strong model antigen for naïve T cell responses, was not able to prime immune responses in most patients. In addition, the immunophenotyping data revealed a decrease in the percentage of naïve CD8⁺ T cells and a skewed phenotype towards memory, as well as an increase expression of immunosuppressive molecules, such as CD200 and PD-1 in most patients. This would have a suppressive effect in priming tumour specific T cell responses and thus explains why it is so difficult to mount a naïve response in these patients using protocol similar to the one used for healthy donors.

Along with the defects on both antigen presenting and effector mechanisms, the lack of appropriate stimulation protocol and low numbers of CD3⁺ T cells obtained from each sample made it difficult to fully investigate T cell responses against Bax in CLL patients. However, by developing appropriate *in vitro* stimulation strategies to overcome the tolerance mechanisms, such as the use of CD40-activated B cells as APC, addition of antibodies to block immunosuppressive effects and/or the immunomodulatory drug lenalidomide to recover antigen presentation, there is the potential to improve the detection of both naïve and memory peptide-specific T cells. The Bax-specific T cell clone was able to recognise CLL cells from most of the HLA-A2⁺ patients tested, thus implying that Bax-derived peptides are present in these malignant cells. Therefore it will be important to fully investigate the frequency of Bax specific T cells in patients, so as to evaluate their potential utility in an immunotherapeutic setting.

Following on from this, it would be interesting to screen for T cell responses against Bax epitopes in HLA-matched osteosarcoma patients and women with varying severity of

cervical disease due to the fact that Bax-specific T cell clone was able to kill osteosarcoma cell lines, HPV-transformed cervical cancer cell lines, but not HPV-negative cervical cancer cells. Infection with high risk HPVs is associated with the development of high grade cervical intraepithelial neoplasia (CIN) and cervical cancer (Walboomers *et al.*, 1999; zur Hausen, 1996). A study including women with incident HPV infection, low grade CIN, high grade CIN or cervical cancer would give an indication as to how frequently responses can be detected against these epitopes in the different disease states. By studying these cohorts over an extended period of time it would also be possible to track changes in the immune responses to these epitopes that may correlate with disease clearance or progression. Together with the screening of patients, it would also be helpful to obtain tumour biopsies during the course of disease to analyse Bax expression and its degradation, so it would be possible to correlate T cell responses with the degree of Bax degradation. It would be predicted that Bax degradation would increase as disease progresses and possibly increased frequencies of Bax-specific T cells would be detected.

One benefit of identifying Bax epitopes is that, armed with the knowledge of the HLA-A2 restriction, it will be possible to directly monitor the appropriate donors/patients to identify the frequency and nature of immune responses in an epitope-specific context. This knowledge also allows the design and generation HLA class I-peptide tetramers, which is currently being considered as the next step for this research. Tetramers are a helpful immunological tool for monitoring peptide-specific T cell responses, which can be used to facilitate precise phenotyping of peptide-specific T cells by flow cytometry and have the ability to detect low frequency T cells directly *ex vivo* from PBMC. So, it will of great interest to design a tetramer for Baxp613:HLA-A2 for monitoring T cell responses directly *ex vivo* and after stimulation *in vitro* in CLL patients.

Based on the reactivity of the T cell clone KSBV17 (Chapter 4), Bax could be regarded as a broad cancer antigen and Bax peptides as substrate for a possible immunotherapy for multiple human cancers. To our knowledge, no other tumour antigen has this unique pattern towards CLL, osteosarcoma and HPV-transformed cervical cancer. Although several conventional therapies exist for these different cancers, they still remain incurable and thus the development of new immunotherapeutic approaches for these cancers is warranted to improve the survival and quality of life of patients.

Bax epitopes identified in this study offer the opportunity to design novel immunotherapies for multiple cancers. However, the possibility that this self-antigen would exhibit a level of autoimmunity in the host immune system is a concern. It has been reported for certain self-antigens that anti-tumour responses can also result in autoimmunity. For example, autoimmune reactions against melanocytes causing vitiligo can occur during melanoma treatment (Yee *et al.*, 2000). Studies of immunity against CEA, an oncoprotein also expressed in normal colon epithelium and in most gut carcinomas, raised more serious concerns about autoimmune side effects. Vaccination for an anti-tumour response resulted in severe intestinal autoimmunity associated with weight loss and mortality in mice (Bos *et al.*, 2008).

However, a number of previous reports using self-antigens, such as the ubiquitous self-protein p53 (wild-type), revealed that tolerance can be overcome without any autoimmune damage to normal tissue, yielding CTLs of sufficient avidity to recognise tumour cells expressing endogenous antigen (van der Burg *et al.*, 2002; Vierboom *et al.*, 1997; Vierboom *et al.*, 2000). It has been shown that potentially autoreactive wild-type p53-specific CTLs can eradicate established tumours and persist in mice without autoimmune tissue damage (Vierboom *et al.*, 1997; Vierboom *et al.*, 2000). A explanation for the tumour selectivity could be that the normal tissues lack danger signals and have insufficient quantities of peptides display by the MHC class I molecules and be immunologically ignored, thus

might be protected against the destruction by the autoreactive peptide-specific CTLs (Vierboom *et al.*, 1997). Several other self-proteins that are ubiquitously expressed have been tested in clinical trials, such as HER2/neu, hTERT and survivin (Brunsvig *et al.*, 2006; Honma *et al.*, 2009; Peoples *et al.*, 2005; Tsuruma *et al.*, 2004). These clinical trials have shown that vaccination against these self-proteins was feasible and safe, without any serious autoimmunity reaction being observed.

Although the possibility of an autoimmune attack was not formally ruled out in this present study, it was possible to show that the Bax-specific T cell clone had no specific cytotoxic activity towards normal healthy cells. It is proposed that in normal cells Bax is largely confined in the cytosol in an inactive stable form and not efficiently targeted for proteasomal degradation. A recent report has shown that only when Bax undergoes conformational changes and translocation to the mitochondria, it triggers ubiquitin recognition and Bax proteasomal degradation in cancer cells (Yu *et al.*, 2008). Thus, it is likely that there will not be sufficient peptides for MHC class I pathway on normal cells for recognition by CD8⁺ T cells. A distinctly higher degradation rate of Bax resulting in higher density of the CTL epitopes of interest on MHC class I of the tumour cells should allow the tumour cells to be distinguished from normal cells for T cell recognition. Another possibility is that tumour cells have different proteasomes compared with normal cells and can process epitopes differentially. Nevertheless, it will be of great importance to test Bax in pre-clinical *in vivo* models to exclude the possibility of autoimmunity. This could be done by adoptive transfer of autologous Bax-specific T cells grown *ex vivo* into HLA-A2/Kb transgenic mice to observe whether any autoimmunity incident develops.

The findings from this study argue that potential tumour antigens should not be restricted to stable self-proteins that are over-expressed, and that self-proteins that are short-lived and have an increased rate of degradation in tumours should also be taken into

consideration. Similar to Bax, p53 degradation by the proteasome appears to be correlated to enhanced processing of p53 epitopes and increased T cell recognition (Sirianni *et al.*, 2004; Vierboom *et al.*, 2000). This study together with those findings constitutes the ultimate illustration of a potential novel category of tumour antigens: unstable self-proteins with an increased degradation rate by the proteasome. Another protein (retinoblastoma protein) with a similar pattern in cancer that could belong to this category is currently being studied in our laboratory.

Definition of novel tumour antigens is essential for their potential use in immunotherapy and also as a way of tracking tumour-specific T cells during the course of treatment and disease. An advantage of the identification of new tumour antigens is that it increases the pool of immunogenic peptides that can be used to recruit a larger, more potent repertoire of T cells targeting the tumour. A rational immunotherapy should satisfy various criteria such as range of application in terms of tumour types addressed, diversity of antigens addressed to ensure disease coverage and prevent tumour escape, and broad coverage of patient populations.

Approaches that target multiple parts of immunity and integrate elements that target tumour escape simultaneously are more likely to result in clinical success. The identification of multiple CTL epitopes from several tumour antigens involved in several cancer traits, such as resistance to apoptosis, abnormal cell cycle regulation and metastasis, represent broadly applicable vaccination targets and should allow the development of various types of immunotherapy (Andersen *et al.*, 2008). Therapeutic vaccines using a mixture of peptides from various antigens alone or pulsed on to DCs would probably be useful to elicit *in vivo* anti-tumour immunity for early stages of cancer or as adjuvant setting in more advanced stages. Combination of anti-cancer vaccination to increase the frequency of tumour-reactive T cells prior, or immediately after, ACT therapy may be another mechanism to boost the

immune system of patients with advanced and metastatic disease that underwent chemotherapy or radiotherapy (Andersen *et al.*, 2008; Copier *et al.*, 2009) .

Since Bax is a ubiquitously expressed self-protein, the choice of patients receiving immunotherapy targeting Bax will be critically dependent on the balance between therapeutic anti-tumour immunity and toxicity. Bax antigen could probably be used for immunotherapy of patients with early stages of diseases. For early and small tumours, only low CTL activities will be required to clear tumour and thus tumour elimination may be accomplished without or before causing any apparent autoimmune symptoms. As for established tumours, strong sustained CTL responses will be required to complete remission of large tumours, and thus may induce strong CTLs responses against the tumour with potential autoimmune reactions (Ludewig *et al.*, 2000). Furthermore, targeting multiple tumour antigens seems important to augment the efficacy and diversity of antitumor immunity, and provide coverage of various tumours of any particular type. An interesting approach would be to incorporate proteins, such as p53, Bax and other proteins from Bcl-2 family, in a multi-epitope setting. Since alterations of these proteins contribute to apoptosis resistance through different mechanisms, targeting these proteins may represent a more efficient strategy to achieve potent anti-tumour immune response, tumour regression and prevent escape in clinical settings.

The results in this study have defined several parameters likely to be important for cancer immunotherapies. These revealed that the selection of appropriate epitopes from Bax protein is relevant to tumour immunity, and imply that not only self-protein that overexpressed in cancer can provide a repertoire of peptide epitopes that are actually presented by tumour cells. It has been proposed that proteins that regulate apoptosis may be widely applicable targets for immunotherapy and strategies designed to target regulators of apoptosis have gained considerable interest for treatment of cancer (Andersen *et al.*, 2005). Since dysregulation of Bax is an essential factor for resistance to apoptosis in several cancers,

Bax derived epitopes may be able to circumvent tumour evasion and thus could be potential immunotherapeutic targets.

In conclusion, this present study provided a first insight into the relationship between expression of Bax protein, CTL recognition of Bax-derived epitopes and tumour antigenicity. A reverse immunology approach demonstrated the ubiquitously expressed Bax as a novel tumour antigen. Several tumour cells naturally processed and presented Bax immunogenic epitopes and induce responses from Bax specific T cells, supporting Bax as a target for anti-cancer T cell immunity.

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