

**Characterisation of T cell Defects in  
Acute Myeloid Leukaemia**

**Helen Rifca Le Dieu**

A thesis submitted for the degree of Doctor of Philosophy,  
Queen Mary University of London

February 2009

**Centre for Medical Oncology  
Institute of Cancer**

## **Dedication**

To Bob. I made it.

## **Acknowledgements**

The work described in this thesis was performed while I was a clinical research fellow in the Cancer Research UK Medical Oncology Unit at St. Bartholomew's Hospital, London. Funding was initially provided by Cancer Research UK (CRUK) until September 2006 when I was awarded a Medical Research Council (MRC) Clinical Research Training Fellowship for 30 months.

I would like to thank my supervisor, John Gribben for giving me the opportunity to spend this time delving into a fascinating field. It may not always have been easy and often it was anything but, however, as he frequently predicted, I did indeed learn a great deal through the process. Jude Fitzgibbon, I thank for his support and pastoral care and Professor Lister, for the feeling of cohesiveness he fosters amongst the clinical fellows. It made a very good working environment.

I also acknowledge David Taussig, who provided so much help, support and advice throughout the duration of this project.

I wish to thank Gullu Gorgun from the Dana Farber Cancer Institute, Boston, Massachusetts with whom I spent four weeks in July 2005. She taught me basic lab techniques and provided protocols for RNA extraction and cell separation as well as introducing me to that world-class institution.

There are many individuals whose help has been crucial to me in this project. They are listed below.

Sameena Iqbal and Karin Summers taught me how to process peripheral blood specimens and freeze down PBMCs.

Derek Davies team in the flow cytometry lab at Cancer Research UK, 44 Lincoln's Inn Fields taught me how to do flow cytometry. Support was

subsequently provided by Linda Hammond from Tumour Biology, Charterhouse Square.

Assistance with the Affymetrix GeneChip® Protocol was provided by Olga Yiannikouris, Elodie Noel and Gael Molloy.

Elodie Noel and Rodrigo Proto-Siquera assisted me with the use of GeneSpring and Ingenuity software for analysis of gene expression data.

Jenny Dunne, Karin Summers, Elodie Noel and Shireen Kassam taught me qRT-PCR technique and analysis.

Alan Ramsay taught me the immune synapse assay, how to use the confocal microscope and provided healthy B and T cell samples for these assays.

Farideh Miraki-Moud taught me the whole blood lysis method for flow cytometry described in section 2.7.2 and also the DNA extraction method in section 2.12.2.

Priya Virappane taught me multiplex PCR and how to analyse GeneScan data.

Emmanuela Carlotti helped with planning the T cell clonality experiments.

Jenny Dunne, Tracy Chaplin and Amanda Dixon-McIver were a constant source of advice.

Andy Williams provided IT expertise.

Tony Allen and Bev Cork from Miltenyi Biotec were always on hand, particularly in times of difficulty, to provide advice.

I must also acknowledge my many willing ‘normal’ volunteers as well as the patients who so generously provided blood samples that have enabled this work to be carried out.

And last but not least, my clinical fellow colleagues and particularly Jane and Derville for getting me through this. We certainly had a lot of fun!

## **Abstract**

Understanding the immune system in patients with cancer and how it interacts with malignant cells is critical for the development of successful immunotherapeutic strategies at a time when novel cancer treatment approaches are required. Acute myeloid leukaemia (AML) results in widespread interaction between the malignant cells and T cells and as such, offers an opportunity to study these interactions.

A flow cytometric analysis of T cells in the peripheral blood of patients presenting with AML illustrated that the absolute number of T cells is increased in AML compared with healthy controls. Furthermore, a large population of CD3+56+ cells was identified. These cells are not natural killer T cells but effector T cells that may represent a failing immunosurveillance mechanism. Two technical issues were explored: how to separate T cells from the peripheral blood of newly diagnosed AML patients and the impact of the method of immunomagnetic cell separation on the gene expression profile of healthy T cells. Gene expression profiling was subsequently performed on T cells from AML patients compared with healthy controls. Global differences in transcription were observed suggesting aberrant T cell activation patterns in AML. As differentially regulated genes involved in actin cytoskeletal formation were noted, a functional assessment of the ability of T cells from AML patients to form immunological synapses was performed. This illustrated that although T cells from AML patients can form conjugates with autologous blasts, their ability to form immune synapses and recruit phosphotyrosine signalling molecules to that signalling interface is impaired. Taken together, these findings demonstrate that numerically T cells are plentiful in AML however they are abnormal in terms of the genes they are transcribing and in their interactions with tumour cells. Targeting immunological synapse formation may represent an important means of improving T cell recognition of tumour cells across a range of cancers.

---

## Publications

Peripheral blood T cells in AML patients at diagnosis have abnormal phenotype and genotype and form defective immune synapses with AML blasts.

**Le Dieu R**, Taussig D, Ramsay AG, Mitter R, Lee AM, Lister TA, Gribben JG.  
Blood (submitted)

Positive immunomagnetic selection has minimal impact on the gene expression profile of T cells.

**Le Dieu R**, Mitter R, Gribben JG.  
BMC Genomics (submitted)

Negative immunomagnetic selection of T cells from peripheral blood of presentation AML specimens.

**Le Dieu R**, Taussig D, Lister TA, Gribben JG.  
Journal of Immunological Methods (submitted)

$E\mu$ -TCL1 mice represent a model for immunotherapeutic reversal of chronic lymphocytic leukemia-induced T cell dysfunction.

Gorgun G, Ramsay AG, Holderried TAW, Zahrieh D, **Le Dieu R**, Quackenbush J, Croce CM, Gribben JG.  
PNAS (accepted for publication)

Chronic lymphocytic leukaemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug.

Ramsay AG, Johnson AJ, Lee AM, Gorgün G, **Le Dieu R**, Blum W, Byrd JC, Gribben JG.  
J. Clin. Invest. 2008 Jul; 118(7): 2427-37

Differential gene expression profile identifies the nature of T cell defects in AML patients at diagnosis.

**Le Dieu R**, Ramsay AG, Taussig D, Mitter R, Gribben JG

Blood Nov 2008; 112: 1186 (Abstract)

CD3+/CD56+ Cells, but not Natural Killer T Cells, are Increased in Peripheral Blood of Untreated Patients with Leukemia.

**Le Dieu R**, Taussig D, MacDougal F, Lister TA and Gribben JG.

Blood Nov 2007; 110: 1815 (Abstract)

**Book Chapters:**

Chronic Lymphocytic Leukaemia

**Le Dieu R** and Gribben JG.

Chapter 10. Haemopoietic Stem Cell Transplantation in Clinical Practice. First Edition 2008. Editors: J. Treleaven, A.J. Barrett

Allogeneic Stem Cell Transplantation for Non-Hodgkin's and Hodgkin's Lymphoma.

**Le Dieu R** and Gribben JG.

Chapter 14. The Lymphomas. Second Edition 2006. Editors: G. Canellos, T.A. Lister, B. Young

**Reviews:**

Transplantation in Chronic Lymphocytic Leukaemia.

**Le Dieu R** and Gribben JG.

Current Hematologic Malignancy Reports 2007 Jan, 2(1):56-61

Vaccine- and immune-based therapy in chronic lymphocytic leukaemia

**Le Dieu R** and Gribben JG.

Semin Oncol. 2006 Apr; 33(2):220-9

CD40 activation: lessons for HIV immunotherapy from malignancies?

**Le Dieu R** and Gribben JG.

J HIV Ther. 2005 Sep;10(3):51-5.

## **Individual Contributions**

I performed all the work described in this thesis with the following exceptions.

This work would not have been possible without the tremendous facility of the Medical Oncology Tissue Bank and Clinical Database developed by Professor Andrew Lister and maintained by his staff.

Help with the identification of AML patient specimens was provided by Sameena Iqbal and Finlay MacDougall.

AML patient data was provided by Finlay MacDougall.

David Taussig, MRC Clinician Scientist, assisted with the development and optimisation of the AML blast depletion cocktail.

Richard Mitter from the Bioinformatics & Biostatistics Service at Cancer Research UK, 44 Lincoln's Inn Fields provided bioinformatics support.

Tracy Chaplin, Senior Scientific Officer, performed the Affymetrix U133Plus2 GeneChip® hybridisations.

Graham Clark from the Equipment Park at Cancer Research UK, 44 Lincoln's Inn Fields performed the GeneScanning analysis.

FACS experiments detailed in section 2.13.1 were carried out by staff from the Flow Cytometry Lab, Cancer Research UK, 44 Lincoln's Inn Fields.

Immune synapse data was also analysed by Abigail Lee and Alan Ramsay.

## Table of Contents

<b>Dedication .....</b>	<b>2</b>
<b>Acknowledgements.....</b>	<b>3</b>
<b>Abstract.....</b>	<b>6</b>
<b>Publications.....</b>	<b>7</b>
<b>Individual Contributions .....</b>	<b>9</b>
<b>Table of Contents .....</b>	<b>10</b>
<b>List of Figures.....</b>	<b>15</b>
<b>List of Tables .....</b>	<b>18</b>
<b>Abbreviations .....</b>	<b>20</b>
<b>Chapter 1 .....</b>	<b>25</b>
<b>Introduction.....</b>	<b>25</b>
1.1    T Cells .....	25
1.1.1    White Blood Cells.....	25
1.1.2    Lymphocytes.....	26
1.1.3    The T Cell Receptor and T Cell Subsets .....	29
1.1.4    T Cell Development.....	31
1.1.5    The Diversity of the Immune Response.....	33
1.1.6    T Cell Activation.....	35
1.2    Cancer Immunoediting .....	38
1.2.1    A History of Immunosurveillance.....	38
1.2.2    Schreiber’s ‘Three E’ Hypothesis .....	40
1.2.3    Tumour Escape Mechanisms .....	41
1.3    Acute Myeloid Leukaemia (AML).....	45
1.3.1    Classification.....	45
1.3.2    Epidemiology and Aetiology .....	48
1.3.3    Prognostic Stratification.....	49
1.3.4    Treatment .....	50
1.3.5    Outcomes .....	51
1.3.6    Haematopoietic Stem Cell Transplantation (SCT).....	52
1.3.7    Molecular Targeted Therapies .....	54
1.4    The Immune System in AML .....	55
1.4.1    Costimulatory Molecules .....	56
1.4.2    Secretion of Inhibitory Molecules.....	57
1.4.3    Regulatory T Cells .....	58
1.4.4    NK Cells .....	59

1.4.5	Dendritic Cells .....	59
1.4.6	Fibroblasts.....	59
1.5	Curing AML by Immune Manipulation.....	60
1.5.1	Vaccination .....	60
1.5.2	Cytokines .....	64
1.5.3	Antibodies.....	65
1.5.4	Adoptive T cell Immunotherapy .....	66
1.6	Aims of this Thesis .....	69
<b>Chapter 2 .....</b>		<b>70</b>
<b>Materials and Methods .....</b>		<b>70</b>
2.1	Ethical Considerations .....	70
2.2	Samples.....	70
2.3	Cell Freezing and Thawing.....	71
2.4	Separation of PBMCs from Whole Blood (Density Gradient Centrifugation) .....	71
2.5	Cell Counting.....	72
2.6	Cell Separation Protocols .....	72
2.6.1	Separation of CD4 and CD8 T Cells from Healthy PBMCs by Negative Immunomagnetic Selection .....	72
2.6.2	Separation of CD4 and CD8 T Cells by Positive Immunomagnetic Selection .....	74
2.6.3	Separation of CD4 and CD8 T Cells from Presentation AML Specimens by Negative Selection.....	76
2.7	Flow cytometry.....	77
2.7.1	Surface Staining .....	77
2.7.2	Whole Blood Lysis Method .....	78
2.7.2	Intracytoplasmic Staining .....	79
2.7.3	Intracytoplasmic Staining for Cytokines.....	79
2.7.4	Flow Cytometric Acquisition and Analysis .....	80
2.8	Cytospins .....	83
2.9	RNA Extraction .....	84
2.9.1	Assessment of RNA Quality and Quantity for the Purpose of Microarrays.....	86
2.10	Gene Expression Microarrays (Affymetrix UK Ltd.).....	87
2.10.1	First Strand cDNA Synthesis .....	89
2.10.2	Second Strand cDNA Synthesis.....	89
2.10.3	Clean-up of Double Stranded cDNA .....	90
2.10.4	Synthesis of Biotin-labelled cRNA by In Vitro Transcription (IVT).....	90
2.10.5	Clean-up of Biotin-labelled cRNA.....	91
2.10.6	Fragmentation of cRNA.....	92
2.10.7	Preparation of the Hybridisation Cocktail.....	92
2.10.8	Hybridisation, Washing, Staining and Scanning.....	92
2.10.9	Microarray Analysis.....	93

2.11	Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).....	94
2.11.1	cDNA Synthesis from RNA.....	94
2.11.2	qRT-PCR Set-up.....	96
2.11.3	qRT-PCR Analysis.....	98
2.12	T Cell Receptor Gene Clonality Assay.....	99
2.12.1	Fluorescence Activated Cell Sorting of 3+4+, 3+8+ and 3+56+ Populations.....	99
2.12.2	DNA Extraction (Qiagen DNaseasy).....	100
2.12.3	Multiplex PCR.....	101
2.12.4	GeneScanning.....	104
2.13	Cell Conjugation Assay.....	105
2.13.1	Cell Conjugation.....	105
2.13.2	Immunofluorescence Confocal Microscopy.....	106
2.13.3	Image Analysis.....	106
<b>Chapter 3</b>	<b>.....</b>	<b>108</b>
<b>Characterisation of T Cells from AML Patients</b>	<b>.....</b>	<b>108</b>
3.1	Introduction.....	108
3.2	Aims.....	113
3.3	Materials and Methods.....	114
3.3.1	Samples.....	114
3.3.2	Flow Cytometry.....	116
3.3.3	Statistics.....	116
3.3.4	Assessment of TCR Gene Rearrangements.....	116
3.3.5	Correlation with Clinical Data.....	119
3.4	Results.....	120
3.4.1	Total T Cells in AML.....	120
3.4.2	CD56-expressing T Cells in AML.....	124
3.4.3	CD3+56+ Cells are not $\gamma/\delta$ T Cells.....	129
3.4.4	Extended Phenotype of CD3+56+ Cells.....	130
3.4.5	Expression of Activation Markers by CD3+56+ Cells.....	132
3.4.6	CD3+56+ Cell Expression of Intracellular Cytotoxic Granules.....	134
3.4.7	Cytokine Production by CD3+56+ Cells.....	136
3.4.8	Assessment of Clonality of CD3+56+ Cells.....	139
3.4.9	Comparison of Absolute Numbers of CD3+56+ Cells at Presentation and Remission of AML.....	143
3.4.10	Change in Absolute Numbers of CD3+56+ Cells in a Single Patient.....	144
3.4.11	Correlation of Absolute Total CD3 and CD3+56+ Cell Numbers with Patient Characteristics.....	145
3.5	Discussion.....	151
<b>Chapter 4</b>	<b>.....</b>	<b>154</b>

<b>Isolating T cells from AML presentation specimens .....</b>	<b>154</b>
4.1 Introduction .....	154
4.2 Aims .....	155
4.3 Materials and Methods .....	155
4.4 Results .....	156
4.5 Discussion.....	172
<b>Chapter 5 .....</b>	<b>173</b>
<b>The Impact of the Method of Cell Selection on Gene Expression Profile....</b>	<b>173</b>
5.1 Introduction .....	173
5.2 Aims .....	175
5.3 Materials and Methods .....	175
5.3.1 Samples .....	175
5.3.2 RNA.....	176
5.3.3 Quality Control (QC) Data for Affymetrix U133Plus2 GeneChips® .....	177
5.3.4 Analysis of Microarray Data .....	180
5.3.5 Validation of Microarray Data by qRT-PCR .....	182
5.4 Results .....	185
5.4.1 Gene Expression Microarray .....	185
5.4.2 qRT-PCR Validation.....	191
5.5 Discussion.....	194
<b>Chapter 6 .....</b>	<b>196</b>
<b>Gene Expression Profile of AML T Cells.....</b>	<b>196</b>
6.1 Introduction .....	196
6.2 Aims .....	197
6.3 Materials and Methods .....	197
6.3.1 AML Samples .....	197
6.3.2 Cell Selection.....	199
6.3.3 RNA.....	199
6.3.4 Quality Control Data for Affymetrix U133Plus2 GeneChips® .....	201
6.3.5 Healthy Samples .....	203
6.3.6 Analysis of Microarray Data .....	203
6.3.7 Validation of Microarray Data by qRT-PCR .....	206
6.3.8 Protein Validation of Gene Expression Changes .....	209
6.4 Results .....	211
6.4.1 Gene Expression Microarray .....	211
6.4.2 qRT-PCR Validation.....	230
6.4.3 Protein Validation .....	233
6.4 Discussion.....	236
<b>Chapter 7 .....</b>	<b>241</b>

---

<b>Immunological Synapse Formation in AML Patients .....</b>	<b>241</b>
7.1 Introduction .....	241
7.2 Aims .....	242
7.3 Materials and Methods .....	242
7.4 Results .....	248
7.4.1 Do Blasts Form Immune Synapses with Healthy T cells? .....	248
7.4.2 Do AML T Cells Interact with Autologous Blasts? .....	249
7.5 Discussion.....	252
<b>Chapter 8 .....</b>	<b>254</b>
<b>Discussion.....</b>	<b>254</b>
<b>Future Work.....</b>	<b>264</b>
<b>Appendix A: Company Addresses.....</b>	<b>265</b>
<b>Appendix B: Antibodies.....</b>	<b>268</b>
<b>Appendix C: Common Differentially Expressed Genes in AML and CLL</b>	
<b>Datasets .....</b>	<b>271</b>
CD4 .....	271
CD8 .....	272
<b>References .....</b>	<b>275</b>

## List of Figures

Figure 1.1: Haematopoiesis.....	26
Figure 1.2: A Peripheral Blood Lymphocyte .....	28
Figure 1.3: A Large Granular Lymphocyte .....	28
Figure 1.4: Structure of Class I and II MHC Molecules .....	31
Figure 1.5: Germline Organisation of Human TCR Locus .....	34
Figure 1.6: The Two-Signal Hypothesis for T Cell Activation .....	36
Figure 1.7: Intracellular Signalling Events during T Cell Activation.....	37
Figure 1.8: Myeloblasts.....	46
Figure 1.9: Survival in AML.....	49
Figure 1.10: MRC AML15 Flowchart for Non-APL AML Treatment .....	54
Figure 2.1: Method used for Cell Counts .....	72
Figure 2.2: Flow Cytometry Gating Strategy .....	81
Figure 2.3: Location of CountBright™ Counting Beads on Fluorescence Dot Plots .....	82
Figure 2.4: Absolute Cell Counts using CountBright™ Beads.....	83
Figure 2.5: Typical Absorbance Plot for RNA on the Nanodrop Spectrophotometer .....	85
Figure 2.6: Electropherogram of Good Quality RNA .....	87
Figure 2.7: GeneChip® Eukaryotic Labelling Assay for Expression Analysis.....	88
Figure 2.8: Expected Bands for 1Kb DNA Ladder .....	96
Figure 2.9: BIOMED-2 Primers.....	103
Figure 3.1: A Large Population of CD3+CD56+ cells in AML Patients .....	109
Figure 3.2: Gates Used for Flow Sorting Cell Populations .....	117
Figure 3.3: Bar Charts Illustrating Total T Cells in AML.....	122
Figure 3.4: CD4 and CD8 Cells in AML .....	123
Figure 3.5: CD3+56+ Cells in AML .....	124
Figure 3.6: The Components of a Tetramer .....	125
Figure 3.7: Scatter Plots of True NKT Cells .....	126
Figure 3.8: Basic Phenotype of CD56-expressing CD3 Cells.....	127
Figure 3.9: Surface Phenotype of True NKT Cells .....	128
Figure 3.10: TCR Phenotype of CD3+56+ Cells .....	129
Figure 3.11: Activation Marker Expression on CD3+56+ Cells Compared with CD3+56- Cells	133
Figure 3.12: Size and Granularity Characteristics of CD3+56+ Cells .....	134
Figure 3.13: Alteration in CD56 Expression During Cytokine Release Experiment .....	136
Figure 3.14: Change in FSC/SSC Characteristics of CD3+56+ Cells During Cytokine Release Experiment.....	137
Figure 3.15: Autofluorescence of CD3+56+ Cells After Culture.....	138
Figure 3.16: Effect of Stimulation on CD56 Expression by T Cells .....	139

Figure 3.17: Typical GeneScanning Traces for CD3+56+ Cells.....	140
Figure 3.18: Absolute Numbers of CD3+56+ Cells in Remission .....	144
Figure 3.19: Absolute Number of CD3+56+ Cells at Presentation, Remission and Relapse .....	145
Figure 3.20: Correlation between FAB Type and Absolute T Cell Number. ....	146
Figure 3.21: Correlation between Absolute CD3+56+ Cell Count in AML Patients with Presentation Total White Cell Count .....	147
Figure 3.22: Correlation between Age and Total Number of CD3+56+ Cells .....	148
Figure 3.23: Correlation between Sex and Total Number of CD3+56+ Cells .....	149
Figure 3.24: Correlation between Risk of Relapse and Absolute Number of CD3+56+ Cells .....	150
Figure 4.1: Flow Cytometric Analysis of PBMCs from a Diagnostic AML PB Specimen .....	156
Figure 4.2: Experimental Design for Isolation of Unlabelled CD4 or CD8 T Cells .....	157
Figure 4.3: Depletion of Myeloblasts by CD33 Positive Selection.....	158
Figure 4.4: Negative Selection of CD4 and CD8 T Cells from Healthy Peripheral Blood .....	159
Figure 4.5: Cytospin of T Cell Positive Fraction .....	161
Figure 4.6: Depletion of CD33+ve Myeloblasts using x1, x2 and x10 CD33 Microbeads .....	162
Figure 4.7: The Impact of the Use of 2%HAG on Isolated Cell Purity.....	163
Figure 4.8: CD16 Expression on Healthy CD56-expressing NK Cells.....	165
Figure 4.9: Successful T cell Isolation from AML Presentation PB by Negative Immunomagnetic Selection. ....	166
Figure 4.10: Identity of Contaminating Cells in Isolated CD8 Specimens .....	167
Figure 4.11: Impact of the Addition of CD16 to the CD56 Negative Custom Cocktail.....	168
Figure 4.12: Impact of Omission of 2% HAG Incubation step on Removal of CD16-expressing Cells.....	169
Figure 4.13: Effect of CD36 and CD11c Antibodies on Depletion of Unwanted Cells .....	170
Figure 5.1: Comparison of Positive <i>versus</i> Negative Selection for Cell Separation .....	174
Figure 5.2: Expression of Affymetrix Human GAPDH Controls over 16 Samples .....	179
Figure 5.3: RNA Digestion Plot.....	180
Figure 5.4: Unsupervised Clustering.....	181
Figure 5.5: $\beta$ actin PCR for Confirmation of cDNA Synthesis.....	184
Figure 5.6: TaqMan® Assay Relative Efficiency Plots .....	185
Figure 5.7: Supervised Clustering of CD8 Samples.....	186
Figure 5.8: qRT-PCR Validation.....	193
Figure 6.1: Gene Expression Changes in CD4 and CD8 T Cells in CLL.....	197
Figure 6.2: Worked Example of the Method for Determining Samples for Microarray Analysis	198
Figure 6.3: Fragmented Biotinylated cRNA with 1Kb Ladder .....	201
Figure 6.4: NUSE Analysis of All Samples .....	204
Figure 6.5: Pseudo-images of GeneChip® Data .....	205
Figure 6.6: $\beta$ actin PCR for Confirmation of cDNA Synthesis.....	207
Figure 6.7: Plot of Log Input RNA <i>versus</i> $\Delta C_T$ for <i>ACTN1</i> and <i>GAPDH</i> .....	209
Figure 6.8: Unsupervised Clustering of All samples and All Probesets.....	212

Figure 6.9: Venn Diagram Illustrating the Distribution of Differentially Regulated Probesets ....	214
Figure 6.10: Supervised Analysis: AML <i>versus</i> Healthy T cells .....	215
Figure 6.11: Common Genes in AML and CLL T Cell Datasets .....	216
Figure 6.12: CD4 AML <i>v</i> Healthy - Location and Function of Differentially Expressed Genes ..	224
Figure 6.13: CD8 AML <i>v</i> Healthy - Location and Function of Differentially Expressed Genes ..	224
Figure 6.14: TCR Signalling Pathway for CD4 AML <i>v</i> Healthy analysis. ....	226
Figure 6.15: TCR Signalling Pathway for CD8 AML <i>v</i> Healthy Analysis .....	227
Figure 6.16: Actin Cytoskeleton Signalling Pathway for CD4 AML <i>v</i> Healthy Analysis .....	227
Figure 6.17: qRT-PCR Validation of Microarray Data .....	233
Figure 6.18: CD4 T Cells - Protein Validation of Microarray Data .....	234
Figure 6.19: CD8 T Cells - Protein Validation of Microarray Data .....	235
Figure 7.1: Immunofluorescence Images of Cell Conjugates. ....	245
Figure 7.2: Immunofluorescence Images of Immune Synapses .....	246
Figure 7.3: Immunofluorescence Images of Phosphotyrosine (p-Tyr) Signalling .....	247
Figure 7.4: Healthy T Cells can Form Immune Synapses with AML Blasts .....	248
Figure 7.5: AML T Cells can Form Cell Conjugates with Autologous AML Blasts .....	249
Figure 7.6: Immune Synapse Formation between AML T Cells and Autologous AML Blasts ....	250
Figure 7.7: Phosphotyrosine Signalling at the Immune Synapse between AML T Cells and Autologous AML Blasts .....	251
Figure 7.8: AML Blasts can Form Immune Synapses with Healthy NK Cells .....	252
Figure 8.1: T Cells in a Bone Marrow Trephine from a Patient Presenting with AML .....	258

## List of Tables

Table 1.1: Classification of Acute Myeloid Leukaemia .....	47
Table 1.2: Outcomes in AML .....	51
Table 1.3: Characteristics of LAK and CIK Cells.....	67
Table 2.1: Cell Capacities of MACS® Columns .....	73
Table 2.2: Cell Capacities of StemSep™ Columns.....	76
Table 2.3: Pump Set-Up for Use with StemSep™ Columns.....	77
Table 2.4: List of TaqMan® Gene Expression Assays .....	97
Table 2.5: qRT-PCR Formulae .....	99
Table 3.1: Differences between NKT cells and CD3+56+ Cells .....	113
Table 3.2: Characteristics of AML Patients .....	114
Table 3.3: Characteristics of Healthy Volunteers.....	115
Table 3.4: Initial Staining Protocol .....	116
Table 3.5: Data for 3+4+56- Populations.....	118
Table 3.6: Data for 3+8+56- Populations.....	118
Table 3.7: Data for 3+56+ Populations .....	119
Table 3.8: Data for Blast Populations .....	119
Table 3.9: Total T Cells in AML.....	121
Table 3.10: Extended Phenotype of CD3+56+ Cells .....	131
Table 3.11: Expression of Cytotoxic Granules.....	135
Table 3.12: Expression of Cytotoxic Granules in the CD3+SSC <sup>High</sup> Population.....	135
Table 3.13: TCR Gene Clonality Results .....	141
Table 3.14: Comparison of Clonality in Different T cell Populations in AML Samples .....	142
Table 3.15: Comparison of Clonality of T Cell Populations Between AML and Healthy Samples .....	142
Table 4.1: T cell Isolation Experiments .....	160
Table 4.2: Expression of Cell Surface Markers on AML Blasts .....	163
Table 4.3: Blast Antibody Cocktail Optimization Experiments.....	164
Table 4.4: Successful CD4 and CD8 T Cell Isolation from Presentation PB from 6 AML Patients .....	171
Table 5.1: Characteristics of Healthy T Cell Samples.....	176
Table 5.2: Quality and Quantity of T Cell RNA Obtained.....	177
Table 5.3: Quality Control Data for Affymetrix U133Plus2 GeneChips® .....	178
Table 5.4: Genes Validated by qRT-PCR .....	183
Table 5.5: Availability of Microarray RNA Samples for qRT-PCR.....	183
Table 5.6: List of 114 Differentially Expressed Genes in Positively Selected CD8 Cells .....	190
Table 6.1: Characteristics of AML Patients .....	199

Table 6.2: Quality and Quantity of RNA for CD4 T Cells.....	200
Table 6.3: Quality and Quantity of RNA for CD8 T Cells.....	200
Table 6.4: Quality Control Data for CD4 T Cells .....	202
Table 6.5: Quality Control Data for CD8 T Cells .....	202
Table 6.6: Characteristics of AML and Healthy Donor T Cell Datasets .....	203
Table 6.7: Genes Chosen for Validation of Microarray Data.....	206
Table 6.8: Samples Available for qRT-PCR.....	206
Table 6.9: Choice of Positive Control for each TaqMan® Assay .....	209
Table 6.10: Cell Surface Molecules used for Protein Validation of Gene Expression Changes ...	210
Table 6.11: Staining Protocol for Flow Cytometric Validation of Microarray Data.....	211
Table 6.12: Differentially Expressed Probesets Identified Using Different Filtering Parameters.	213
Table 6.13: Differentially Expressed Genes Found on Supervised Analysis of the AML T Cell Samples Only.....	217
Table 6.14: CD4 AML v Healthy - Top 10 Up-regulated (red) and Down-regulated (blue) Genes .....	219
Table 6.15: CD8 AML v Healthy - Top 10 Up-regulated (red) and Down-regulated (blue) Genes .....	220
Table 6.16: CD4 AML v Healthy – Genes of Potential Relevance .....	222
Table 6.17: CD8 AML v Healthy - Genes of Potential Relevance.....	223
Table 6.18: CD4 AML v Healthy - Top Functions and Pathways Identified by Ingenuity .....	225
Table 6.19: CD8 AML v Healthy - Top Functions and Pathways Identified by Ingenuity .....	225
Table 6.20: PubMed Analysis of Gene Function .....	229
Table 7.1: Characteristics of AML Patients .....	243
Table 7.2: Experimental Set-up: Do Blasts Form Immune Synapses with Healthy T Cells?.....	244
Table 7.3: Experimental Set-up: Do AML T cells Form Immune Synapses with Autologous Blasts?.....	244

## Abbreviations

A	absorbance
ACD-A	acid citrate dextrose - A
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
ANOVA	analysis of variance
API	activator protein 1
APC	allophycocyanin or antigen presenting cell
APL	acute promyelocytic leukaemia
ATRA	all-trans retinoic acid
BP	base pair
BSA	bovine serum albumin
CAR	coxsackie and adenoviral receptor
CBF	core binding factor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CDR3	complementarity-determining region 3
CEBPA	CCAAT enhancer binding protein alpha
CIK	cytokine-induced killer cells
CLL	chronic lymphocytic leukaemia
CMAC	7-amino-4-chloromethylcoumarin
CML	chronic myeloid leukaemia
CR	complete remission
CRUK	Cancer Research UK
C <sub>T</sub>	cycle threshold
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen-4
cRNA	complementary ribonucleic acid
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DEPC	diethyl pyrocarbonate
DFCI	Dana-Farber Cancer Institute
DLI	donor lymphocyte infusion
DMSO	dimethylsulphoxide
DN	double negative
DNA	deoxyribonucleic acid

---

DTT	dithiothreitol
EBV	Epstein-Barr virus
EDTA	ethylenediamine tetra-acetate
ELISA	enzyme-linked immunosorbant assay
FAB	French-American-British
FACS	fluorescence-activated cell sorting
FBC	full blood count
FC	fold change
FCS	foetal calf serum
FDR	false discovery rate
FITC	fluorescein isothiocyanate
FLT3	FMS-like tyrosine kinase 3
FOXP3	forkhead box P3
FPD	familial platelet disorder
FSC	forward light scatter
FUSE	far upstream element
GITR	glucocorticoid-induced tumour necrosis factor receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GO	gemtuzumab ozogamicin
GrB	granzyme B
GVHD	graft- <i>versus</i> -host disease
GVL	graft- <i>versus</i> -leukaemia effect
HAG	human anti-globulin
HLA	human leucocyte antigen
ID	induction death
iDC	immature dendritic cell
IDO	indoleamine 2,3-dioxygenase
IFN $\gamma$	interferon-gamma
IL-2	interleukin 2
IL-4	interleukin 4
IL-10	interleukin 10
IL-15	interleukin 15
ImC	immature cell
IP3	inositol triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
ITD	internal tandem duplication
IVT	<i>in vitro</i> transcription
JNK	jun N-terminal kinase
KIR	killer immunoglobulin-like receptor
KLH	keyhole limpet haemocyanin

---

LAK	lymphokine-activated killer cells
LDDC	leukaemia -derived dendritic cell
LGL	large granular lymphocyte
LRF	leukaemia research fund
MAPK	mitogen-associated protein kinase
MART-1	melanoma antigen recognized by T- cells 1
MAS5	microarray suite version 5.0
MDSC	myeloid derived suppressor cell
MFI	mean fluorescence intensity
MGG	May-Grunwald-Giemsa stain
MGUS	monoclonal gammopathy of uncertain significance
MHC	major histocompatibility complex
MLL	mixed lineage leukaemia
MPO	myeloperoxidase
MRC	Medical Research Council
mTPR	mammalian target of rapamycin
MUD	matched unrelated donor
NCAM	neural cell adhesion molecule
NCR	natural cytotoxicity receptor
NFAT	nuclear factor of activated T cells
NF $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cell
NKT	natural killer T cell
NOD-SCID	nonobese diabetic – severe combined immune deficiency
NPM	nucleophosmin
NT	natural T cell
NTP	nucleotide triphosphate
NUSE	normalized unscaled standard errors
OFA-iLRP	oncofetal antigen-immature laminin receptor
PARP	poly (ADP-ribose) polymerase
PB	peripheral blood
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PERCP	peridinin-chlorophyll protein
PFA	paraformaldehyde
PKC	protein kinase C
PMA	phorbol myristate acetate

---

PRAME	preferentially expressed antigen of melanoma
p-Tyr	phosphotyrosine
QC	quality control
qRT-PCR	quantitative real-time polymerase chain reaction
RAG	recombinase-activating gene
RAR $\alpha$	retinoic acid receptor alpha
RCF	relative centrifugal force
RD	resistant disease
RHAMM	receptor for hyaluronic acid mediated motility
RIN	RNA integrity number
RMA	robust multi-array average
RNA	ribonucleic acid
RPM	rotations per minute
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
sAg	superantigen
SAPE	streptavidin phycoerythrin
SCID	severe combined immune deficiency
SCT	stem cell transplantation
SD	standard deviation
SE	standard error
SMAC	supramolecular activation cluster
SNP	single nucleotide polymorphism
SSC	side light scatter
TAA	tumour associated antigen
TAM	tumour-associated macrophages
TAP	transporter associated with antigen processing
TCL-1	T cell leukaemia – 1
T <sub>CM</sub>	central memory T cell
TCR	T cell receptor
T <sub>EM</sub>	effector memory T cell
TGF- $\beta$	transforming growth factor beta
T <sub>H1</sub>	T helper 1 cell
T <sub>H2</sub>	T helper 2 cell
T <sub>H17</sub>	T-helper 17 cell
TNF $\alpha$	tumour necrosis factor alpha
T <sub>regs</sub>	regulatory T cells
TRM	transplant related mortality
VEGF	vascular endothelial growth factor

WHO	world health organisation
WT1	Wilms' tumour 1

# Chapter 1

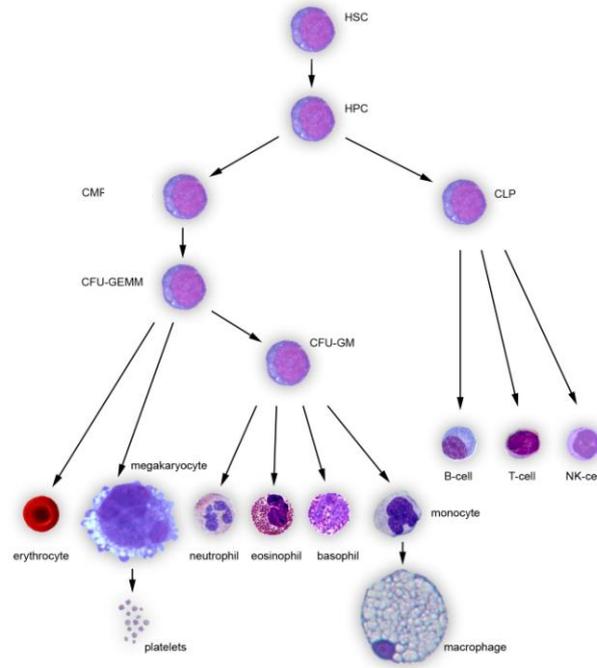
## Introduction

Cancer is characterised by an uncontrollable proliferation of cells that have the ability to invade and metastasize. Original descriptions were found on Egyptian papyri dating from 1600BC on which the author stated “there is no treatment” (Breasted 1922). Since that time prognosis has improved radically. However, despite rapid recent advances in genomics, pharmacology and biotechnology, further improvements in cancer survival remain small. To achieve the aim of a cure, alternative methods of cancer therapy need to be pursued. One such avenue is to harness the immune system.

### 1.1 T Cells

#### *1.1.1 White Blood Cells*

T lymphocytes form one part of the white blood cell (leucocyte) population found in human peripheral blood. On the basis of nuclear shape, leucocytes are defined as either polymorphonuclear (granulocytes) or mononuclear cells. While granulocytes are made up of neutrophils, eosinophils and basophils, mononuclear cells are the monocytes and lymphocytes (**Figure 1.1**).



**Figure 1.1: Haematopoiesis**

HSC: haematopoietic stem cell; HPC: haematopoietic progenitor cell; CMP (CFU-S): common myeloid progenitor; CLP: common lymphoid progenitor; CFU-GEMM: colony-forming unit-granulocytes, erythroblasts, macrophages, megakaryocytes (From Google Images).

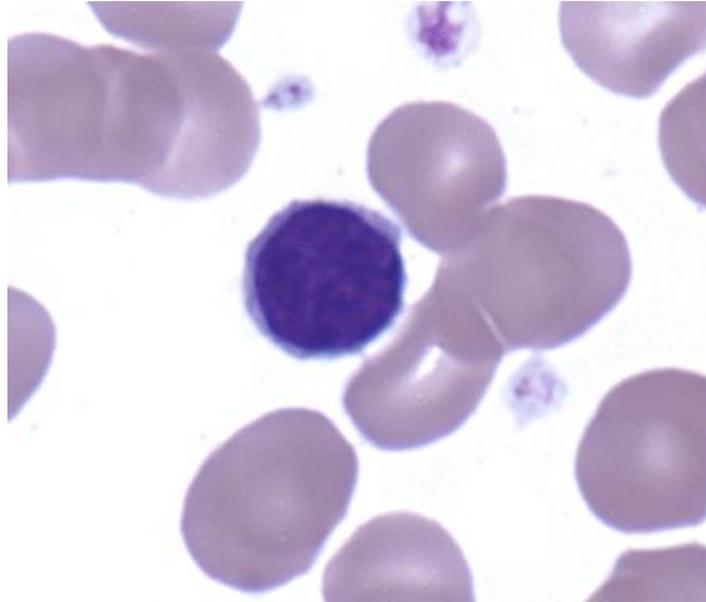
In the peripheral blood, lymphocytes make up 20-40% of all leucocytes with a typical absolute count in a Caucasian adult being  $1.0-3.2 \times 10^9/L$ . In the bone marrow, they make up 16.1% of the cellular component (95% range 6.0-26.2%). These cells vary in diameter from 10 to  $16\mu\text{m}$  and characteristically have scanty cytoplasm and a round nucleus with condensed chromatin (Bain 2002, Bain 2001) (**Figure 1.2**).

### 1.1.2 Lymphocytes

There are 3 major subsets of lymphocytes; T cells (thymus-derived), B cells (bone marrow-derived) and NK (natural killer) cells. In the peripheral blood, around 80% of circulating lymphocytes are T cells, 10-15% are B cells and ~10% are NK cells whereas in the bone marrow B cells predominate within the precursor cell subset and T cells amongst mature lymphocytes (Hoffbrand 2002). In general it is not possible to distinguish the subsets of lymphocytes morphologically. However,

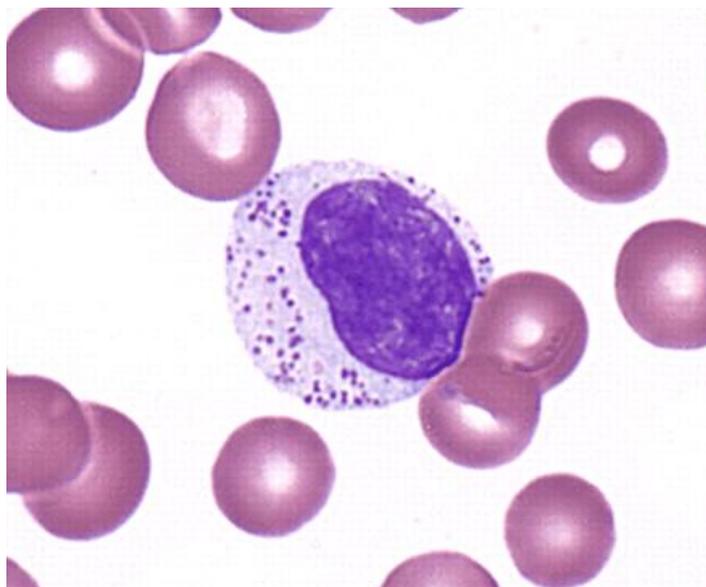
activated T cells and NK cells are within the population of ‘large granular lymphocytes’ (LGL) characterised by more abundant cytoplasm and prominent azurophilic granules (**Figure 1.3**).

Lymphocytes are crucial for protection against disease. This is most clearly shown by genetic disorders in humans that result in their absence. The commonest cause of X-linked severe combined immune deficiency (SCID) is a mutation in the common gamma chain ( $\gamma_c$ ) resulting in widespread failure of interleukin signalling with the result that there are low or absent T and NK cells and non-functional B cells. Unless transplanted, these children die within one year due to severe recurrent infections (Buckley 2000).



**Figure 1.2: A Peripheral Blood Lymphocyte**

(ASH Image Bank)



**Figure 1.3: A Large Granular Lymphocyte**

(ASH Image Bank)

The immune system is composed of two major arms; the adaptive and innate responses. The adaptive immune response has a number of fundamental features: *specificity* – distinct antigens will elicit distinct responses, *diversity* – responses can be made to a vast number of antigens, *memory* – resulting in enhanced responses to repeated exposure to the same antigen, *specialisation* – responses are tailored to the defence required, *self-limitation* – responses are triggered by antigen to destroy antigen and therefore wane over time and lastly, *non-reactivity to self* or tolerance. T and B cells make up the adaptive immune system with T cells being responsible for cell-mediated immunity and B cells for humoral immunity via the production of antibodies. This response is complemented by the innate immune response which is non-specific and has no memory but has the advantage of rapid, maximal response on exposure to antigen largely via pattern recognition receptors such as toll-like receptors and mannose receptors. NK cells form part of the cellular innate immune response that also includes phagocytes (macrophages, neutrophils, dendritic cells (DCs)), mast cells, basophils and eosinophils. NK cells distinguish self from non-self on the basis of surface expression of the major histocompatibility complex (MHC) Class I molecule via inhibitory killer immunoglobulin-like receptors (KIRs) (Abbas 2000).

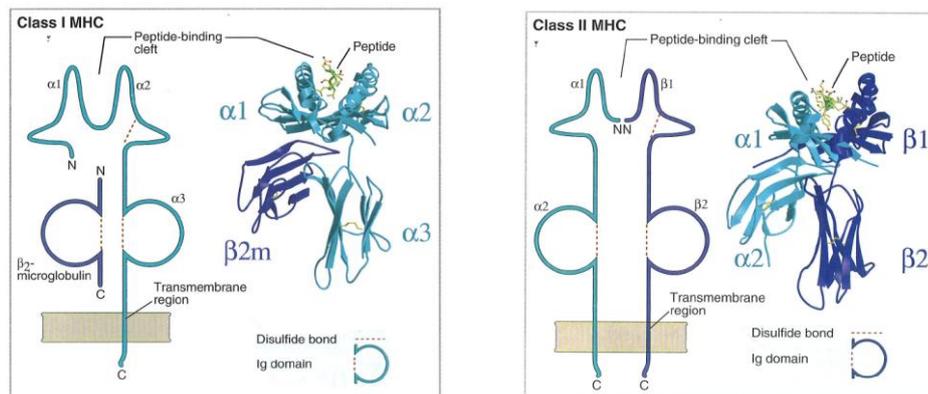
### ***1.1.3 The T Cell Receptor and T Cell Subsets***

T cells are characterised by the expression of a heterodimeric T cell receptor (TCR). In more than 90% of cases, it is made up of alpha and beta glycoprotein chains with fewer than 10% of circulating T cells expressing a TCR made up of gamma and delta chains. However,  $\gamma/\delta$  T cells make up the majority of the intraepithelial lymphocyte population within the gut and other epithelial mucosa. This unconventional T cell subset borders between the adaptive and the innate immune response (Hayday 2000). Further subclassification of T cells is on the basis of expression of cluster of differentiation (CD) surface proteins. A protein is given a CD number if it identifies a particular lineage or differentiation stage of the cell, has a defined structure and is recognised by a group or ‘cluster’ of monoclonal antibodies. Thus T cells are characterised by the expression of CD3,

CD4 or CD8 whereas B cells express CD19, CD20 and CD22 and NK cells express CD16 and CD56 but not CD3.  $\alpha/\beta$  T lymphocytes consist of two major subsets, helper and regulatory T cells that express CD4 and cytotoxic T cells that express CD8. CD4 cells predominate in the peripheral blood whereas CD8 cells make up the dominant T cell population in the bone marrow and the gut. Cytotoxic CD8 T lymphocytes (CTLs) destroy cells expressing their cognate antigen through release of the effector molecules perforin and granzyme B. Helper CD4 cells differentiate into  $T_H1$ ,  $T_H2$  or  $T_H17$  subsets depending on the cytokine signals received with each secreting a different selection of cytokines to assist a local adaptive immune response (Zhu and Paul 2008). Regulatory CD4 cells ( $T_{regs}$ ) maintain immunological tolerance by switching off T-cell mediated immunity when it is no longer required. There are two main subsets – the naturally occurring ( $CD4+CD25+FOXP3+$ ) generated within the thymus and the adaptive ( $Tr1$  or  $T_H3$ ) that arise in the periphery during an immune response (Sakaguchi 2004).

The function of the T cell receptor is to recognise antigen; that is, any molecule that can prompt the generation of antibody. T cell receptors recognise peptide fragments of protein. However recognition is only possible when these peptides are appropriately displayed or presented to them on the surface of an antigen-presenting cell (APC) in association with an MHC molecule. There are two types of MHC heterodimers. Class I molecules can be found on almost all nucleated cells. They present endogenous peptides sampled from the cellular cytosol and are recognised by CD8 T cells. This function is crucial for the killing of intracellular microbes such as viruses which may infect any cell within the body. CD4, T helper cells, recognise Class II MHC molecules which are only found on professional APCs (DCs, B lymphocytes and macrophages) and which present exogenous peptides that have been sampled from the extracellular compartment by the process of endocytosis. MHC class I molecules are designated HLA (human leucocyte antigen)-A, HLA-B and HLA-C in humans. They are made up of a polymorphic  $\alpha$  chain encoded on the MHC locus on chromosome 6 and a non-polymorphic subunit called  $\beta$ 2-microglobulin. Peptides of between 8 and 11 residues can be presented within the peptide binding groove. MHC Class II

molecules (HLA-DR, DP and DQ) are composed of an  $\alpha$  and a  $\beta$  polypeptide chain both encoded on the MHC locus. These molecules accommodate slightly larger peptides of 10-30 residues (**Figure 1.4**).



**Figure 1.4: Structure of Class I and II MHC Molecules**

(From (Abbas 2000))

Each MHC molecule can present multiple peptides but cannot distinguish between those that are foreign and those that are self. Binding specificity is determined by polymorphic residues known as anchor residues located within the peptide-binding domain that are complementary to residues on the bound peptide. This association is a low affinity interaction with a slow on-rate and very slow off rate which allows for peptide-MHC complexes to persist for long enough to be recognised by an antigen-specific T cell (Germain 1994).

### ***1.1.4 T Cell Development***

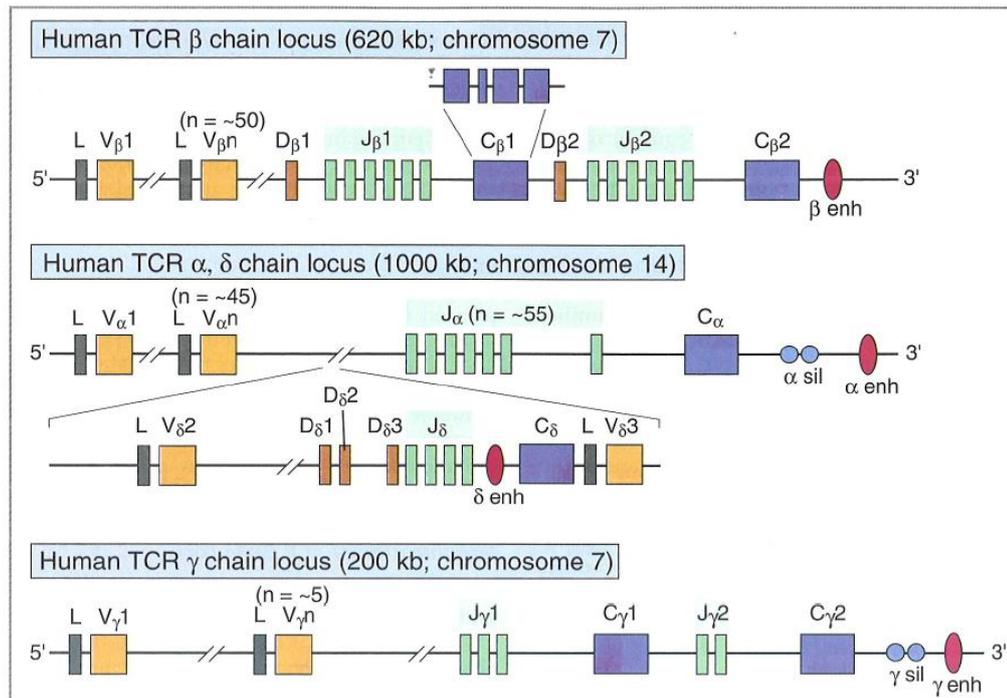
All leucocytes are derived from a common cell of origin, a pluripotent stem cell that is located within the bone marrow. From here, the immature T cells (CD4-CD8-) migrate to the thymus to undergo maturation as thymocytes. As they

migrate from the cortex to the medulla of the thymus, the double-negative (CD4-CD8-) T cells gradually become double-positive (CD4+CD8+) and finally single-positive (CD4+CD8- or CD4-CD8+). Around 98% of thymocytes are deleted during their passage through the thymus through the processes of positive and negative selection. The immature T cells are exposed to thymic epithelial cells expressing self-antigens in association with an MHC molecule. T cells that recognise and avidly bind to these self antigens are deleted in a process of negative selection (selects against T cells reactive to self antigen resulting in tolerance to self) whereas those T cells that bind self MHC molecules with only low affinity are positively selected (selects for functional cells capable of interacting with MHC molecules) (Germain 2002). T cells that pass both levels of selection leave the thymus as naïve lymphocytes and enter the peripheral blood circulation. Once mature, naïve lymphocytes survive for long periods of time; they have been shown to divide only once every 3.5 years in humans (McLean and Michie 1995). From the post-capillary venules, the naïve lymphocytes migrate into the peripheral lymphoid organs; the lymph nodes, the spleen and the mucosal and cutaneous lymphoid tissue. These organs concentrate foreign antigens that have entered the body via the skin, gastrointestinal or respiratory tracts. Here T cells home to the paracortical areas of the lymph nodes and the periarteriolar sheaths surrounding the central arterioles within the spleen. Within the lymphoid organs, the naïve cells recognise antigens presented by professional APCs (DCs, macrophages or B cells) and subsequently differentiate into the effector (cytotoxic or helper) and memory cells of the adaptive immune response. Differentiated lymphocytes subsequently return to the peripheral blood via the efferent lymphatic system and the thoracic duct in the process of lymphocyte recirculation. From here, effector cells home to the site of antigen entry within the peripheral tissues and destroy those cells expressing the appropriate foreign antigen. Memory cells can be divided into central memory ( $T_{CM}$ ) or effector memory ( $T_{EM}$ ) categories and can be either CD4 or CD8 positive. Central memory cells express L-selectin and CCR7 in contrast to effector memory cells. In addition,  $T_{CM}$  produce interleukin-2 (IL-2) rather than the cytokines interferon-gamma ( $IFN\gamma$ ) and interleukin-4 (IL-4) that are produced by  $T_{EM}$  (Sallusto, *et al* 2004). Memory cells take up residence in normal tissue in preparation for the next

exposure to antigen allowing a more rapid response to secondary exposure (recall). In contrast to naïve and recently activated lymphocytes, memory cells may survive in a quiescent state without exposure to antigen for many years. Historically, naïve and memory T cells have been distinguished by the expression of the cell surface markers CD45RA (naïve) and CD45RO (memory). It is now clear that this is inaccurate and other combinations of markers have been reported (De Rosa, *et al* 2001). In this study the combination of the cell surface markers CD45RA and CD27 has been used to phenotypically distinguish effector, naïve and memory CD8 T cells (Hamann, *et al* 1997, Hamann, *et al* 1999). At any one time, 20-50% of all lymphocytes are within the peripheral circulation, the remainder being within the lymphoid organs (von Andrian and Mackay 2000).

### ***1.1.5 The Diversity of the Immune Response***

It is estimated that the immune system of an individual can recognise between  $10^7$  and  $10^9$  different antigens. This vast lymphocyte repertoire is possible due to the variability in the antigen recognition sites on lymphocytes – the T and B cell antigen receptors (TCR/BCR). The genes for the four proteins of the TCR are encoded in three different loci within the genome. The TCR  $\beta$  chain locus is on chromosome 7, the  $\delta$  chain locus is contained within the  $\alpha$  chain locus on chromosome 14 and the  $\gamma$  chain locus on chromosome 7. Each germline TCR locus is made up of multiple copies of at least three different types of gene segments; V (variable), J (joining), C (constant) and D (diversity) separated by non-coding DNA (**Figure 1.5**).



**Figure 1.5: Germline Organisation of Human TCR Locus**

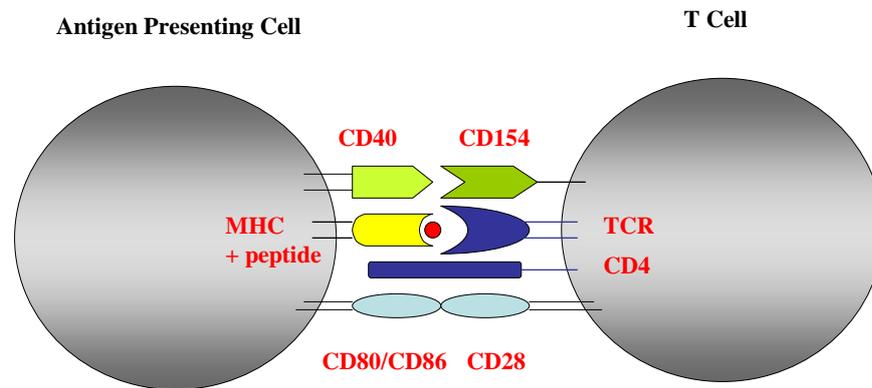
(From (Abbas 2000))

Transcription of this germline DNA will not give rise to antigen receptor proteins. It is only within developing lymphocytes that different V, D and J regions are brought together by rearrangements within the DNA and functional antigen receptor genes are created. This process is known as somatic recombination and is the means by which random individual V, J and D (when present) are brought together to form a single V(D)J gene that will encode the variable region of the receptor. The lymphocyte-specific recombinase responsible for this process is a complex of two proteins encoded by recombination-activating gene 1 and recombination-activating gene 2 (RAG-1 and RAG-2). The C region is joined post-transcription resulting in an mRNA transcript that on translation produces one of the chains of the antigen receptor. The association with a different, randomly generated V region on the other receptor chain introduces further combinatorial diversity. Another mechanism by which diversity is introduced is junctional diversity. At the time of VDJ joining, enzymes randomly remove or add nucleotides at the junctions of gene segments. These areas (complementarity-determining or CDR3 regions) therefore show the greatest variability and are also

the most important for determining the specificity of antigen binding. The rearrangement of TCR genes is completed during the double-positive maturation stage just before the thymocytes enter the medulla. In this way, every T lymphocyte clone expresses a unique TCR capable of recognising an individual peptide. However, although the potential number of molecules that can be expressed is very large, it is estimated that each individual only expresses a fraction of the potential repertoire ( $\sim 10^7$ ) (Davis and Bjorkman 1988).

### ***1.1.6 T Cell Activation***

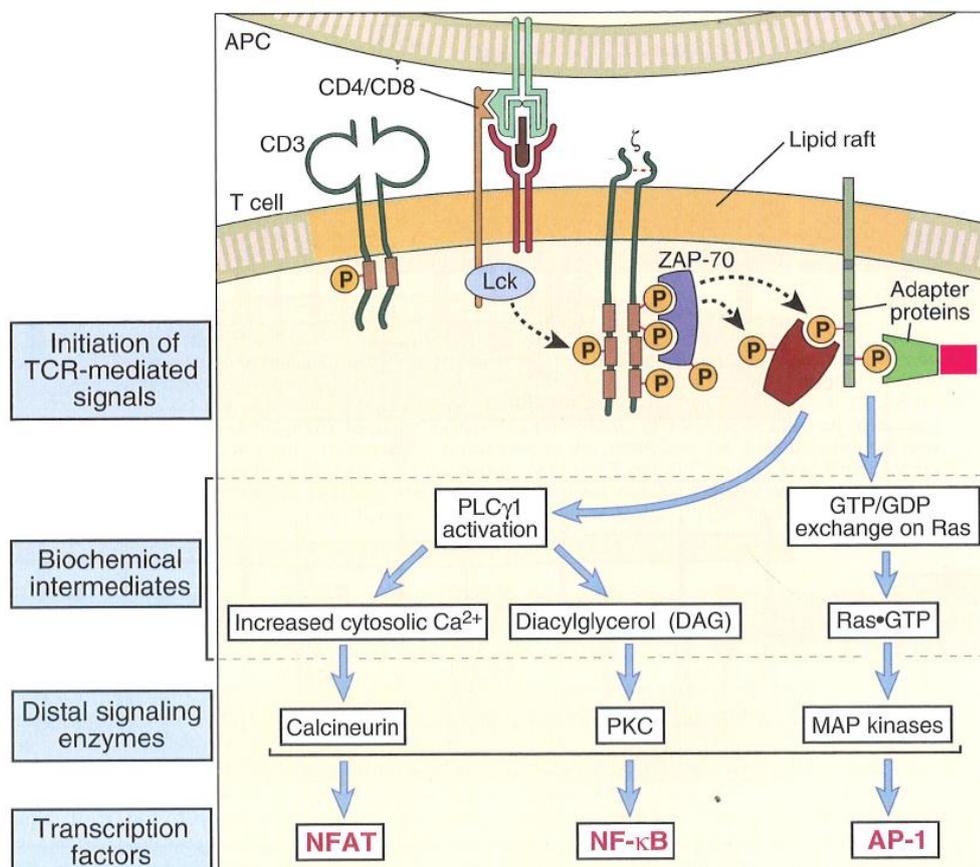
A T cell becomes activated when it recognises the antigen. This happens when the TCR binds to antigen in association with an MHC molecule on the surface of an APC. This ensures that only a T cell with a TCR specific to the antigen is activated. However, this alone is insufficient to activate the cell. To license the T cell to respond to the antigen, a second signal must occur. If no second signal is given, the T cell becomes anergic, or unresponsive and will be more difficult to activate in the future. The second signal is provided by costimulation. The only costimulatory receptor constitutively expressed on T cells is CD28. This binds to CD80/CD86 on APCs. Once this costimulatory signal has been provided, a range of other costimulatory receptors are expressed on the T cell surface such as CD40L (CD154), ICOS and PD-1 which reinforce the activation signal (**Figure 1.6**).



**Figure 1.6: The Two-Signal Hypothesis for T Cell Activation**

The point of physical contact between a T cell and an APC is known as the immunological synapse. Once antigen recognition has occurred, actin polymerises within the cytoskeleton resulting in cellular polarisation and rapid mobilisation of the TCR complex, CD4 or CD8 coreceptors, costimulatory receptors, enzymes and adaptor proteins to the centre of the synapse to form supramolecular activation clusters (SMAC). Integrins such as LFA-1 remain on the periphery to maintain stability (Dustin and Cooper 2000). The TCR not only consists of the  $\alpha/\beta$  heterodimer but also the protein CD3. One of its homodimers, CD3 $\zeta$  consists of 6 immunoreceptor tyrosine-based activation motifs (ITAMs). On activation, these ITAM motifs are phosphorylated by Lck, a Src family tyrosine kinase that is associated with the cytoplasmic tails of CD4 and CD8. This results in the recruitment of ZAP-70 to the TCR complex. ZAP-70 is critical for sustaining the signalling cascade initiated by TCR recognition of antigen. The tyrosine kinases Lck and ZAP-70 go on to phosphorylate tyrosine residues on numerous other molecules including CD28, LAT and SLP-76 culminating in an aggregation of signalling complexes around the TCR. Phosphorylated LAT recruits SLP-76 to the membrane which in turn recruits PLC $\gamma$ , Vav1, Itk and potentially PI3K. LAT

is also responsible for activating the Ras-MAP kinase signalling pathway. Both PLC $\gamma$  and PI3K act on phosphatidylinositol 4,5-bisphosphate on the inner surface of the cell membrane to release diacylglycerol (DAG) and inositol triphosphate (IP<sub>3</sub>). DAG binds to and activates various protein kinase Cs (PKC) especially PKC $\theta$  in T cells. This is important for activating the transcription factors nuclear factor kappa B (NF $\kappa$ B) and activator protein 1 (AP1). IP<sub>3</sub> activates receptors within the endoplasmic reticulum resulting in intracellular calcium release. The calcium activates calcineurin which in turn causes the translocation of the transcription factor nuclear factor of activated T cells (NFAT) into the nucleus. Here it regulates the transcription of numerous genes, most importantly, the cytokine IL-2 which promotes the proliferation of the activated T cell. Once activated, the T cell will differentiate, proliferate and secrete cytokines (Acuto and Cantrell 2000) (**Figure 1.7**).



**Figure 1.7: Intracellular Signalling Events during T Cell Activation**

(From (Abbas 2000))

## 1.2 Cancer Immunoediting

The immune system has the potential to prevent cancer in three ways. Firstly, it can eliminate virally infected cells and so prevent virus-induced tumours. Secondly, an inflammatory microenvironment promotes tumorigenesis therefore rapid resolution of infection and termination of the immune response is critical in cancer prevention. Finally, the immune system can recognise transformed cells when they arise due to their expression of novel antigens, either tumour-specific antigens (molecules unique to cancer cells) or tumour-associated antigens (molecules expressed differently by cancer cells and normal cells). This process is known as immunosurveillance. Although it has long been established that the immune system protects the individual from infection by exogenous organisms, the existence of cancer immunosurveillance has been more hotly debated.

### 1.2.1 A History of Immunosurveillance

The theory that the immune system can recognise and eliminate primary tumour cells without external intervention was first postulated by Paul Ehrlich in 1900 (Ehrlich 1900). However, it took over 50 years for the field of immunology to develop sufficiently far to allow this hypothesis to be experimentally tested. In the 1950s, the first step was taken when Medawar and colleagues used non-inbred strains of mice to demonstrate that the immune response can recognise and destroy allogeneic transplanted tumours thus establishing the critical role of the cellular components of the immune system in mediating graft rejection (Billingham, *et al* 1953, Billingham 1956). It was then illustrated that tumour cells are themselves immunologically distinct from normal cells by the demonstration that mice could be immunised against transplanted syngeneic tumours induced by chemical carcinogens or viruses. This work established the existence of ‘tumour-specific antigens’ (Klein 1966, Old 1964). These discoveries were incorporated into the formal hypothesis of ‘cancer immunosurveillance’ made by Sir Frank Macfarlane Burnet and Lewis Thomas (Burnet 1957, Thomas 1959). Burnet stated:

*“It is by no means inconceivable that small accumulations of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence”.*

The natural challenge to this hypothesis was a demonstration that hosts with impaired immune function had an increased incidence of spontaneous or chemically induced tumours. A number of groups set about addressing this question using thymectomised or genetically athymic (nude) mouse models (Grant and Miller 1965, Stutman 1974, Stutman 1975). Unfortunately their results were discordant and inconclusive and in 1982 Thomas noted “the greatest trouble with the idea of immunosurveillance is that it cannot be shown to exist in experimental animals” (Thomas 1982). However, with the discovery of NK cells and the realisation that nude mice possess detectable populations of  $\alpha/\beta$  T cell receptor bearing lymphocytes, it became clear that no conclusions could be reliably drawn from the experiments previously performed. The development of the RAG-1 and RAG-2  $-/-$  mouse corrected this problem. These mice lacked the recombinase activating gene 1 or 2 which are required for repairing the breaks in double-stranded DNA and are expressed exclusively in lymphocytes. These mice failed to rearrange lymphocyte antigen receptors and completely lacked NK, T and B cells. DNA repair mechanisms in all other cell compartments were normal (Shinkai, *et al* 1992). When these mice were injected with chemical carcinogens, they developed sarcomas more rapidly and with greater frequency than wild-type animals. The same was true for spontaneous tumour growth. For the first time, the impact of loss of immune function on tumour development could be seen (Shankaran, *et al* 2001).

In humans, evidence for immunosurveillance is now accumulating. Perhaps the strongest data comes from reports of spontaneously regressing melanoma lesions associated with a clonal expansion of T cells (Ferradini, *et al* 1993, Zorn and Hercend 1999a, Zorn and Hercend 1999b). However, these reports are rare and it is unclear why spontaneous regression should occur in melanoma in particular. More generally, reviews of transplant registries have demonstrated that

immunocompromised individuals post-transplant have a higher risk of developing a variety of malignancies with non-viral aetiologies with the most recent detailing a 7.1 fold increased incidence compared with the non-transplant population (Birkeland, *et al* 1995, Roithmaier, *et al* 2007). Another study, from Japan looked at the cytotoxicity of peripheral blood mononuclear cells (PBMCs) from 3625 healthy individuals. Over an 11 year follow-up, those individuals whose lymphocytes had shown a high or medium level of cytotoxicity had a lower risk of developing cancer of any type compared with those whose lymphocytes had shown low cytotoxicity as the start of the study (Imai, *et al* 2000). The immune system also influences the outcome of cancer once a diagnosis has been made. Data from melanoma has suggested a positive correlation between the presence of tumour-infiltrating lymphocytes and increased survival (Clemente, *et al* 1996). Similar observations have been made in colon cancer (Galon, *et al* 2006), follicular lymphoma (Dave, *et al* 2004, Lee, *et al* 2006), cervical cancer (Piersma, *et al* 2007), breast cancer (Kohrt, *et al* 2005) and urothelial cancers (Sharma, *et al* 2007). Multiple myeloma provides a clinical model of immunoediting. In its pre-malignant stage, monoclonal gammopathy of uncertain significance (MGUS), T cells from the bone marrow show vigorous responses to the autologous pre-malignant cells. These responses are not seen in patients with multiple myeloma (Dhodapkar, *et al* 2003b). It is therefore now becoming clear that evaluation of the immune response in and around the tumour should be included at the time of the initial diagnosis to aid prognostic evaluation and guide treatment.

### **1.2.2 Schreiber's 'Three E' Hypothesis**

The question that then arises is – why do tumours occur in immunocompetent individuals? Schreiber and colleagues have proposed the 'Three E's' hypothesis of cancer immunoediting. The immune system functions to protect the host but also to sculpt the developing tumour. Early immunosurveillance results in the first E, 'elimination' the evidence for which is detailed above. Secondly, 'equilibrium' occurs when the cells have transformed but are held in check by the immune system. During this time, a Darwinian selection process takes place whereby the immune system selects for less immunogenic tumour cells in the course of the antitumour immune response. This is aided by the intrinsic genetic instability of

tumour cells. The targets that are often affected are tumour associated antigens (TAAs), MHC molecules and components of the IFN $\gamma$  signalling pathway. Evidence for this part of the process was more difficult to obtain but was recently demonstrated in a mouse model by Schreiber's group (Koebel, *et al* 2007). The third E is 'escape' and represents the point when the tumour starts to proliferate uncontrollably and becomes clinically apparent as a result of acquired insensitivity to immunological detection (Dunn, *et al* 2002).

### ***1.2.3 Tumour Escape Mechanisms***

Malignant cells have evolved numerous mechanisms for suppressing and therefore escaping the immune response both within the local tumour microenvironment and systemically (Rabinovich, *et al* 2007). One of the most common ways that a tumour can hide from the immune system is by downregulating its antigen presentation machinery. Mutation of the  $\beta$ 2-microglobulin gene results in complete loss of MHC Class I expression whereas transcriptional regulation of the gene can cause decreased expression. Genetic instability within the tumour can result in selective loss of individual HLA alleles or mutations of the transporter associated with antigen processing (TAP) or molecules within the immunoproteasome (LMP2, LMP7). Mutations and/or deletions within the tumour DNA may also mean that the initial tumour antigen is no longer expressed (antigen-loss variants) and therefore cannot be recognised by cytotoxic T cells (Khong and Restifo 2002). As well as failing to appropriately present TAA, tumour cells can fail to deliver adequate costimulatory signals necessary for T cell activation by lack of expression of the costimulatory molecules CD80 and CD86 (Chen, *et al* 1992, Townsend and Allison 1993). The consequences of T cell recognition of tumour antigen would then be anergy rather than activation (Schwartz 2003). Tumours may also evolve to express T cell inhibitory molecules B7-H1 (Dong, *et al* 2002), HLA-G (Malmberg, *et al* 2002) or HLA-E (Derre, *et al* 2006).

Tumour-induced T cell tolerance may be induced by inadequate presentation of TAAs by tumours themselves but also by APCs (cross-priming) (Sotomayor, *et al*

2001). The context of antigen presentation by APCs is critical. When dendritic cells present antigen in a non-inflammatory microenvironment, as exists around tumours, it results in T cell tolerance rather than T cell priming (Steinman, *et al* 2003). Tumours further inhibit DC maturation and function by secreting such molecules as IL-10 (Gerlini, *et al* 2004) and vascular endothelial growth factor (VEGF) (Gabilovich, *et al* 1996). Stat3 (Cheng, *et al* 2003) and SOCS1 (Evel-Kabler, *et al* 2006) have been recognised as important signalling molecules in DCs in regulating the delicate balance between inducing T cell tolerance and T cell priming. Aside from the microenvironment, several other abnormalities have been noted in DCs in patients with cancer. Firstly, fewer numbers of these cells are found in the peripheral blood of cancer patients (Almand, *et al* 2000). Secondly, those DCs that are recruited to tumour sites appear to be functionally immature with low expression of the costimulatory molecules CD80 and CD86 (iDCs) (Nestle, *et al* 1997, Troy, *et al* 1998). Furthermore, a number of studies have demonstrated that a form of regulatory DC, known as plasmacytoid DCs (pDC) are more likely to accumulate in tumour sites. These pDCs can effectively present antigen to T cells but instead of priming result in anergy (Hartmann, *et al* 2003, Vermi, *et al* 2003, Zou, *et al* 2001). Therefore, although tumour cells, despite being self, should be visible to the immune system they have developed mechanisms of inhibiting DCs so they become invisible.

As well as hiding from the immune system, malignant cells can disable it in a number of ways. Tumours have been shown to secrete a variety of immunosuppressive factors such as transforming growth factor  $\beta$  (TGF $\beta$ ), soluble Fas ligand (Houston, *et al* 2003), prostaglandin E2 (Akasaki, *et al* 2004) and sialomucins (Agrawal, *et al* 1998). Modulation of tryptophan metabolism by the enzyme indoleamine 2,3-dioxygenase (IDO) also contributes to immunosuppression. It causes a shortage of tryptophan resulting in proliferation arrest in T cells (Munn, *et al* 1999). IDO was previously recognised for its role in inducing maternal tolerance to fetal antigens (Munn, *et al* 1998). It was then demonstrated that IDO is also expressed by tumour cells. When immunogenic tumours were engineered to overexpress IDO they grew more aggressively and were less likely to be associated with an activated T cell infiltrate (Uyttenhove, *et*

*al* 2003). Another means of tumour escape is via protein-glycan interactions. The level of expression of galectin-1 by tumours correlates with aggressiveness (Liu and Rabinovich 2005) and there is accumulating evidence that this is via its inhibitory effect on T cell effector functions. Galectin-1 sensitises to T cells to FasL-induced death (Matarrese, *et al* 2005), induces T cell apoptosis (Perillo, *et al* 1995), blocks proximal TCR signalling (Chung, *et al* 2000) and suppresses the secretion of pro-inflammatory cytokines (Rabinovich, *et al* 1999).

The recruitment and expansion of CD4+CD25+ T<sub>regs</sub> represents a systemic method of tumour escape. These cells comprise about 5-10% of all peripheral T cells and may be identified by the expression of the intracellular forkhead box P3 (FOXP3) transcription factor in combination with glucocorticoid-induced TNF receptor (GITR), cytotoxic T lymphocyte antigen-4 (CTLA-4) (Sakaguchi 2004) and absent expression of CD127 (Seddiki, *et al* 2006). The mechanism by which T<sub>regs</sub> induce immunosuppression is unclear but direct contact with CD4+CD25+ T<sub>regs</sub> causes long-lasting anergy and production of IL-10 by CD4+CD25- T cells (Dieckmann, *et al* 2002). IL-10 inhibits cytokine production from T cells and exerts anti-inflammatory and suppressive effects on most types of haemopoietic cells. IL-10 also potently inhibits antigen-presenting cells (Mocellin, *et al* 2001). Curiel and colleagues were the first to demonstrate that CD4+CD25+FOXP3+ T<sub>regs</sub> suppress tumour-specific T cell immunity and contribute to cancer growth *in vivo* with cell numbers inversely correlated with overall survival of patients with ovarian carcinoma (Curiel, *et al* 2004). Increased numbers of these cells in the peripheral blood have been observed in breast and pancreatic cancer (Liyanage, *et al* 2002), head and neck cancers (Chikamatsu, *et al* 2007), acute myeloid leukaemia (AML) (Wang, *et al* 2005), chronic lymphocytic leukaemia (CLL) (Beyer, *et al* 2005) and myeloma (Beyer, *et al* 2006).

Another type of suppressor cell shown to induce tumour-specific T cell tolerance is the myeloid-derived suppressor cell (MDSC) or immature cell (ImC) (Kusmartsev, *et al* 2005). These are bone marrow derived precursors of macrophages, granulocytes and DCs that express CD33 but lack markers of mature myeloid or lymphoid cells including MHC Class II that are thought to

induce T cell anergy by direct cell contact via the TCR (Almand, *et al* 2001). In the mouse these cells are thought to inhibit the differentiation of APCs through the production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Kusmartsev and Gabrilovich 2003). ImCs have been found to be increased in the peripheral blood of patients with cancer and correlate with stage of disease. On surgical removal of tumours, the number of ImCs dropped within 3-4 weeks (Almand, *et al* 2000). Macrophages themselves may also contribute to suppression of tumour immunity. Tumour-associated macrophages (TAMs) have been identified that have an polarised T2 phenotype that promotes cancer progression and metastasis (Mantovani, *et al* 2002) and this can be induced *in vitro* by cancer cells (Hagemann, *et al* 2006).

Defects have also been demonstrated directly within the T cells themselves that prevent them responding appropriately. Impaired proximal T cell signalling with reduced expression of CD3 $\zeta$  and the tyrosine kinases Lck and Fyn was noted in tumour-infiltrating lymphocytes in mouse models (Koneru, *et al* 2005, Mizoguchi, *et al* 1992), a finding that has now been reproduced in humans in multiple cancer types (Kurt, *et al* 1998, Nakagomi, *et al* 1993, Reichert, *et al* 1998, Wang, *et al* 1995).

The downstream effect of the escape mechanisms detailed above is anergic T cells that fail to respond to tumour cells adequately. This has been demonstrated in mouse models by experimental evidence from two research groups that independently showed that antigen-specific CD4 cells become tolerant during tumour growth *in vivo* (Bogen 1996, Staveley-O'Carroll, *et al* 1998). There is now also a body of data from patients demonstrating defective T cell function in a variety of solid tumours (Alexander, *et al* 1993, Miescher, *et al* 1986, Yoshino, *et al* 1992) and the haematological malignancies AML (Buggins, *et al* 1999), acute lymphoblastic leukaemia (ALL) (Nash, *et al* 1993), myeloma (Frassanito, *et al* 2001) and CLL (Scrivener, *et al* 2003).

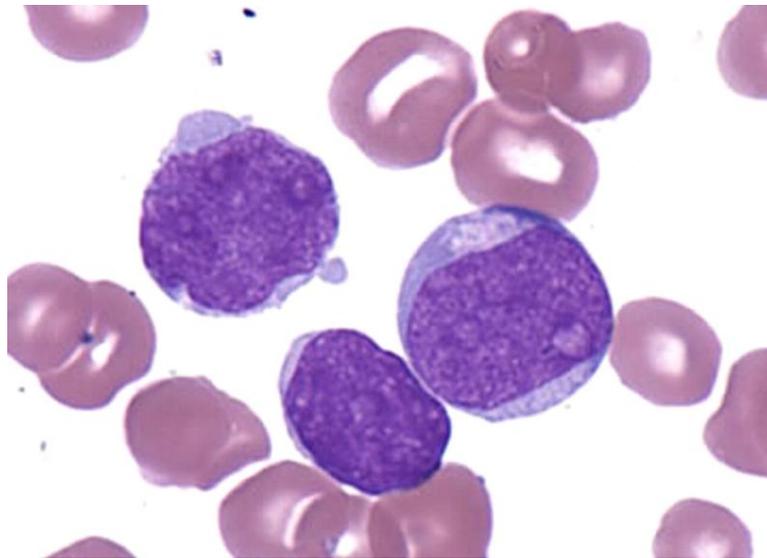
This data illustrates that there is now overwhelming evidence that the immune system has a vital role to play in the development of cancer. If an attempt is to be made to treat or prevent cancer by immune manipulation a detailed understanding

of how T cells and tumours interact is required. For example, the most immunogenic TAA identified *in vitro* may be no use as a vaccine if the corresponding T cells within the patient are effectively exhausted whereas a weaker antigenic target might be able to draw on reserves of previously unstimulated T cells which in the presence of appropriate adjuvants could result in an effective immune response. In addition, the efficacy of T cell infusions as adoptive immunotherapy may be limited in the presence of tumour.

### 1.3 Acute Myeloid Leukaemia (AML)

#### 1.3.1 Classification

The name leukaemia, from the Greek ‘white blood’, was first coined by the German histopathologist Rudolf Virchow in 1847 as a result of the microscopic appearance of what seemed to be pus in the bloodstream of affected individuals (Virchow 1847). This followed the original, largely ignored, clinical description of the disease by Velpeau in 1827 (Velpeau 1827). AML is a heterogeneous aggressive disorder in which malignant transformation results in the accumulation within the bone marrow of early haematopoietic progenitors, called myeloblasts (blasts) (**Figure 1.8**) arising from a leukaemic stem cell (Bonnet and Dick 1997). These myeloblasts have a characteristic immunophenotype: they express markers typical of haemopoietic precursors – CD34, HLA-DR, TdT and CD45 in addition to myeloid lineage markers CD13, CD33, CD15, cytoplasmic myeloperoxidase (MPO) and CD117. A diagnosis of AML is made when myeloblasts make up more than 20% of the cells within the bone marrow (Jaffe 2001). AML was originally classified on the basis of the morphology and cytochemistry of the blasts (French-American-British (FAB)) (Bennett, *et al* 1976, Bennett, *et al* 1985) but more recently the World Health Organisation (WHO) classification has incorporated immunophenotypic, cytogenetic and clinical features in addition to morphology in order to provide a more clinically relevant classification (Jaffe 2001) (**Table 1.1**).



**Figure 1.8: Myeloblasts**

(ASH Image Bank)

FAB Classification	WHO Classification
<p><b>M0:</b> undifferentiated myeloblastic</p> <p><b>M1:</b> myeloblastic without differentiation</p> <p><b>M2:</b> myeloblastic with maturation</p> <p><b>M3:</b> hypergranular promyelocytic</p> <p><b>M3-variant:</b> hypogranular bilobed promyelocytes</p> <p><b>M4:</b> myelomonocytic</p> <p><b>M5a:</b> monoblastic</p> <p><b>M5b:</b> monocytic</p> <p><b>M6:</b> erythroleukaemia</p> <p><b>M7:</b> megakaryoblastic</p>	<p><b>AML with recurrent cytogenetic translocations</b></p> <ul style="list-style-type: none"> <li>• AML with t(8;21)(q22;q22), AML1(CBF<math>\alpha</math>)/ETO</li> <li>• Acute promyelocytic leukaemia (AML with t(15;17)(q22;q11-12) and variants, PML/RAR<math>\alpha</math>)</li> <li>• AML with abnormal bone marrow eosinophils (inv(16)(p13q22) or t(16;16)(p13;q22) CBF<math>\beta</math>/MYH11)</li> <li>• AML with 11q23 (MLL) abnormalities</li> </ul>
	<p><b>AML with multilineage dysplasia</b></p> <ul style="list-style-type: none"> <li>• With prior myelodysplastic syndrome</li> <li>• Without prior myelodysplastic syndrome</li> </ul>
	<p><b>AML and myelodysplastic syndromes, therapy-related</b></p> <ul style="list-style-type: none"> <li>• Alkylating agent-related</li> <li>• Topoisomerase type II inhibitor-related</li> <li>• Other types</li> </ul>
	<p><b>AML not otherwise categorised</b></p> <ul style="list-style-type: none"> <li>• AML minimally differentiated</li> <li>• AML without maturation</li> <li>• AML with maturation</li> <li>• Acute myelomonocytic leukaemia</li> <li>• Acute monoblastic and monocytic leukaemia</li> <li>• Acute erythroid leukaemia</li> <li>• Acute megakaryoblastic leukaemia</li> <li>• Acute basophilic leukaemia</li> <li>• Acute panmyelosis with myelofibrosis</li> <li>• Myeloid sarcoma</li> </ul>

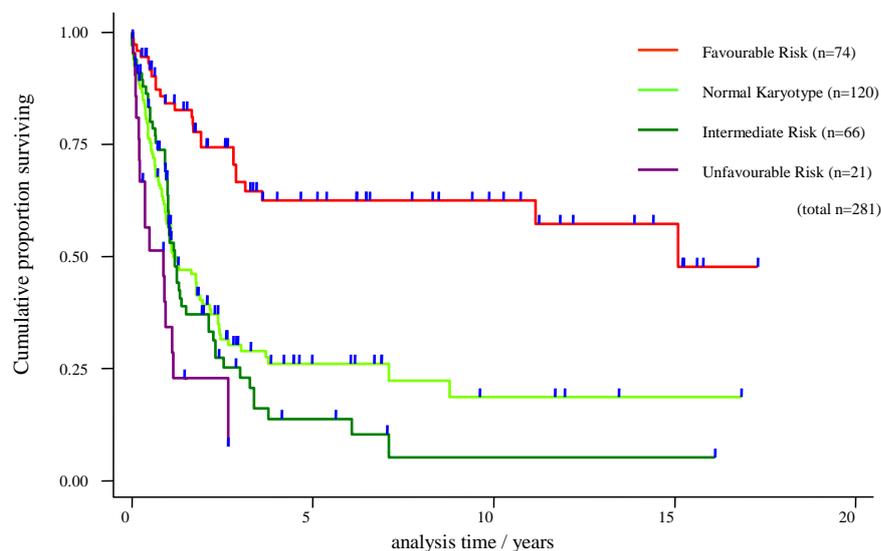
**Table 1.1: Classification of Acute Myeloid Leukaemia**

### 1.3.2 Epidemiology and Aetiology

AML occurs in all age groups but becomes increasingly common with advancing age with a median age of presentation of 68. Data from the Office of National Statistics from 2005 for England document an incidence of AML of 4.2 per 100,000 people for men and 3.4 per 100,000 of the population for women across all age groups. Of the 1049 new cases of AML in men and 868 in women recorded in England in 2005 more than half were in the over 60 age group (<http://www.statistics.gov.uk/>). Obvious aetiological factors may only be identified in around 1% of cases. These include viruses, ionising radiation, cytotoxic chemotherapy and benzene (Sandler and Ross 1997). The majority of AML is thought to be due to sporadic errors during cell division in haematopoietic stem cells resulting in chromosomal translocations. Transcription factors important for normal haematopoiesis are frequently targeted such as core binding factor (*CBF*), retinoic acid receptor alpha (*RAR $\alpha$* ) and mixed lineage leukaemia (*MLL*) genes (Class II mutations). The consequence of these translocations is loss-of-function and impaired haematopoietic differentiation. However, evidence from mouse models and twin studies now suggests that these mutations alone are insufficient to cause AML. Acquired activating mutations in tyrosine kinases such as *FLT3* and *C-KIT* or their downstream effector molecules such as *N-RAS* and *K-RAS* (Class I mutations) confer a proliferative and/or survival advantage without resulting in any block to differentiation. It is thought that only patients who develop both Class I and Class II mutations over time will develop the clinical phenotype of AML (Kelly and Gilliland 2002) although this view has recently been challenged (Liso, *et al* 2008). Individuals can also inherit such mutations that predispose them to the development of AML. Perhaps the best characterised is the autosomal dominant mutation in the transcription factor *RUNX1* that results in familial platelet disorder with propensity to myeloid malignancy (FPD/AML). The median incidence of AML/MDS in individuals who inherit this mutation is 35% (Owen, *et al* 2008).

### 1.3.3 Prognostic Stratification

The dominant clinical feature of AML is bone marrow failure; anaemia, neutropenia and thrombocytopenia, and the disease is rapidly fatal if untreated. At diagnosis patients are stratified into prognostic risk groups according to the cytogenetic abnormalities found within the leukaemic cells (Grimwade, *et al* 1998). Patients with t(8;21), t(15;17) and inv(16) whether alone or in conjunction with other abnormalities have a relatively favourable prognosis in that they are less likely to have primary resistant disease and have a higher overall survival and a lower relapse risk. Patients with complex cytogenetic changes (>5 unrelated changes within one clone), -5/del(5q), abnormalities of 3q or -7 whether alone or in conjunction with intermediate or other adverse risk abnormalities have an unfavourable/adverse outcome. They are less likely to achieve a complete remission (CR) with induction chemotherapy, have a lower overall survival and higher relapse rate. All other patients are classed as intermediate risk and include those with a normal karyotype (**Figure 1.9**).



**Figure 1.9: Survival in AML**

Overall survival of previously untreated AML patients diagnosed < 60 with respect to cytogenetic risk category (1988-2005) (St. Bartholomew's Hospital data).

The so-called ‘normal karyotype’ group makes up the majority of patients and consequently there is much interest in stratifying them further by the identification of new prognostic mutations. Those identified thus far include CCAAT enhancer binding protein alpha (*CEBPA*) (Leroy, *et al* 2005), nucleophosmin (*NPM1*) (Falini, *et al* 2005), FMS-like tyrosine kinase 3 (*FLT3*) internal tandem duplication (ITD) (Kottaridis, *et al* 2001, Thiede, *et al* 2002) and there is increasing evidence for Wilms’ tumour 1 (*WT1*) (King-Underwood, *et al* 1996, Summers, *et al* 2007, Virappane, *et al* 2008). The aim of this stratification is to aid treatment decisions.

### **1.3.4 Treatment**

Despite major advances in the understanding of the biology of AML over the last 20 years, pharmacological agents used in the treatment of AML have changed very little. These drugs include the cytotoxic anthracycline antibiotic, daunorubicin which inhibits the enzyme topoisomerase II that is critical for effective DNA synthesis; mitoxantrone which is structurally related to daunorubicin; the podophyllotoxin, etoposide, another topoisomerase II inhibitor and the antimetabolite cytarabine (ara-C), an analogue of the naturally occurring nucleoside 2’deoxycytidine that allows it to be incorporated into DNA and RNA but its main cytotoxic action is to inhibit DNA polymerase (Rang). Alone, each of these agents only has a small impact on the disease. It is in their combination that their strength lies. The combination DA (daunorubicin and ara-C) resulted in a CR rate of 60-65% when initially introduced and became the template for modern drug regimens in AML (Yates, *et al* 1973).

Therapy involves initial induction chemotherapy to induce a morphological CR defined as <5% myeloblasts present in a normocellular bone marrow. This is followed by multiple courses (between two and three) of consolidation chemotherapy to eradicate minimal residual disease and maintain the remission. Each course induces a profound, though transient bone marrow hypoplasia with the requirement for transfusional support with packed red cells and platelets and the risk of neutropenic sepsis. In older patients or those with existing co-

morbidities, this curative treatment regimen may be inappropriate. The alternative is to provide supportive care with blood products, antibiotics and low dose ara-C (Burnett, *et al* 2007). All AML subtypes are treated in the same way apart from acute promyelocytic leukaemia (APL) associated with the t(15;17). In this case, all patients are given all-trans retinoic acid (ATRA), a derivative of vitamin A, which causes differentiation of the immature promyelocytes into mature granulocytes.

### 1.3.5 Outcomes

Despite these intensive treatment regimens, the outcome for individuals who develop AML is not good with the greatest risk being relapse. Several multicentre prospective studies have now shown that around 80% of patients under the age of 60 will enter CR of whom 40-50% will survive 5 years. Relapses generally occur within three years of initial treatment. Once a relapse has occurred, around 40% of patients will achieve a second remission but this is often short-lived with only 10-15% surviving long-term (Treleaven 2009) (**Table 1.2**). Varying the combination of existing chemotherapeutic agents is unlikely to have a large impact on these overall survival figures and hope rests with alternative therapeutic strategies.

Group	No. of Patients	CR (%)	ID (%)	RD (%)	Relapse Risk at 5yr (%) (SE)	Survival at 5yr (%) (SE)
<b>Favourable</b>	377	91	8	1	35 (2.8)	65 (2.5)
<b>Intermediate</b>	1,072	86	6	8	51 (1.8)	41 (1.5)
<b>Adverse</b>	163	63	14	23	76 (4.5)	14 (2.8)

**Table 1.2: Outcomes in AML**

CR rates, reasons for failure, relapse risk and survival by hierarchical cytogenetic risk group

CR: complete remission, ID: induction death, RD: refractory disease, SE: standard error. (From (Grimwade, *et al* 1998)).

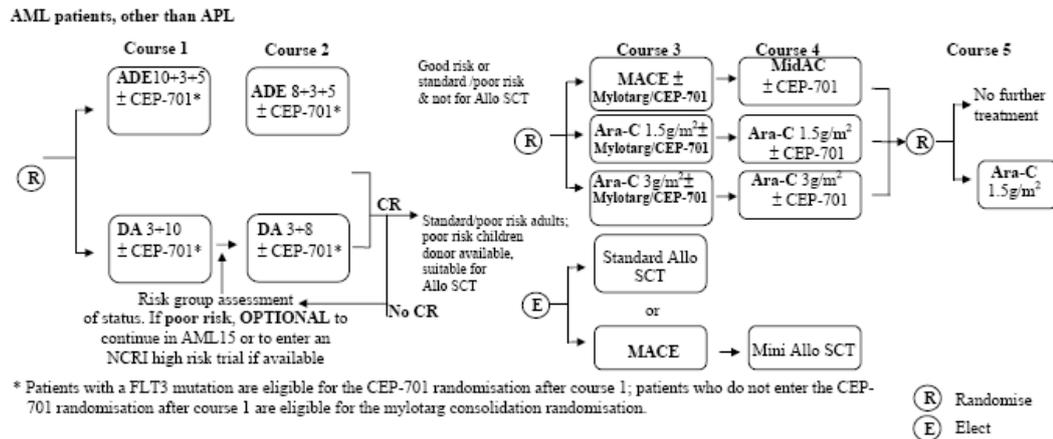
### **1.3.6 Haematopoietic Stem Cell Transplantation (SCT)**

Once a patient has relapsed, their prognosis is awful. The current role of stem cell transplantation in AML is therefore to prevent this complication. There is accumulating evidence that AML can be cured with allogeneic stem cell transplantation (Clift, *et al* 1987, Gale, *et al* 1996). This procedure involves ablating an individual's haemopoietic and immune system by chemo/radiotherapy rendering them aplastic and then replacing it with stem cells harvested from another individual. These allogeneic stem cells repopulate the bone marrow. Immunocompetent donor T cells transferred with the graft can recognise and destroy residual malignant cells in the graft-*versus*-leukaemia effect (GVL). However, these healthy T cells can also recognise normal host cells resulting in the major side effect of graft-*versus*-host disease (GVHD), a life-threatening disorder affecting the skin, gut and liver (Billingham 1966). Attempting to separate the GVHD from the GVL effect is a major research focus. The incidence of GVHD and other complications result in significant transplant-related mortality (TRM) of 20-25% which means that, despite the antileukaemic efficacy of allogeneic transplantation, it is not without risk. Autologous stem cell transplantation by definition allows no GVL effect and relies purely on the chemo/radiotherapy to eradicate residual malignancy and as a result has a lower TRM of around 5%. An early concern was the risk of re-infusing malignant cells with the stem cell graft as a result of which purging techniques were developed.

Four major study groups, EORTC-Gimema, GOELAM, US Intergroup and UK MRC performed pivotal studies in the 1980s and 90s in an attempt to address the issue of which treatment modality, allogeneic transplant, autologous transplant or chemotherapy alone, was best in CR1 (Burnett, *et al* 2002, Cassileth, *et al* 1998, Harousseau, *et al* 1997, Zittoun, *et al* 1995). In the autograft comparison, in all cases, a reduced risk of relapse was seen but no survival benefit could be demonstrated due to transplant-related mortality effects and the ability to salvage patients who relapsed after chemotherapy. With regards to allogeneic transplantation, a donor *versus* no donor analysis showed there was no significant survival difference at four years in any of the four trials. These studies have been criticised however on the basis that 30% of patients with a donor did not go on to

receive an allograft. A more recent meta-analysis looked at studies that evaluated the outcome of patients with AML in CR1 by availability of a donor using an intention-to-treat approach regardless of treatment actually received to try to eliminate the selection bias demonstrated previously (Yanada, *et al* 2005). This analysis did demonstrate an overall survival advantage for patients with donors. It also showed for the first time the effect of cytogenetic risk group on outcome from allogeneic transplant with an advantage observed for poor-risk patients with donors. Allogeneic transplant conferred no benefit to favourable-risk patients but for the intermediate risk group the situation was unclear.

Therefore, the current strategy in the UK is to use autologous transplantation in AML in patients with APL in second molecular remission, older patients who would otherwise have been considered for allogeneic transplant and younger patients who lack a sibling or unrelated donor (Apperley 2008). Due to their high risk of relapse, poor risk patients in first CR, less than 35 years with good performance status should be treated with allogeneic stem cell transplantation. If a suitable sibling donor is unavailable, efforts should be made to use an alternative donor; matched unrelated (MUD), cord blood or haploidentical (Apperley 2008). It remains an ongoing topic of debate as to the most suitable treatment for intermediate risk patients. Further identification of molecular markers should aid stratification and treatment decisions. Currently, if patients in this category are to be transplanted in CR1, it should only be in the context of a clinical trial (Treleaven 2009). The current MRC AML 15 study aims to answer this question (**Figure 1.10**).



**Figure 1.10: MRC AML15 Flowchart for Non-APL AML Treatment**

In view of the treatment intensity, allogeneic transplant is only an option for young, fit individuals. In recent years, there has been a push towards less intensive conditioning regimens in order to broaden the applicability of this form of treatment. The reduced intensity non-myeloablative allogeneic transplant relies on an immunosuppressive conditioning regimen to allow donor stem cells to engraft and then makes use of donor lymphocyte infusions later on to encourage complete engraftment. Rather than using a myeloablative conditioning regimen to get rid of residual disease, this type of transplant relies on the GVL effect. Despite a lack of prospective randomised controlled trial data, reduced intensity transplants have been widely adopted and now account for 27% of allogeneic stem cell transplants in Europe, a rise from <1% 3 years previously (Gratwohl, *et al* 2002). However despite the early reduction in TRM because of the less intense conditioning, long-term morbidity and mortality remain high, largely due to GVHD and relapse. Currently no AML-specific prospective trials comparing results of reduced intensity protocols with standard myeloablative conditioning regimens have been published.

### 1.3.7 Molecular Targeted Therapies

More targeted treatments are currently being investigated in AML (Burnett and Knapper 2007). These include FLT3 inhibitors such as CEP-70 and farnesyl transferase inhibitors such as tipifarnib. Farnesyl transferases catalyse post-

translation modification of RAS and other proteins involved in signal transduction in proliferating cells. C-KIT inhibitors such as dasatinib are being evaluated in the 30% of CBF leukaemias that have a C-KIT mutation. Potential epigenetic changes in AML are being targeted through the investigation of demethylating agents and histone deacetylase inhibitors. Interest has also focused on targeting the CD34<sup>+</sup>CD38<sup>-</sup> leukaemic stem cell (Bonnet and Dick 1997) using the monoclonal antibody IL3-R $\alpha$  (Yalcintepe, *et al* 2006) and mammalian target of rapamycin (mTOR) inhibitors (Recher, *et al* 2005). Alone, none of these agents alone show significant efficacy and are therefore all being evaluated in combination with existing drug regimens.

#### **1.4 The Immune System in AML**

There is accumulating evidence of the power of the immune system to cure AML. Spontaneous remissions in association with concurrent infections have been reported (Fozza, *et al* 2004, Ifrah, *et al* 1985, Maywald, *et al* 2004, Trof, *et al* 2007). A study in the New England Journal of Medicine from 1971 looked at the 'immune competence' (as assessed by delayed type hypersensitivity skin testing, antibody response to immunisation with keyhole limpet haemocyanin (KLH) and lymphocyte proliferative response to stimulation with the mitogen phytohaemagglutinin) in 25 AML patients pre and post chemotherapy and found that those with normal immune competence were more likely to achieve a complete or partial response to therapy (Hersh, *et al* 1971). The emergence of anti-tumour CTLs was associated with the maintenance of remission in children with AML treated with chemotherapy alone or with autologous transplants (Montagna, *et al* 2006). However, it is in the allogeneic setting that the ability of healthy T cells to eliminate residual leukaemic blasts in the graft-*versus*-leukaemia effect is really apparent (Horowitz, *et al* 1990). This data suggests that it is possible for a healthy immune system to control AML. However, before successful immunotherapeutic interventions can be made in this disease it is crucial to understand what impact the leukaemic blasts have on the immune system that allows them to escape immunosurveillance mechanisms.

The microenvironment in AML is immunosuppressive. This was first noted in the 1970s when autologous tumour cells were shown to inhibit the proliferation of remission lymphocytes stimulated with alloantigen. This suppressive effect could be inhibited by ultrasonic disintegration of the blasts, exposing the blasts to UV light or by adding them more than twenty-four hours after stimulation of the lymphocytes (Taylor, *et al* 1979). Several mechanisms may be important in the induction of this effect and numerous cell types have been implicated.

#### **1.4.1 Costimulatory Molecules**

Despite expressing dendritic cell-like surface antigens such as CD11c, HLA-DR, CD86 and CD83, myeloblasts are thought to be poor antigen presenting cells. This is because their expression of MHC Class I and the co-stimulatory molecules CD40 (which enhances the efficacy of antigen presentation) and CD80 is low (Brouwer, *et al* 2000, Vollmer, *et al* 2003, Whiteway, *et al* 2003). Expression of these molecules can be induced by 24 hours in culture and results in enhanced recognition by T cells. However, even when costimulatory molecule expression is induced in leukaemia cell lines and a T cell proliferative response obtained, T cell production of the 'anti-cancer' T-helper 1 (T<sub>H</sub>1) type cytokine profile is still inhibited. Thus up-regulation of co-stimulatory molecule expression alone is not sufficient for anti-tumour immunity (Buggins, *et al* 1999). Of note, in blocking experiments, no single co-stimulatory or adhesion molecule plays a crucial role in the recognition of leukaemic blasts by cytotoxic T cells. Instead it is the combination that is required (Brouwer, *et al* 2000).

The heterogeneous nature of AML is demonstrated in that not all FAB types act as poor APCs. Cell lines derived from AML blasts with the inv(16) cytogenetic abnormality are able to induce activation and proliferation of autologous and allogeneic T cells which are then capable of lysing inv(16) positive blasts whereas cell lines derived from other FAB types can only induce T cell activation (Banat, *et al* 2003). This allows speculation that improved antigen presentation to the immune system may be one mechanism for the improved prognosis in these patients.

#### **1.4.2 Secretion of Inhibitory Molecules**

The possibility that AML myeloblasts secrete an inhibitory serum factor was postulated by Lim and colleagues in 1991 (Lim, *et al* 1991). Twenty-seven patients with AML were shown to have impaired cytotoxic function of CD3<sup>+</sup> T cells despite increased expression of cytoplasmic cytotoxic markers. The presence of such an inhibitory molecule was later confirmed. T cells were purified from the peripheral blood or bone marrow of healthy donors. These were then cultured either alone or in the presence of primary AML cells placed in a cell culture insert that prevented contact with the T cells. T cell activation was induced with either anti-CD3/anti-CD28 or phorbol myristate acetate (PMA)/ionomycin and subsequent T cell activation and proliferation then assessed. It was found that the presence of the AML cells inhibited the production of IL-2 and IFN $\gamma$  by the T cells and this inhibition occurred in the absence of cell contact. The nature of this secreted inhibitory factor was investigated by pre-incubating the AML blasts with ganglioside synthesis inhibitors, nitric oxide inhibitor, indomethacin and neutralising antibodies to TGF $\beta$  and IL-10, none of which were able to reverse the inhibitory effect. Furthermore, an enzyme-linked immunosorbant assay (ELISA) for VEGF levels in AML cell supernatants was undetectable. The fact that this inhibitor was a protein was established as trypsinisation of the supernatant prior to addition to AML cells destroyed the inhibitory effect. In addition, pre-incubation of the AML cells with the inhibitors of golgi function, brefeldin A or sodium monesin, also overcame the inhibitory effect. The consequences to T cell signalling of incubation with tumour supernatant were firstly, prevention of nuclear translocation of NF $\kappa$ B and NFATc thus preventing their function as transcription factors for the production of cytokines and up-regulation of cell surface receptors and secondly, inhibiting entry of T cells into the cell cycle by preventing phosphorylation of pRb and p130 and preventing induction of p107 and c-myc. The group concluded that inhibition of NF $\kappa$ B by the protein produced by AML cells would be sufficient to account for all the effects noted (Buggins, *et al* 2001). A further late event in T cell immunosuppression by AML cells is the loss of CD3 $\zeta$  chain which of note is also important for the transduction of activating signals in NK cells (Buggins, *et al* 1998, Moretta, *et al* 2001). These

findings were independently confirmed and extended to show that AML blasts also inhibit the proliferative activity of NK cells (Orleans-Lindsay, *et al* 2001).

The microenvironment in AML is also anti-apoptotic (Milojkovic, *et al* 2004). Not only does the tumour supernatant delay apoptosis of both resting and stimulated T cells, it also has this effect on peripheral B cells, monocytes and neutrophils as well as promoting the survival of other AML myeloblasts. The mechanism postulated for this is a reduction in cleavage of pro-caspase-3, -8, -9 and the caspase substrate poly (ADP-ribose) polymerase (PARP) perhaps as a result of up-regulated bcl-2 (which is down-regulated in anti-CD3/anti-CD28 activated T cells). These effects may be mediated by inhibition of c-myc or by prevention of phosphorylation of pRb.

It is interesting to note that other myeloid diseases display similar characteristics. However, in chronic myeloid leukaemia (CML) and myelodysplasia the immunosuppressive effect upon T cells correlated with disease stage. The higher the blast count, the greater the T cell immunosuppressive effect seen and the lower the level of c-myc (Milojkovic, *et al* 2005).

Although it has been established that the immunosuppressive molecule secreted by blasts is a protein, to date its identity remains unknown.

### **1.4.3 Regulatory T Cells**

The peripheral blood of patients with AML contains an increased population of CD4+ CD25+ regulatory T cells (4.1% +/- 1.8% in AML patients compared with 2.0% +/- 0.5% in healthy donors) (Wang, *et al* 2005). Although, these T<sub>regs</sub> have a similar immunophenotype and cytokine secretion pattern to T<sub>regs</sub> from healthy individuals they have enhanced apoptotic and proliferative capabilities compared with T<sub>regs</sub> from healthy individuals. Furthermore, they suppress the proliferation and secretion of T<sub>H1</sub> type cytokines (IL-2 and IFN $\gamma$ ) but improve the secretion of IL-10. Thus this population of cells may be contributing to the evasion of the immune response by the tumour.

#### **1.4.4 NK Cells**

AML blasts, in common with many other tumour cells, down-regulate MHC molecules and are thus targets for NK cell attack. The anti-tumour activity of NK cells has been demonstrated in mouse models where they have been shown to inhibit radiation-induced lymphomagenesis (Datta 1996) and to control the tumorigenesis induced by human T-cell leukaemia virus (Feuer, *et al* 1995, Stewart, *et al* 1996). In human cancer, it has been shown in CML that NK cell number and function progressively decrease during the spontaneous course of the disease (Pierson and Miller 1996) and that both recover on treatment with interferon- $\alpha$  (Pawelec, *et al* 1995). In AML, impaired NK cell activity and lack of production of the NK stimulatory cytokines IL-1 $\beta$ , IFN $\gamma$  and IL-2 have been associated with early relapse (Lowdell, *et al* 2002, Tajima, *et al* 1996).

NK cells also express natural cytotoxicity receptors (NCRs). Engagement of these receptors strongly enhances NK-mediated cytolytic activity. In contrast to healthy donors, in most patients with AML the majority of NK cells have a low NCR surface density which correlates with weak cytolytic activity against autologous leukaemic cells (Costello, *et al* 2002b).

#### **1.4.5 Dendritic Cells**

In AML, circulating DCs exhibit the cytogenetic abnormalities of the leukaemic blasts suggesting they have arisen from the leukaemic clone. Furthermore abnormalities have been noted in the ratio of myeloid DCs to the regulatory pDCs. *Ex vivo* pDCs were also found to have impaired function (Mohty, *et al* 2001). Thus normal cross-priming responses may be inhibited.

#### **1.4.6 Fibroblasts**

The role of the bone marrow microenvironment in supporting leukaemic cell growth is becoming evident. Leukaemia cell apoptosis was shown to be inhibited by co-culture of AML cells in direct contact with a fibroblast cell line (Garrido, *et*

al 2001). It has subsequently been shown that there is bidirectional cytokine cross-talk between leukaemic blasts and fibroblasts resulting in increased proliferation, anti-apoptotic signaling and increased local levels of the pro-angiogenic IL-8 (Ryningen, *et al* 2005).

## **1.5 Curing AML by Immune Manipulation**

Current therapies for AML rely on drugs that target proliferating cells and therefore they also have profound effects on normal dividing cells the result of which is often life-threatening side effects. Interest has focused on immunotherapy as it has the potential to be the most tumour-specific ‘magic bullet’ that can be devised. It can take the form of active immunity whereby the host’s own weak immune response to tumour is augmented either by vaccinations with tumour cells or antigens or the use of cytokines. Alternatively, tumour – specific antibodies or tumour-specific cytotoxic T cells (adoptive cellular therapy) can be administered as a form of passive immunity.

### **1.5.1 Vaccination**

A requirement for the development vaccines is the identification of TAAs. This has been made technically possible through the introduction of serological screening of cDNA expression libraries with autologous serum (SEREX) (Sahin, *et al* 1995) and microarray technologies. AML-specific TAAs identified by these techniques include receptor for hyaluronic acid mediated motility RHAMM/CD168 (Greiner, *et al* 2002), the cancer-testis antigens MPP11 (Greiner, *et al* 2003) and PASD1 (Guinn, *et al* 2005), Proteinase 3 and preferentially expressed antigen of melanoma (PRAME) (Greiner, *et al* 2000). Other antigens identified as possible targets for immunotherapy due to their overexpression in AML include CD33 (Bae, *et al* 2004), BCL-2 (Andersen, *et al* 2005), oncofetal antigen-immature laminin receptor (OFA-iLRP) and WT1 (Bergmann, *et al* 1997).

Naturally occurring cytotoxic T cells directed towards TAAs in AML patients have been identified in the cases of proteinase 3 and WT1 (Scheibenbogen, *et al* 2002), RHAMM/CD168 (Greiner, *et al* 2005) and PRAME (Rezvani, *et al* 2008).

Identification of these TAAs has allowed one of the earliest vaccine approaches to be utilised; immunisation with purified tumour antigen in the form of peptide along with an adjuvant to improve immunogenicity. Phase I clinical trials have already been reported for WT1 and RHAMM/CD168 that demonstrated clinical responses in association with an increase in frequency of antigen-specific CTLs (Oka, *et al* 2004) (Schmitt, *et al* 2008). Others are underway for the PR1 peptide (derived from proteinase 3) (Heslop, *et al* 2003) and the Hammersmith Hospital has initiated a study combining vaccines for WT1 and PR1 (K. Rezvani; Leukaemia Research Fund (LRF) Immunotherapy Day, Institute of Child Health 12.11.08).

Vaccination strategies have also been investigated using the antigenic DNA rather than peptide. Naked DNA in association with some form of adjuvant is injected into muscle or skin employing a method to improve transfection rates such as a gene gun or electroporation (Heslop, *et al* 2003). DNA is subsequently taken up by muscle or skin cells, processed and the resultant protein shed as antigen to be taken up by local APCs and presented on both Class I and Class II molecules. The advantage of this approach is that all arms of the immune response are activated. Such a strategy has been investigated for the treatment of APL. This form of AML is characterised by the expression of the fusion oncoprotein PML-RAR $\alpha$  which represents a tumour specific antigen. Mice with APL that were vaccinated with naked DNA from PML-RAR $\alpha$  in association with the tetanus toxin fragment C survived longer than unvaccinated mice. Increased antibody and IFN $\gamma$  production as well as relatively higher numbers of CD4 and CD8 T cells were seen in responding animals (Padua, *et al* 2003).

An alternative form of vaccination that avoids the requirement for identification of TAAs is to use autologous tumour cells that have been 're-activated' to improve their antigen-presenting capacity. Gamma-irradiation of myeloblasts

induces the expression of co-stimulatory molecules. The blasts are then effective stimulators of T cell proliferation *in vitro* (Vereecque, *et al* 2004a). This technique was used in an early clinical trial at St. Bartholomew's Hospital (Powles, *et al* 1977). More recently, autologous blasts have been genetically modified *ex vivo* to express costimulatory molecules such as CD80 alone (Hirst, *et al* 1997) or in combination with stimulatory cytokines such granulocyte-macrophage colony-stimulating factor (GM-CSF) (Stripecke, *et al* 2000) or IL-2 (Chan, *et al* 2005). Genes may be transferred by electroporation or lipofection but both these methods result in low gene transfer frequencies and high cell death. A much more effective strategy is the use of viral vectors. Both retroviral and adenoviral vectors have been tried but the gene transduction rate was found to be poor as AML blasts fail to cycle in culture and AML cells only rarely express the coxsackie and adenovirus receptor (CAR) required by adenoviruses for infection. As lentiviral vectors can infect non-dividing cells they have proved much more successful (Chan, *et al* 2006). This method results in tumour cells becoming potent stimulators of autologous and allogeneic T cells and NK cells. A clinical trial is now underway at King's College Hospital in which AML patients in first remission post allogeneic stem cell transplant will receive irradiated autologous tumour cells modified to express CD80 and IL-2 (Chan, *et al* 2006).

An alternative method of reactivation is to culture blasts with the cytokines IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor –alpha (TNF $\alpha$ ). This converts them into effective APCs with costimulatory molecule expression – so-called 'leukaemia-derived dendritic cells (LDDC) (Cignetti, *et al* 1999). These have been generated from patients at diagnosis and reinjected as vaccine therapy once the patients had achieved complete remission in a phase I/II trial involving 22 patients (Roddie, *et al* 2006). Anti-leukaemic T cell responses were seen but the technique is not broadly applicable as only around a quarter of patients have AML cells that can successfully differentiate into LDDC.

Rather than using leukaemia-derived DCs, DCs of non-leukaemic origin can be used. These can be loaded *ex vivo* with tumour antigen and then injected as

cellular vaccines. There are numerous ways that antigen can be loaded onto DCs; if the antigen is known DCs can be pulsed with the peptide of interest itself, if it is unknown, DCs can be pulsed with tumour cell lysate, apoptotic tumour cell bodies or tumour-derived RNA or DNA. Alternatively, DCs can be fused directly to tumour cells. These techniques have resulted in tumour regressions in animal models and numerous clinical trials in solid tumours are underway in the USA (Galea-Lauri 2002). In AML, several groups have used these methods to demonstrate the generation of anti-leukaemic CTLs *in vitro* using DCs pulsed with autologous peptides eluted from AML blasts (Delluc, *et al* 2005), irradiated autologous blasts (Fujii, *et al* 1999, Spisek, *et al* 2002), a MUC1-derived peptide (Brossart, *et al* 2001) and fusion hybrids (Klammer, *et al* 2005). A window between 20 and 60 days post chemotherapy has been demonstrated for generating autologous monocyte-derived DCs from AML patients (Royer, *et al* 2008) and the potential for using monocyte-derived DCs from an HLA-matched allogeneic donor established (Lee, *et al* 2008). One case report illustrated the use of a WT1 and KLH pulsed donor-derived DC vaccine administered in a patient who relapsed post allogeneic stem SCT. Immune responses were observed towards the KLH but not the WT1 and the leukaemia clinically progressed (Kitawaki, *et al* 2008).

Despite the widespread interest in vaccine therapy in cancer, it has yet to demonstrate a real therapeutic benefit. In a review of 1,306 vaccine treatments only a 3.3% overall response rate was observed (Rosenberg, *et al* 2004). Possibly one of the biggest disappointments in the field of haematology are the recently discussed results of a phase III trial of anti-idiotypic vaccines in follicular lymphoma that showed no improvement in progression-free survival (Levy 2008). What could be the reasons for these poor results? Some vaccines only manage to recruit CD8 T cells and it is becoming increasingly apparent that CD4 help is crucial for effective vaccination strategies therefore antigens chosen must also be able to be presented by class II MHC molecules. Another potential reason for this could be that anti-tumour CTL responses induced by immunization are being suppressed by T<sub>regs</sub>. Such TAA-specific T<sub>regs</sub> have been identified for WT1 in AML patients (Lehe, *et al* 2008). Investigators have attempted to eliminate T<sub>regs</sub>

using IL-2 conjugated to diphtheria toxin (ONTAK) with some improvement in tumour-specific T cell responses (Dannull, *et al* 2005). As CD25 is also present on effector T cells, more selective depletion strategies may be appropriate. Anti-CTLA-4 has been used as part of vaccination strategies in solid tumours with objective clinical responses but with the side effect of auto-immunity (Phan, *et al* 2003). Finally, as discussed by Professor Ron Levy in the context of his follicular lymphoma trial (LRF Immunotherapy Meeting, Institute of Child Health 12.11.08) and mirroring the data from 1971 by Hersh and colleagues (Hersh, *et al* 1971) it would seem that some individuals are better able to mount immune responses than others and these patients do show better survival. The challenge lies in identifying those patients who are more likely to respond to vaccination protocols or alternatively finding methods to improve the immune responses of those who currently do not mount effective responses to tumour vaccines.

### **1.5.2 Cytokines**

As well as engineering vaccines to carry cytokines in addition to antigen, cytokines may be administered systemically. The cytokine IL-2 has a critical role in the activation and proliferation of B and T cells as well as the ability to generate lymphokine-activated killer cells (LAK) that can lyse leukaemia cells *in vitro* (Oshimi, *et al* 1986). The first reports of the use of IL-2 to prevent relapse in patients with AML in CR showed inconsistent efficacy and often severe toxicity (vascular leak syndrome) associated with high doses (Cortes, *et al* 1999, Macdonald, *et al* 1990). When the first phase II study reported two treatment-related deaths and failed to show superiority of IL-2 treatment over no treatment, interest waned (Blaise, *et al* 1997). Low dose regimens have more recently been trialled in both children and adults over the age of 60 in CR, neither of which have demonstrated efficacy (Baer, *et al* 2008, Lange, *et al* 2008). However a phase III trial involving 320 patients has demonstrated improved leukaemia-free survival in patients who received IL-2 maintenance therapy (Brune, *et al* 2006). This discrepant result could be due to the addition of histamine dihydrochloride, an agent added for its potential to protect leukaemia-reactive T and NK cells from phagocytes.

The combination of IL-2 with IL-12 has also been evaluated *in vitro*. Together these cytokines have been shown to induce lysis of blasts resistant to classic LAK effects at doses of IL-2 much lower than previously required (Vitale, *et al* 1998). However no clinical trials using this combination have been reported.

There is also a case report indicating the efficacy of IFN $\alpha$  in AML. Treatment was commenced in second chemotherapy-induced CR and the patient remained in remission for up to two years (Lowdell, *et al* 1999).

### **1.5.3 Antibodies**

CD33 is widely expressed by myeloblasts but also leukaemic stem cells making it an attractive target for immunotherapy (Taussig, *et al* 2005). It has been targeted by the immuno-conjugate gemtuzumab ozogamicin (GO; Mylotarg). This is an IgG<sub>4</sub> humanised monoclonal antibody conjugated to the cytotoxic agent calicheamicin. As a single agent it was found to be safe and to result in a CR rate of 26% in patients in first relapse (Larson, *et al* 2005, Sievers, *et al* 2001). It has subsequently been investigated in an upfront setting in combination with conventional chemotherapy in the elderly (Amadori, *et al* 2004) and in young AML patients (Kell, *et al* 2003) with encouraging results. The current MRC AML 15 trial is randomising patients to induction therapy with or without GO and also randomising to examine whether GO is beneficial in consolidation. A non-conjugated humanised CD33 monoclonal antibody (lintuzumab) has also been reported to cause objective responses including CRs as monotherapy in elderly patients at diagnosis with minimal side effects and the potential for outpatient therapy (Raza 2007). However, a potential problem with CD33 as a target is that it is also expressed on normal haematopoietic stem cells (Taussig, *et al* 2005) – a possible explanation for the prolonged cytopenias already noted with the use of Mylotarg (Kell, *et al* 2003, Sievers, *et al* 2001).

Other antibodies that are of interest include CD44, an adhesion molecule expressed on AML blasts that appears capable of inducing differentiation in AML cell lines and in some cases inhibits proliferation and stimulates apoptosis via a

direct effect on leukaemic stem cells (Charrad, *et al* 2002, Charrad, *et al* 1999, Jin, *et al* 2006). A fully humanised anti-FLT3 antibody has also been developed that inhibits both wild-type and ITD-mutant FLT3 and prolongs survival of leukaemia-bearing mice (Li, *et al* 2004).

#### **1.5.4 Adoptive T cell Immunotherapy**

This technique involves the *ex vivo* expansion of cytotoxic T cells and their subsequent infusion. Various cell types and *ex vivo* manipulations have been used in clinical trials of adoptive immunotherapy. Most frequently utilized are unmanipulated donor T cells (donor lymphocyte infusions – DLI) post allogeneic SCT to augment GVL effects and eliminate residual disease. However, a side effect of infusing alloreactive cells is to induce GVHD. In attempt to limit this problem, donor T cells have been engineered with a suicide gene such as herpes simplex virus 1- thymidine kinase (HS-tk) gene. When a severe GVHD reaction is seen, these alloreactive T cells can be specifically eliminated by the administration of ganciclovir (Bonini, *et al* 1997). Unfortunately however the tk gene product is immunogenic resulting in immune destruction of the engineered lymphocytes therefore alternative suicide genes are being investigated. Autologous T cells can also be manipulated to improve their recognition of tumour cells. T cells can be nonspecifically activated *ex vivo* by incubating peripheral blood mononuclear cells with anti-CD3 and anti-CD28 monoclonal antibodies conjugated to beads. This results in T cell activation and has dramatically improved T cell defects *in vitro* in CLL (Bonyhadi, *et al* 2005). Infusion of T cells treated in this way has been the subject of early clinical trials (Porter, *et al* 2006). Two other activated T cell types can be generated *ex vivo*; cytokine-activated killer (CIK) cells and lymphokine-activated killer (LAK) cells (**Table 1.3**).

Cell Type	Origin	CD3	CD56	Culture Conditions	MHC-restriction	<i>In vitro</i> activity and clinical efficacy
<b>LAK</b>	NK cell	-	+	Culture PBMCs in IL-2 at 1000 U/ml for 3-5 days	No	Cytolytic against NK resistant targets, 20-30% response rate when given with IL-2 in renal cell carcinoma and melanoma patients
<b>CIK</b>	T cell	+	+	Culture PBMCs in IFN $\gamma$ , OKT3, IL-2 for 3-4 weeks	No	Activity against autologous and allogeneic tumour cells. Limited information on clinical trials

**Table 1.3: Characteristics of LAK and CIK Cells**

(Adapted from (Linn and Hui 2003)).

Most work on LAK cells has been in solid tumours where these cells have been generated *ex vivo* then infused with IL-2 with modest effect (Rosenberg, *et al* 1985) although their use post autologous transplant in AML has also been reported (Benyunes, *et al* 1993). 3+56+ CIK cells are thought to be an *in vitro* phenomenon derived from 3+56- T cells in the peripheral blood. At the end of culture CIK cells have potent cytolytic activity against tumour targets and are better at protecting against tumour development in SCID mice transplanted with human lymphoma cells compared with LAK cells with the advantage of not requiring the additional use of IL-2 (Lu and Negrin 1994). The feasibility of infusing autologous CIK cells post autologous SCT in AML has recently been reported in 13 patients (Arai 2007).

Autologous T cells with cytotoxicity against autologous AML cells have been cloned from patients in cytological remission (Csako, *et al* 1980). Therefore the prospect of generating anti-leukaemic CTLs to treat residual disease would seem a possibility. Unfortunately the generation and expansion of such cell lines remains technically difficult particularly due to the poor immunogenicity of leukaemic blasts although they have been achieved in mouse models (Boyer, *et al* 1997). Antigen-specific CTLs may also be generated. One of the most convincing examples of this has been achieved by infusing polyclonal Epstein-Barr Virus

(EBV)-specific T cell lines or CD8 T cell clones to treat patients with post-transplant EBV-associated lymphoproliferative disease (Khanna, *et al* 1999, Savoldo, *et al* 2000). Finally, T cells can be transduced with genetically engineered TCRs. The potential of this technique has recently been demonstrated in metastatic melanoma. Autologous lymphocytes were genetically engineered to express the genes encoding the alpha and beta chains of the TCR specific for MART-1, a melanoma TAA. These were then re-infused into 15 patients after maximum lymphodepletion. Durable engraftment was seen at levels greater than 10% of peripheral blood lymphocytes for at least two months post infusion. In two patients, high sustained levels of engineered cells could be detected at one year post infusion and this was associated with objective clinical regression of metastatic lesions (Morgan, *et al* 2006). A similar method has also been attempted in AML with T cells engineered specific to the WT1 antigen (Tsuji, *et al* 2005) which were able to eliminate leukaemia in a non-obese diabetic-severe combined immune deficiency (NOD-SCID) mouse model (Xue, *et al* 2005). A phase I clinical trial is soon to open in patients with AML and CML run by the Royal Free Hospital (E. Morris; LRF Immunotherapy Day, Institute of Child Health 12.11.08). A NK cell line, YT, has also been modified by gene transfer to express a humanised chimaeric TCR specific for CD33. These cells were then able to specifically lyse a human AML cell line. As NK cells do not have the MHC specificity requirements that CTLs have, this cell line potentially could represent an unlimited resource for immunotherapy in AML (Schirrmann and Pecher 2005).

## 1.6 Aims of this Thesis

New therapeutic strategies are being developed with the aim of reducing the occurrence of relapse in AML patients. Despite the initial promise of immunotherapy, clinically thus far results have been disappointing. However, for any immunological treatment to have a chance of success, be it vaccination or adoptive T cell transfer, a primary requirement is to understand the host immune system, how it reacts to malignancy and by extension how it will respond to intervention. Mouse models have suggested a situation of chronic T cell exhaustion or anergy at the time of tumour escape but these results require validation in human cancers. Up to this point, work in AML has concentrated on examining the effects of tumour cells *ex vivo* on healthy or remission T cells.

This project aims to look back inside the patient and characterise T cell defects that may be present in patients at the time of diagnosis with AML by flow cytometry, gene expression profiling and functional studies. If defects can be identified there is potential to seek methods of reversing them with the end result of providing an effective autologous T cell response to cancer.

## Chapter 2

### Materials and Methods

Addresses of all the companies listed below can be found in **Appendix A**.

#### 2.1 Ethical Considerations

Ethical approval for the study entitled ‘The impact of the tissue microenvironment and immune system on haematological malignancies’ was sought and obtained from the East London and The City HA Local Research Ethics Committee 3. REC reference number 05/Q0605/140. This covered the use of both stored samples and the acquisition of new samples of human biological material from patients with haematological malignancy and healthy volunteers.

#### 2.2 Samples

Frozen PBMCs from patients with AML at the time of diagnosis were obtained from the Tissue Bank collected and maintained by the Medical Oncology Department of St. Bartholomew’s Hospital. All patients signed consent forms to allow storage of specimens for research purposes. The Tissue Bank is maintained according to the Human Tissue Act 2004 (deemed license no. 12199). Samples were chosen on the basis of diagnosis, time of sampling, number of cryovials stored and whether other ancillary information (such as single nucleotide polymorphism (SNP) genotyping) was available. Much of the preliminary work was possible through the use of a large apheresis peripheral blood collection from a single patient at diagnosis. This allowed storage of around 30 cryovials of  $5 \times 10^7$  PBMCs each for use in the early stages of this project.

Friends, family and staff from the Medical Oncology unit at St. Bartholomew’s Hospital provided blood samples when healthy volunteers were required. All were appropriately counselled and consented. For cell conjugation experiments, where

larger numbers of T cells were required, healthy T cells were retrieved from buffy coats supplied by the National Blood Service.

### **2.3 Cell Freezing and Thawing**

Human cells were frozen by adding freeze mix (fetal calf serum (FCS) (PAA Laboratories Ltd.) + 10% dimethylsulphoxide (DMSO) (Fisher Scientific)) dropwise to a cell pellet. A maximum of  $5 \times 10^7$  cells were frozen in 1.8mls freeze mix per 2ml cryovial. Cryovials were left in Nalgene freezing containers (containing isopropanol) at  $-80^{\circ}\text{C}$  overnight to allow gradual cooling before transfer to liquid nitrogen for long-term storage. On removal, cells were thawed rapidly at  $37^{\circ}\text{C}$ . The contents of the cryovial were then transferred to a 15ml falcon tube. 450 $\mu\text{l}$  10% DNAase I (Sigma Aldrich) was then added dropwise to remove cellular debris followed by an equal volume of pure FCS. Samples were then centrifuged at 1300rpm for 5minutes at  $4^{\circ}\text{C}$  prior to further procedures.

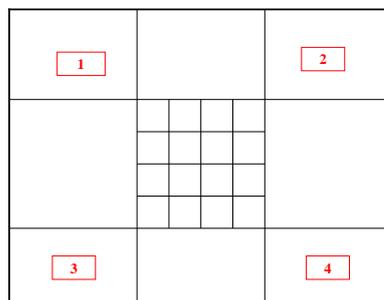
### **2.4 Separation of PBMCs from Whole Blood (Density Gradient Centrifugation)**

This protocol was used for processing peripheral blood samples from healthy volunteers and buffy coats.

Venous blood was taken using standard technique and placed into an equal volume of Roswell Park Memorial Institute medium (RPMI) (CRUK Media Production Lab) +10% acid citrate dextrose-A (ACD-A) anticoagulant. Samples were then layered on top of 5mls of Lymphoprep (Axis-Shield) in 15ml falcon tubes. The tubes were spun at 1500rpm for 25 minutes at room temperature (RT) with the brake off. The interface layer representing live mononuclear cells was carefully removed with a pastette and washed twice with phosphate-buffered saline (PBS)+2%FCS prior to cell counting.

## 2.5 Cell Counting

Cell counts and viability assessment were performed using a haemocytometer and Trypan Blue (Sigma Aldrich) exclusion. Ten  $\mu\text{l}$  of cells diluted in trypan blue were added to the chamber of the haemocytometer. Cells were examined under a x10 objective using a Willovert inverted phase contrast microscope (Hund). Live and dead cells (blue) were counted separately to give an estimate of viability. The live cell count was subsequently used in calculations (**Figure 2.1**).



**Figure 2.1: Method used for Cell Counts**

Areas 1, 2, 3, 4 were counted then the result divided by 4 (X).

$X \times 10^4 \times \text{dilution} = \text{no. of cells / ml.}$

Later on in the project, the department obtained an automated cell counter (Vi-cell XR – Beckman Coulter) on which live cell counts were obtained.

## 2.6 Cell Separation Protocols

### 2.6.1 Separation of CD4 and CD8 T Cells from Healthy PBMCs by Negative Immunomagnetic Selection

MACS® columns, magnet, reagents and cell isolation cocktails (Miltenyi Biotec Ltd.) were used in this protocol. The buffer used was PBS pH 7.2, 0.5% bovine serum albumin (BSA) and 2mM ethylenediamine tetra-acetate (EDTA) prepared by diluting MACS BSA stock solution 1:20 with autoMACS™ Rinsing Solution.

PBMCs were isolated from whole blood by density gradient centrifugation as detailed above. Cells were counted and aliquots of  $1 \times 10^6$  cells removed for flow cytometry. The samples were then centrifuged at 1300rpm for 10 minutes and the subsequent pellets resuspended in 40 $\mu$ l buffer per  $10^7$  total cells. Ten  $\mu$ l of biotin-labelled antibody cocktail (CD4+ or CD8+ T Cell Isolation Kit II) per  $10^7$  total cells were then added. The samples were mixed by pipetting then incubated for 10 minutes at 4-8 $^{\circ}$ C. Thirty  $\mu$ l of buffer per  $10^7$  total cells were then added followed by 20 $\mu$ l of anti-biotin microbeads per  $10^7$  total cells. The samples were then incubated for a further 15 minutes at 4-8 $^{\circ}$ C and subsequently washed in 10-20 times the labelling volume of buffer. The cells were then resuspended in 500 $\mu$ l buffer. The columns available for cell separation are listed below (**Table 2.1**)

Column	Max. No. of Labelled Cells	Max. No. of Total Cells
MS (recommended for positive selection)	$10^7$	$2 \times 10^8$
LS (recommended for positive selection)	$10^8$	$2 \times 10^9$
LD (recommended for depletion)	$10^8$	$5 \times 10^8$

**Table 2.1: Cell Capacities of MACS<sup>®</sup> Columns**

Cells were separated on an LS column placed in the magnetic field of a MACs separator and prepared by rinsing with 3ml buffer. This effluent was discarded. The cell suspension was applied to the column and washed through with a total of 9mls of buffer. The effluent was collected as the CD4 or CD8 positive fraction. The column was then removed from the magnetic field and placed on top of a fresh centrifuge tube. Five mls of buffer were added to the column and a plunger used to push the buffer through the column. This fraction represented the CD4 or CD8 negative fraction.

Every attempt was made to keep cells at 4<sup>0</sup>C during the separation procedure; columns were stored at 4<sup>0</sup>C prior to separation procedures and cells and buffer were kept on ice at all times.

Post separation, cell counts were performed on both negative and positive fractions and aliquots of 1 x 10<sup>6</sup> cells removed for flow cytometric analysis of cell purity. Cells were subsequently spun down at 1300rpm for 5 minutes at 4<sup>0</sup>C and then either frozen for future use in functional assays or resuspended in 1ml of Trizol (Invitrogen Ltd.) (for up to 10 x 10<sup>6</sup> cells) then stored at -80<sup>0</sup>C for future RNA extraction.

### ***2.6.2 Separation of CD4 and CD8 T Cells by Positive Immunomagnetic Selection***

MACS® columns, magnet, reagents and cell isolation cocktails (Miltenyi Biotec Ltd.) were used in this protocol. The buffer used was PBS pH 7.2, 0.5% BSA and 2mM EDTA prepared by diluting MACS BSA stock solution 1:20 with autoMACS™ Rinsing Solution.

#### **Healthy PBMCs**

PBMCs were isolated from whole blood by density gradient centrifugation as detailed above. Cells were counted and aliquots of 1 x 10<sup>6</sup> cells removed for flow cytometry. The PBMCs were then washed twice with 10mls of buffer before resuspension in 80µl of buffer per 10<sup>7</sup> cells. Twenty µl of CD4 or CD8 Microbeads (Miltenyi Biotec Ltd.) per 10<sup>7</sup> cells were added, mixed well then incubated for 15 minutes at 4-8<sup>0</sup>C. Cells were then washed with 1-2ml buffer per 10<sup>7</sup> cells and spun at 1300rpm for 10 minutes at 4<sup>0</sup>C. Labelled cells were resuspended in 500µl of buffer. Magnetic separation of labelled cells was performed as described previously, in this instance using an MS column as the number of labelled cells was lower than when performing negative selection. Columns were prepared by rinsing with 500µl of buffer and, after the cells had been applied, washed with 3 x 500µl of buffer. Magnetically labelled CD4 or CD8

cells were eluted from the column after removing it from the magnet and placing it in a fresh 15ml falcon tube. One ml of buffer was applied to the column and the cells were flushed out by applying the supplied plunger to the column. The CD4/8 negative fraction was spun down and resuspended in freeze mix for long-term storage. A cell count was performed on the CD4/8 positive fraction, aliquots were removed for flow cytometry and the remaining cells pelleted for subsequent RNA extraction.

### **AML PBMCs**

Certain modifications were made to the protocol above to improve the purity and yield of T cells separated from AML PBMCs.

- 1) After thawing, cells were put through a density gradient centrifugation step to remove dead cells.

- 2) Once the live mononuclear cells had been recovered and washed, they were passed through a 70 $\mu$ m cell strainer (BD) to remove cell clumps.

- 3) After counting, cells were resuspended in 2% human anti-globulin (HAG) (Sigma Aldrich) at a concentration of 80 $\mu$ l per 10<sup>7</sup> cells (rather than buffer) and incubated on ice for 20 minutes to allow blockade of non-specific Fc receptors.

- 4) Cell separation was performed on LS columns because of their higher cell capacity. To improve T cell purity, the column was washed with double the recommended volume of buffer (i.e. with 18mls of buffer).

- 5) To improve cell yield, the negative T cell fraction was subsequently spun down, resuspended in the same volume as prior to the first cell separation and then passed over a second LS column which was also washed with double the recommended volume of buffer. Positive T cell fractions from the 2 columns were then combined, spun down, resuspended in 1ml of buffer, counted, aliquots removed for flow and finally resuspended in 1ml Trizol (Invitrogen Ltd.) and stored at -80<sup>0</sup>C for future RNA extraction.

### 2.6.3 Separation of CD4 and CD8 T Cells from Presentation AML Specimens by Negative Selection

StemSep™ cocktails, columns and pump (StemCell Technologies) were used for this protocol. Both fresh and frozen samples were used.

After thawing and washing, cells were passed over a 70µm cell strainer into a 50ml falcon tube to remove cell clumps. Cells were then counted and aliquots of  $1 \times 10^6$  cells were removed for flow cytometry. Samples were centrifuged at 1300rpm for 5 minutes at 4°C then resuspended in 2% HAG at a concentration of  $5 \times 10^7$  cells/ml. After a 30 minute incubation on ice to allow blockade of Fc receptors, StemSep™ antibody cocktails were added at the recommended volume per ml of cells. Cells were left on ice at 4°C for 30 minutes. Magnetic colloid was then added at a volume of 100µl per ml of cells (rather than 60µl as recommended) and samples were again left for 30 minutes at 4°C with occasional agitation. Magnetic separation of labelled cells was then performed on 0.3” or 0.6” columns depending on total cell number (**Table 2.2**). With AML samples, due to the large size of cells, the optimum number of cells per column was not exceeded.

Column Size	Optimum Number of Cells	Extended Range of Cells
0.6”	$5 \times 10^8$	$10^8 - 1.5 \times 10^9$
0.3”	$5 \times 10^7$	$2 - 8 \times 10^7$

**Table 2.2: Cell Capacities of StemSep™ Columns**

Columns were used in conjunction with StemCell Technologies Blue Magnet and peristaltic pump. Columns were primed with PBS without FCS until the level of the PBS was above the stainless steel matrix of the column and then washed with PBS+2%FCS using the directions below remembering to reverse the pump direction in between (**Table 2.3**).

Column Size	Priming	Washing and loading sample	
	Pump setting	Pump setting	Volume
0.6''	3.0	10.0	25mls
0.3''	1.0	3.0	8mls

**Table 2.3: Pump Set-Up for Use with StemSep™ Columns**

Pump settings and volumes of PBS+2%FCS required for priming and washing StemSep™ columns.

Samples were loaded onto the columns and washed through with PBS+2%FCS at the pump setting and volume listed. At no point were the columns allowed to run dry. Every attempt was made to keep cells at 4<sup>0</sup>C during the separation procedure; the magnet and columns were left at 4<sup>0</sup>C prior to cell separation and solutions and eluted cell fractions were kept on ice. Post separation, cells retained on the column (blasts) could be recovered by removing the column from the magnet, inverting it over a 50ml falcon tube and using a 1ml pipette to pass PBS+2%FCS through the column. All cell fractions were then spun down and resuspended in smaller volumes of PBS+2%FCS suitable for cell counting. Aliquots of 1 x 10<sup>6</sup> cells were removed to allow purity to be assessed by flow cytometry before the cells were used for further experiments. Separated cells were then frozen down for future use in functional assays.

## 2.7 Flow cytometry

This was used to assess purity of T cell fractions after separation procedures and for phenotyping cells. Details of all antibodies used can be found in **Appendix B**.

### 2.7.1 Surface Staining

In general, 0.5 x 10<sup>6</sup> cells were used for controls and 1 x 10<sup>6</sup> cells for samples however; often considerably fewer cells would be used to avoid wasting T cells. Nonetheless, the protocol was not adjusted for fewer numbers of cells. Aliquots of cells were placed in 5ml polystyrene round-bottom tubes (BD) and left on ice until all samples were ready for staining. On all occasions, unlabelled and isotype

controls for the antibodies being used were included. Firstly, cells were washed with 1ml of PBS+2%FCS and spun at 1300rpm for 5 minutes at 4<sup>0</sup>C. Cells were then resuspended in 50µl of PBS+2%FCS or 2% HAG (if not previously exposed to this in the course of the experiment). Cells in 2% HAG were left for 20 minutes on ice. In general, 5µl of each required antibody was then added to the relevant tubes. For certain antibodies a lower volume was added after experience demonstrated that 5µl resulted to staining intensity that was too great. After mixing, samples were incubated at 4<sup>0</sup>C for 30 minutes. Cells were then washed in 1ml PBS+2%FCS and subsequently resuspended in 400µl PBS+2%FCS. 4'6-diamidino-2-phenylindole (DAPI) was added to this final cell suspension at a concentration of 1µl per 5mls (stock=500µg/ml) to allow dead cell exclusion. Samples were left covered in foil at 4-8<sup>0</sup>C until analysis was performed.

### **2.7.2 Whole Blood Lysis Method**

This method was used for phenotyping cells when fresh blood samples were obtained from healthy volunteers or clinic patients.

Peripheral blood was taken directly into BD vacutainer tubes container EDTA as an anticoagulant. 5ml polystyrene round-bottomed tubes were labelled and filled with 4mls of cold ammonium chloride (StemCell Technologies). After gently inverting to mix, 50µl of anticoagulated peripheral blood was added to control tubes and 200µl to sample tubes. Solutions were pipetted up and down to mix. Red cell lysis was allowed to occur while the samples were incubated for 10 minutes on ice. 300µl FCS was then added to each tube and samples were spun at 1300rpm for 5 minutes at 4<sup>0</sup>C to pellet the cells. Cells were resuspended in 50µl 2%HAG and left for 20 minutes on ice. Antibodies were then added to the relevant tubes and incubated at 4<sup>0</sup>C for 30 minutes. After washing with 1ml PBS+2%FCS, cells were resuspended in 300µl PBS+2%FCS+DAPI. For the purpose of cell counting, 50µl Countbright™ absolute counting beads (Invitogen) were added to each sample. These were vortexed for 30 seconds prior to use.

### **2.7.2 Intracytoplasmic Staining**

After staining with surface antibodies, cells were washed with 1ml of PBS+2%FCS. For intracellular staining the Cytofix/Cytoperm plus Fixation/Permeabilisation Kit with BD GolgiPlug (BD) was used. Cells were resuspended in 250µl of Fix/Perm buffer and left for 20 minutes at 4<sup>0</sup>C. Samples were then washed twice with 1ml of 1X Perm/Wash buffer before being resuspended in 50µl Perm/Wash buffer. Intracellular antibodies and isotypes were then added. 0.1µl of anti-perforin antibody and 1µl of anti-granzyme B antibody were used per sample. Following incubation for 45 minutes at 4<sup>0</sup>C, cells were washed three times with 1ml of 1X Perm/Wash buffer. Cells were finally resuspended in 500µl of PBS+2%FCS for flow cytometric analysis. Fixing and permeabilising cells changes their scatter characteristics. Control tubes were therefore always prepared in the same way as the sample tubes.

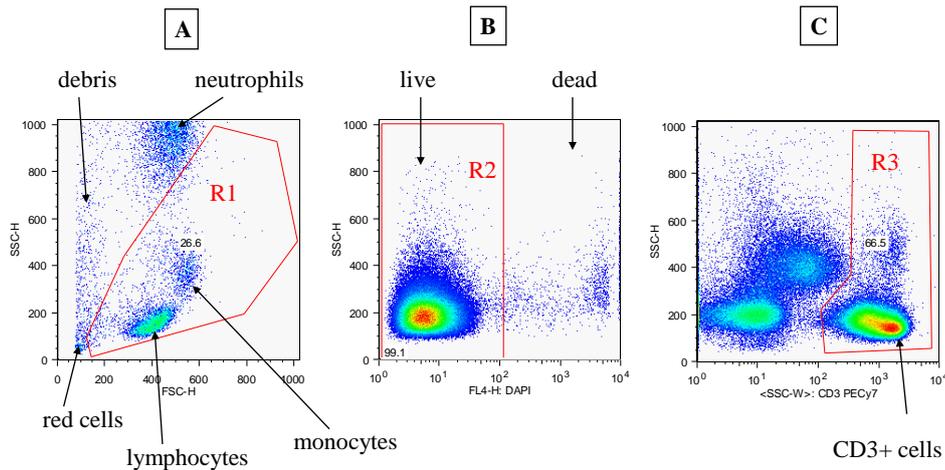
### **2.7.3 Intracytoplasmic Staining for Cytokines**

Frozen cells were thawed, washed, passed over 70µm cell strainers and counted. Samples were divided into those which were to be stimulated and those which were not. Cells were then resuspended in RPMI+10%FCS in 6-well tissue culture plates at a concentration of  $2 \times 10^6$  cells/ml. Fifty ng/ml phorbol myristate acetate (PMA) (Sigma Aldrich), 1µg/ml ionomycin (Sigma Aldrich) and 1µl/ml brefeldin A (BD GolgiPlug) were added to cells undergoing stimulation. To unstimulated samples only 1µl/ml brefeldin A was added. Cells were then incubated for 5 hours at 37<sup>0</sup>C+5%CO<sub>2</sub>. Cells were then harvested by repeated pipetting and washing wells with 5mls PBS+2%FCS. After centrifuging at 1300rpm for 5 minutes at 4<sup>0</sup>C, cells were resuspended in PBS+2%FCS and counted. Aliquots of  $1 \times 10^6$  cells were transferred to labelled 5ml polystyrene round-bottomed tubes. These samples were spun down then resuspended in 50µl 2%HAG and left on ice for 20 minutes. Surface staining antibodies and their appropriate isotype controls were then added and samples incubated at 4<sup>0</sup>C for 30 minutes. Excess antibody was removed by washing with 1ml PBS+2%FCS and then cells were fixed by resuspending them in 100µl of 4% paraformaldehyde (PFA) for 15 minutes at 4<sup>0</sup>C. Cells were subsequently washed with 1ml of PBS+2%FCS twice. Fixed surface

stained cells were then left overnight in the dark at 4<sup>0</sup>C in 1ml of PBS+2%FCS. The following day, cells were spun down at 1300rpm for 5 minutes at 4<sup>0</sup>C and then resuspended in 1ml 1X Perm/Wash buffer (BD). After a 15 minute incubation at 4<sup>0</sup>C, cells were spun down and pellets were resuspended in 50µl of the same 1X Perm/Wash buffer. Intracytoplasmic staining antibodies and their appropriate isotype controls were then added (IL-4 phycoerythrin (PE) = 1.25µl, IFN $\gamma$  fluorescein isothiocyanate (FITC) = 0.5µl, TNF $\alpha$  FITC = 1µl, IL-2 PE = 2.5µl). Samples were left for 30 minutes in the dark at 4<sup>0</sup>C. After incubation, cells were washed twice with 1ml of 1X Perm/Wash buffer before being resuspended in 500µl PBS+2%FCS in preparation for flow cytometric analysis.

#### **2.7.4 Flow Cytometric Acquisition and Analysis**

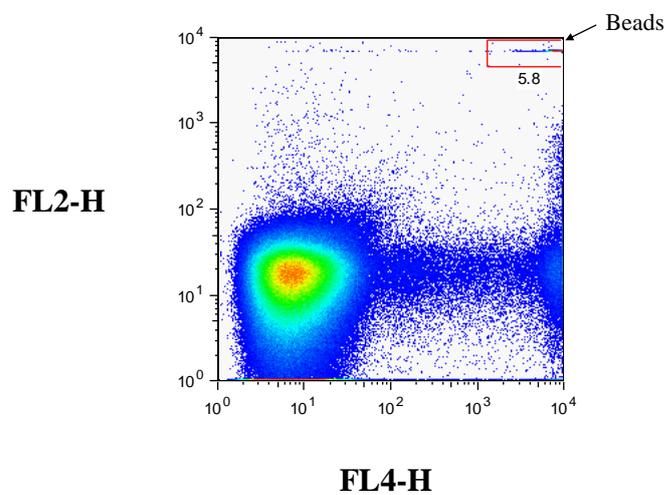
Initially a FACS Calibur (BD) and later a LSR cytometer (BD) were used for flow cytometry. CellQuest software (BD) was used for acquisition and also analysis early on in the project. Subsequently, FlowJo (Tree Star Inc) software was used for analysis. An unlabelled sample was used to set appropriate voltages. This tube was also used to set a gate (R1) over the lymphocyte region as determined by forward (FSC) and side scatter (SSC). A second gate (R2) was set on live cells only as determined by SSC and DAPI. A combination of these two gates was used for all subsequent acquisition. Occasionally a third acquisition gate would be set using R1+R2 gated cells on CD3<sup>+</sup> cells. This gating strategy is illustrated in **Figure 2.2**. Single fluorochrome-labelled samples were used to set appropriate compensation. A minimum of ten thousand events were acquired from R1+R2. During subsequent analysis, gates were set using the isotype controls.



### Figure 2.2: Flow Cytometry Gating Strategy

Scatter plot (A) illustrates the normal positions of cell populations on forward and side scatter plots. R1 indicates the mononuclear cell gate. (B) demonstrates how dead cells are excluded using DAPI. R2 indicates the live cell gate. (C) shows how CD3+ lymphocytes are gated (R3) on cell populations that have already been gated as R1+R2.

For obtaining absolute cell counts, two methods were used. For fresh samples, Countbright™ Absolute Counting Beads (Invitrogen Ltd.) were added to samples as detailed above. These beads are a calibrated suspension of microspheres that fluoresce brightly over a wide range of emission and excitation wavelengths. A known volume of beads is added to a known volume of sample thus the volume of sample analysed can be calculated from the number of bead events recorded and can be used with cell events to determine concentration. A least 1000 bead events were acquired for statistical accuracy. The counting beads appeared in the upper right region of all fluorescence dot plots and were gated on the FL2/FL4 dot plot (Figure 2.3).



**Figure 2.3: Location of CountBright™ Counting Beads on Fluorescence Dot Plots**

The absolute cell number was then calculated using the formula below (**Figure 2.4**).

$$\frac{\mathbf{A}}{\mathbf{B}} \times \frac{\mathbf{C}}{\mathbf{D}} = \text{Concentration of sample as cells}/\mu\text{l}$$

**A** = number of cell events

**B** = number of bead events

**C** = assigned bead count of the lot (beads/50 $\mu\text{l}$ )

**D** = volume of sample ( $\mu\text{l}$ )

#### **Figure 2.4: Absolute Cell Counts using CountBright™ Beads**

Formula for calculating absolute cell number using CountBright™ beads.

To perform absolute cell counts on frozen samples, the bead method was not possible as the cells had been concentrated during the preparation process. Therefore absolute cell count was determined from the full blood count (FBC) performed on the same day using the following calculation: total white cell count  $\times 10^9/\text{L} \times \% \text{ mononuclear gate} \times \% \text{ DAPI gate} \times \% \text{ CD3+ gate} \times \% \text{ +ve cells}$ .

## **2.8 Cytospins**

A Shandon Cytospin 3 (Thermo Fisher Scientific Inc.) was used to perform cytospins of aliquots of 1 to 10000 cells from the CD4 and CD8 positive fractions. Samples were spun at 500rpm for 5 minutes. The area of the glass slide occupied by cells was subsequently demarcated using a diamond marker. Slides were then stained with May-Grunwald-Giemsa (MGG) prior to examination.

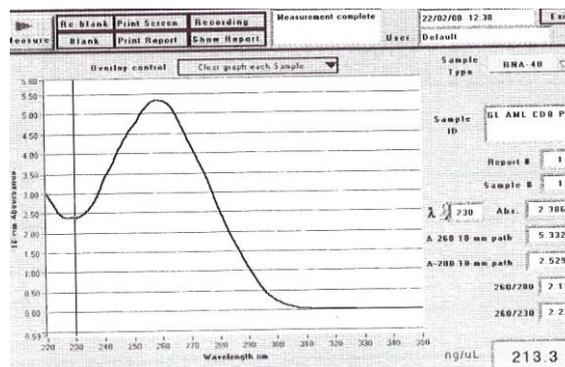
## 2.9 RNA Extraction

Using the standard Trizol (Invitrogen Ltd.) protocol, the quality and quantity of RNA obtained from separated T cell fractions was poor. This appeared to be a property of T cells as when the protocol was used to extract RNA from B cell lines good quality material was obtained. Modifications in the protocol were recommended by Dr. Gullu Gorgun from Dana Farber Cancer Institute (DFCI) and resulted in substantial improvement in the RNA obtained from T cells. These modifications included improving initial phase separation with a gel to reduce phenol contamination and the use of a column to bind the extracted RNA and allow washing, the removal of DNA and subsequent elution. This protocol is detailed below.

One ml Trizol was added to a maximum of  $10^7$  cells and the sample homogenized by pipetting. The sample was allowed to rest at RT for 5 minutes. A MaxTract high density gel tube (Qiagen) was spun for 30 seconds at 12000rpm to bring the gel to the bottom of the tube. The Trizol extract was then added followed by 200 $\mu$ l of chloroform (per 1ml Trizol) and mixed for 15 seconds. The sample was left at RT for 3 minutes followed by a 15 minute spin at 12000rpm at 4°C for 15 minutes. The top (clear) aqueous phase should be above the gel plug and the pink phase should be underneath it. The top phase (approx 600 $\mu$ l) was transferred to a new tube and 1 volume of 95% ethanol (cold) was added and mixed. This mixture was transferred to a Promega spin column (SV Total RNA Isolation Kit) and spun at 12000rpm for 1 minute. The flow through was discarded and the column washed with 500 $\mu$ l SV RNA wash buffer, spun again for 1 minute and the flow through discarded again.

Fifty  $\mu$ l of DNaseI mix was added and the column incubated for 15 minutes at RT. Two hundred  $\mu$ l of DNase STOP buffer was then applied and the column spun for 1 minute at 12000rpm. The flow through was discarded and the column washed with 500 $\mu$ l SV RNA wash buffer, spun for 1 minute at 12000rpm and the flow through discarded once again. The washing step was repeated using 250 $\mu$ l buffer and a 2 minute spin. The collection tube was changed and 30 $\mu$ l of cold

0.1% diethyl pyrocarbonate (DEPC) water was applied to the column. This was allowed to rest for 2 minutes on ice and then spun at 12000rpm for 1 minute. A further 20 $\mu$ l of cold DEPC water was then added, left on ice for 2 minutes then spun at 12000rpm for 1 minute. A 1 $\mu$ l aliquot was used to assess RNA quantity and quality on a Nanodrop spectrophotometer. The absorbance (A) was measured at several different wavelengths; 230, 260 and 280nm. RNA at 1mg/ml has an  $A_{260}$  of 25. Thus quantity can be calculated. The  $A_{260}/A_{280}$  ratio indicates purity and should be between 1.8 and 2.0. Proteins have a peak absorbance around 280nm and will therefore reduce this ratio if present. Absorbance at 230nm indicates phenol contamination. The  $A_{260}/A_{230}$  should be  $>1.8$ . Expected yields of RNA per  $1 \times 10^6$  cultured cells vary with tissue type but range from 1 to 25 $\mu$ g (Figure 2.5).



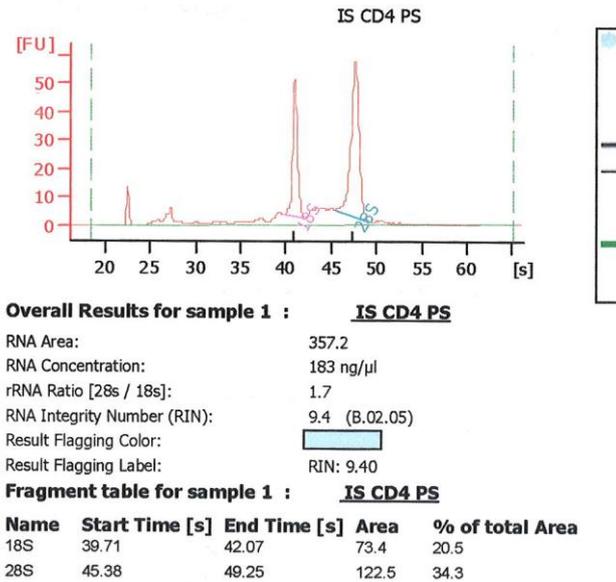
**Figure 2.5: Typical Absorbance Plot for RNA on the Nanodrop Spectrophotometer**

Illustrates good quality RNA.

If the samples had low  $A_{260}/A_{230}$  ratios suggestive of contamination, a second precipitation was performed. A 1/10 volume of 3M sodium acetate and 2.5 volumes of 100% ethanol were added to the sample. After mixing, the sample was left at  $-20^{\circ}\text{C}$  for at least 1 hour and often overnight. The samples were then spun at 12000rcf for 20 minutes at  $4^{\circ}\text{C}$ . The supernatant was carefully removed and the pellet was washed twice with 80% ethanol in DEPC water. The pellet was air-dried for 10 minutes and then resuspended in DEPC water. Quality and quantity was reassessed on the Nanodrop spectrophotometer and the RNA was left at  $-80^{\circ}\text{C}$  until required.

### ***2.9.1 Assessment of RNA Quality and Quantity for the Purpose of Microarrays***

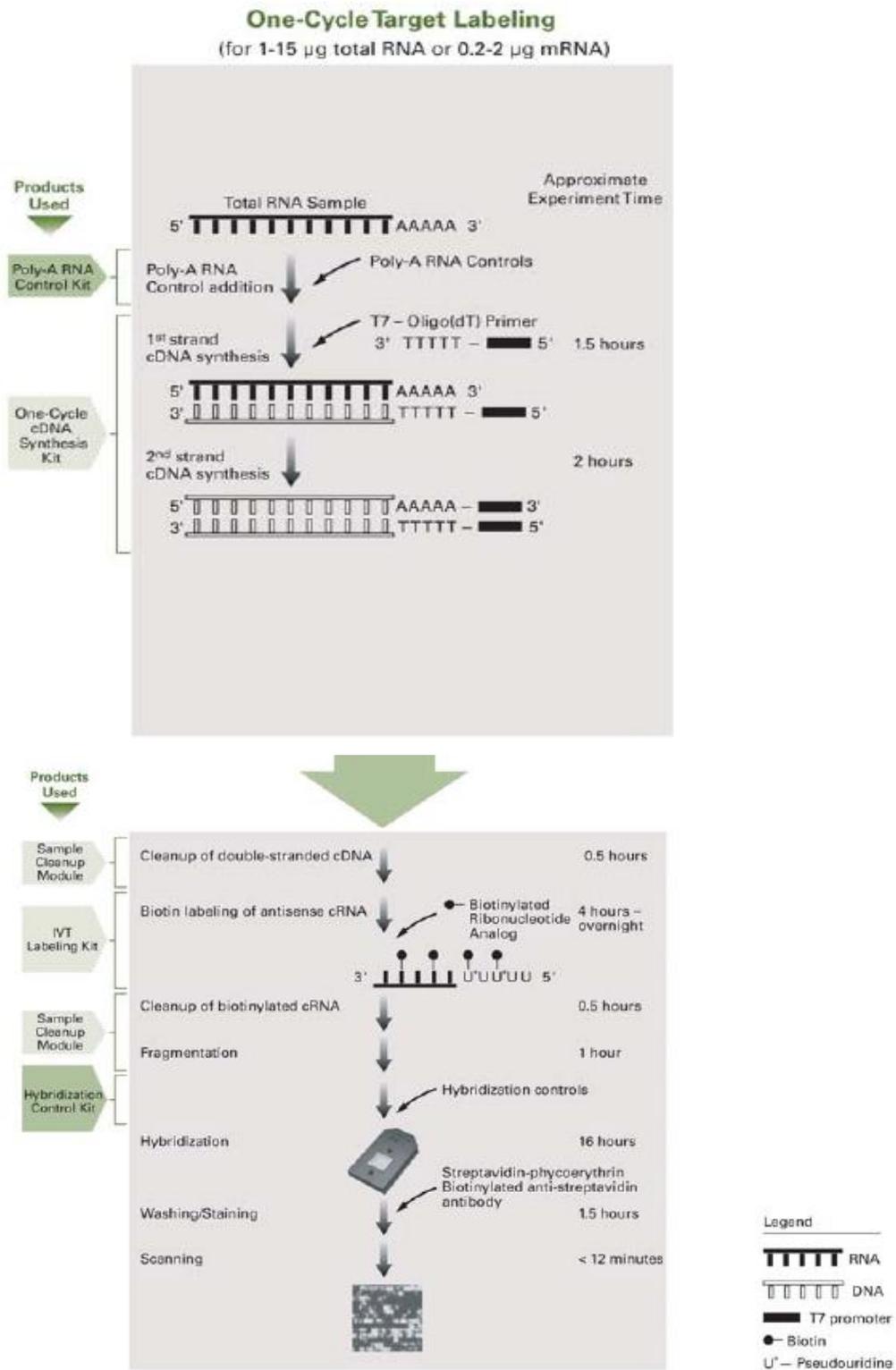
All RNA samples to be used for gene expression microarrays were first checked on the Agilent 2100 Bioanalyzer. This uses a fluorescent assay involving electrophoretic separation to provide qualitative and quantitative assessment of RNA. The Agilent RNA chip contains an interconnected set of microchannels that is used for the separation of nucleic acid fragments based on their size as they are driven through a gel by electrophoresis. The Bioanalyzer measures the amount of fluorescence as the RNA sample is pulsed through the microchannel over time. Gel-dye mix is loaded into the chip using a chip priming station. Up to 12 1 $\mu$ l RNA samples (50-500ng) can be loaded onto the chip together with 1 $\mu$ l of RNA ladder. The software creates an electropherogram which documents fluorescence over time. Smaller molecules are pulsed through the separation channel quicker than larger ones and therefore appear on the left of the electropherogram. High quality RNA should show clear 28S and 18S peaks, low noise between those peaks and minimal low molecular weight contamination as demonstrated in **Figure 2.6**. The RNA Integrity Value (RIN) has a range of 1 to 10 with 1 representing most degraded RNA and 10 most intact. >6 represents quality sufficient for microarrays.



**Figure 2.6: Electropherogram of Good Quality RNA**

## 2.10 Gene Expression Microarrays (Affymetrix UK Ltd.)

The Affymetrix Expression GeneChip® Protocol one-cycle procedure were used to synthesize fragmented biotin-labelled cRNA as recommended by the manufacturer (**Figure 2.7**). Two to five μg of total RNA was used as starting material.



**Figure 2.7: GeneChip® Eukaryotic Labelling Assay for Expression Analysis**

(From Affymetrix Expression Analysis Technical Manual)

### 2.10.1 First Strand cDNA Synthesis

Preparation of RNA/T7-oligo(dT) primer mix

Total RNA (2-5 $\mu$ g)	variable volume
T7-oligo(dT) Primer, 50 $\mu$ M	2 $\mu$ l
DEPC-water	variable volume
<b>Total Volume</b>	<b>12<math>\mu</math>l</b>

After mixing, the samples were incubated at 70°C for 10 minutes and then left on ice for at least 2 minutes.

First Strand cDNA Synthesis Master Mix (prepared on ice)

(5x) First Strand Buffer	4 $\mu$ l
DTT (0.1M)	2 $\mu$ l
dNTPs (10 mM each)	1 $\mu$ l
<b>Total Volume</b>	<b>7<math>\mu</math>l</b>

Seven  $\mu$ l of master mix was added to each tube and the samples were then incubated at 42°C for 2 minutes. At this point 1 $\mu$ l of Superscript II was added to each sample giving a final volume of 20 $\mu$ l. Samples were then left at 42°C for 1 hour. At the end of this period, the samples were allowed to cool down on ice for at least 2 minutes.

### 2.10.2 Second Strand cDNA Synthesis

Second Strand DNA Master Mix (prepared on ice)

DEPC-treated water	91 $\mu$ l
(5x) Second Strand Buffer	30 $\mu$ l
dNTPs (10mM each)	3 $\mu$ l
E.coli DNA Ligase (10U/ $\mu$ l)	1 $\mu$ l
E.coli DNA Polymerase I (10U/ $\mu$ l)	4 $\mu$ l
E.coli RNase H (2U/ $\mu$ l)	1 $\mu$ l
<b>Total Volume</b>	<b>130<math>\mu</math>l</b>

One hundred and thirty  $\mu$ l of master mix was added to each first strand cDNA reaction. After mixing, the samples were incubated at 16°C for 1 hour and 55

minutes. At this time, 2 $\mu$ l of T4 DNA polymerase was added to each and the samples left at 16°C for a further 5 minutes. 10 $\mu$ l of 0.5M EDTA pH8.0 was then added to stop the reaction giving a final volume of 162 $\mu$ l of double-stranded cDNA.

### ***2.10.3 Clean-up of Double Stranded cDNA***

This was performed using the Sample Cleanup Module from Affymetrix that uses a column on which the cDNA binds, is then washed and then eluted. 600 $\mu$ l of cDNA Binding Buffer was added to the 162 $\mu$ l double-stranded cDNA sample and mixed by vortexing for 3 seconds. Five hundred  $\mu$ l of this mix was then applied to the cDNA Cleanup Spin Column placed in a 2 ml Collection Tube. This was then centrifuged for 1 minute at 8000rcf and the flow-through discarded. The spin column was then reloaded with the remaining mixture (262 $\mu$ l) and centrifuged as before. At this point the collection tube was discarded and the column placed in a new 2ml collection tube. To wash the bound cDNA, 750 $\mu$ l of cDNA Wash Buffer was pipetted onto the column which was then spun at 8000rcf for 1 minute. The cap of the spin column was then opened and the samples centrifuged for 5 minutes at maximum speed. The collection tubes were again discarded. The cDNA was eluted by adding 14 $\mu$ l of cDNA Elution Buffer directly onto the spin column membrane and incubating for 1 minute at RT. The columns were centrifuged for 1 minute at maximum speed. The volume of the eluate was assessed and the contents made up to 20 $\mu$ l with DEPC-treated water.

### ***2.10.4 Synthesis of Biotin-labelled cRNA by In Vitro Transcription (IVT)***

An IVT master mix was prepared at RT.

cDNA sample	20 $\mu$ l
10x IVT labeling buffer	4 $\mu$ l
IVT labeling NTP mix	12 $\mu$ l
IVT labeling enzyme mix	4 $\mu$ l
<b>Total Volume</b>	<b>40<math>\mu</math>l</b>

The samples were incubated for 16 hours overnight at 37°C.

### **2.10.5 Clean-up of Biotin-labelled cRNA**

Sixty  $\mu\text{l}$  of DEPC-treated water was added to the *in vitro* transcription reaction. 350 $\mu\text{l}$  of IVT cRNA Binding Buffer was subsequently added and mixed by vortexing for 3 seconds. Two hundred and fifty  $\mu\text{l}$  of 100% ethanol was then added and mixed by pipetting. This made a total sample volume of 700 $\mu\text{l}$ . This was applied to an IVT cRNA Clean-up Spin Column in a 2ml collection tube and spun for 15 seconds at 8000rcf. The flow-through and collection tube were then discarded. Once placed in a fresh collection tube, 500 $\mu\text{l}$  IVT cRNA wash buffer was added to the spin column that was spun at 8000rcf for 15 seconds. This step removes unincorporated dNTPs. Five hundred  $\mu\text{l}$  of 80% ethanol was then applied to the spin column and centrifuged for 15 seconds at 8000rcf. The cap of the column was then opened and the column re-centrifuged this time at maximum speed for 5 minutes. Once the column had been transferred to a fresh 1.5 ml collection tube, 11 $\mu\text{l}$  of DEPC-treated water was added directly onto the membrane of the column. This was then spun for 1 minute at maximum speed. A further 10 $\mu\text{l}$  DEPC-water was applied to the membrane and the spin repeated. This made a total volume of 21 $\mu\text{l}$  of eluted biotin-labelled cRNA.

At this point, 0.5 $\mu\text{l}$  of each sample was removed and made up to 10 $\mu\text{l}$  with DEPC-treated water (1 in 20 dilution). A 1 $\mu\text{l}$  aliquot of this mix was used to check the quality and quantity of cRNA using the Nanodrop spectrophotometer.

Once the quantity of cRNA had been determined, an adjustment to the concentration was made to reflect the possibility of carryover of unlabelled total RNA from the start of the experiment. An assumption of 100% carryover was made.

**Adjusted cRNA yield = cRNA amount after IVT – total starting RNA**

The minimum concentration of cRNA required for fragmentation is 0.6  $\mu\text{g}/\mu\text{l}$ .

### **2.10.6 Fragmentation of cRNA**

Once the cRNA concentrations in each sample had been adjusted, 20µg of cRNA was removed from each. To this, 8µl of 5X fragmentation buffer was added and the volume made up to 40µl with DEPC-treated water. The sample was incubated for 35 minutes at 94°C and then placed on ice. Two µl of the fragmented cRNA was then removed to run on a 1.2% agarose gel for 30 minutes at 100V. The fragmentation should produce a distribution of RNA fragment sizes from approximately 35 to 200 base pairs.

### **2.10.7 Preparation of the Hybridisation Cocktail**

Once fragmented, 15µg of biotin-labelled cRNA (30µl) were added to a hybridisation cocktail prepared as shown.

Control oligoB2	5µl
20X eukaryotic hybridisation controls	15µl
herring sperm DNA	3µl
acetylated BSA	3µl
2X hybridisation buffer	150µl
DEPC-treated water	94µl
<b>Total Volume</b>	<b>300µl</b>

At this point, samples were passed to the Affymetrix operator Mrs. T. Chaplin who performed hybridisation of the samples onto the chips, washing, staining and scanning.

### **2.10.8 Hybridisation, Washing, Staining and Scanning**

Although previous studies in CLL were performed using the Affymetrix U133A GeneChip®, here the Affymetrix U133Plus2 GeneChips® were used as they offer the most comprehensive coverage of the whole human genome. Expression levels of over 47,000 transcripts covering 38,500 well-characterised human genes are analysed on the array. Eleven pairs of oligonucleotide probes are used to measure the level of transcription of each sequence represented.

The Affymetrix GeneChips® were stored at 4°C shielded from light. Prior to hybridisation, they were equilibrated to RT for at least 30 minutes. Meanwhile, the samples in hybridisation cocktail were heated to 95°C for 5 minutes, then cooled to 48°C and finally centrifuged at maximum speed for 5 minutes to precipitate any insoluble material. At this point, the samples were loaded onto the chips and hybridisation allowed to occur by incubating at 45°C for 16 hours in a rotating hybridisation oven set at 60rpm. The following morning, the samples were removed from the chips and replaced with low-stringency wash buffer. The chips were then placed in a GeneChip® 450 Fluidics wash station where they were washed to remove non-hybridised probes and then stained with Streptavidin Phycoerthrin (SAPE) that labels the biotinylated cRNA fragments that have hybridised to the array. Finally, the chips were scanned using the GeneChip® 3000 scanner. This converts the fluorescent signal generated from each bound cRNA fragment into an expression value (raw value) and a flag (present, marginal or absent calls). A report is produced listing these values for each probe set. In addition, certain quality control values are reported which reflect the hybridisation quality. In particular, the noise signal should be less than 4, the scale factor around 1, the background noise less than 100 and around 50% of the probes had to be present, the remaining 50% being absent or marginal. In addition, housekeeping genes were reported as controls. For these, the 3'/5' signal ratio should be less than 1 with values higher than this reflecting degradation in the RNA.

### ***2.10.9 Microarray Analysis***

Various software packages were used for analysis; Genespring GX v7.3 (Agilent Technologies) and Partek Genomics Suite (Partek Inc.) in the Medical Oncology Department and Bioconductor ([www.bioconductor.org](http://www.bioconductor.org)) was used by Richard Mitter, bioinformatician at Cancer Research UK, Lincoln's Inn Fields. Subsequent pathway analysis was done using Ingenuity software (Ingenuity Systems Inc.). Details of specific analyses performed can be found in the relevant chapters.

## 2.11 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Gene expression changes observed in microarray experiments were validated using this technique.

### 2.11.1 cDNA Synthesis from RNA

For qRT-PCR reactions, single-stranded RNA was reverse transcribed into complementary cDNA. One hundred ng of RNA was used as starting material. A no template (water-only) control was always included. The RNA was added to 0.5ml eppendorfs and made up to 11.5µl with molecular biology grade water (Sigma Aldrich). To this, 1.5µl of random hexamers (50µM) (Sigma Aldrich) were added. Samples were heated to 70<sup>0</sup>C for 5 minutes to denature RNA secondary structures then left on ice for 2-5 minutes to allow the primers to anneal to the RNA. Seventeen µl of the following master mix was then added:

M-MLV 5X buffer (Promega UK Ltd.)	6µl
dNTP mix (Amersham)	10µl
M-MLV RT enzyme (Promega UK Ltd.)	1µl (200U)
<b>Total Volume</b>	<b>30µl</b>

Samples were incubated in a PTC-225 thermocycler (MJ Research) at 42<sup>0</sup>C for 1 hour to allow reverse transcription, followed by 95<sup>0</sup>C for 5 minutes to inactivate the enzyme.

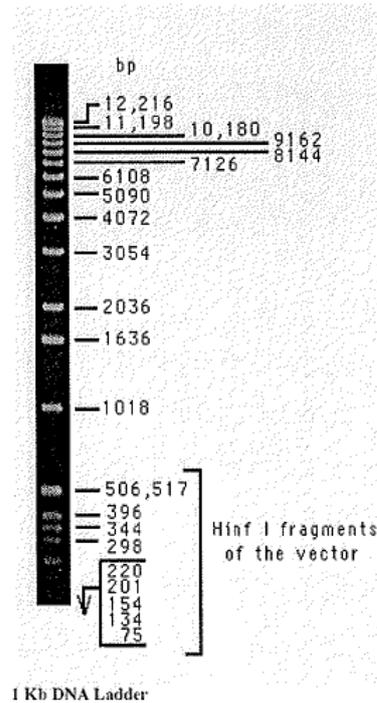
cDNA synthesis was confirmed by performing a standard PCR for the ubiquitously expressed beta-actin gene. Sequences of the primers used were 5'-GCGGGAAATCGTGCGTGCGTGACATT-3' (forward), 5'-GATGGAGTTGAAGGTAGTTTCGTG-3' (reverse) (Sigma Aldrich). The no template control from the cDNA synthesis reaction and a no template control for the beta-actin PCR were included to exclude contamination. Cell line cDNA was also included to ensure consistency of the reverse transcription. Reactions were set up in 0.2ml PCR tubes on ice.

The following master mix was made up per sample:

cDNA	2 $\mu$ l
10X buffer (Invitrogen Ltd.)	2.5 $\mu$ l
dNTPS (10mM) (Amersham)	0.5 $\mu$ l
MgCl <sub>2</sub> (Invitrogen Ltd.)	0.75 $\mu$ l
Forward primer (see above)	1 $\mu$ l
Reverse primer (see above)	1 $\mu$ l
Taq DNA Pol (5u/ml) (Invitrogen Ltd.)	0.2 $\mu$ l
Water (Sigma Aldrich)	17.05 $\mu$ l
<b>Total Volume</b>	<b>25<math>\mu</math>l</b>

The reaction was performed on a PTC-225 thermocycler (MJ Research) using the following programme: 95<sup>0</sup>C for 1 minute, 35 cycles of 95<sup>0</sup>C of 30s (template denaturation), 62<sup>0</sup>C of 30s (primer annealing), 72<sup>0</sup>C for 45s (extension), 4<sup>0</sup>C forever.

Four  $\mu$ l of PCR product with 2 $\mu$ l of loading buffer (bromphenol blue or orangeG – Sigma Aldrich) were checked on a 1.2% agarose (Invitrogen Ltd.) gel (containing ethidium bromide) with 4 $\mu$ l of a 1Kb DNA ladder (Invitrogen Ltd.). Gels were run at 100V for 20-40 minutes. Expected product size was 250-300bp. The band sizes for the DNA ladder used are shown in **Figure 2.8**. Gels were visualised under UV light using a GeneFlash Bio-Imager (Syngene).



**Figure 2.8: Expected Bands for 1Kb DNA Ladder**

### 2.11.2 qRT-PCR Set-up

Reactions were set up in a dedicated hood that was cleaned and UV decontaminated between uses. MicroAmp fast optical 96 well plates (Applied Biosystems) were used. In all cases, Applied Biosystems TaqMan® Gene Expression Assays were used for qRT-PCR probes and primers. Assays were preferentially chosen on the basis of whether they were inventoried and also contained a probe that spanned an exon-exon junction of the associated genes and therefore would not detect genomic DNA (\_m suffix) (Online Selection Guide for TaqMan Gene Expression Assays, Applied Biosystems). **Table 2.4** lists the assays used in this project.

Gene Name	Description	TaqMan® Gene Expression Assay
<i>NR4A2</i>	nuclear receptor subfamily 4, group A, member 2	Hs01118813_ml
<i>CXCR4</i>	chemokine (C-X-C motif) receptor 4	Hs00976734_ml
<i>MS4A3</i>	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	Hs00960994_ml
<i>EEA1</i>	early endosome antigen 1	Hs00185960_ml
<i>ACTN1</i>	actinin, alpha 1	Hs00998100_ml
<i>ATM</i>	ataxia telangiectasia mutated	Hs00175892_ml
<i>FOSB</i>	FBJ murine osteosarcoma viral oncogene homolog B	Hs01547109_ml
<i>JUN</i>	Jun oncogene	Hs99999141_sl
<i>CD48</i>	CD48 molecule	Hs00381156_ml

**Table 2.4: List of TaqMan® Gene Expression Assays**

GAPDH was chosen as the endogenous control (housekeeping gene) for all qRT-PCR reactions. This was after reviewing the expression of standard endogenous controls (18S, GAPDH, B2M and Actin) across all gene expression datasets generated. GAPDH was found to show the least variability across all datasets with the exception of the 5' probeset. This was felt to be irrelevant as the qRT-PCR probe binds to the 3' end of the transcript. The assay used was Human GAPDH Endogenous Control (VIC/TAMRA Probe, Primer Limited), part number 4310884E from Applied Biosystems.

Reactions were set up on ice. Reaction master mix was made up per sample as follows:

TaqMan Universal PCR Master Mix (Applied Biosystems)	10µl
TaqMan Gene Expression Assay (Applied Biosystems)	1µl
Water (Sigma Aldrich)	8µl
<b>Total Volume</b>	<b>19µl</b>

Reaction mix was added to the plate first then 1µl of cDNA was added to each well. Contents were stirred to mix. Plates were sealed with MicroAmp Optical Adhesive Film (Applied Biosystems) and briefly centrifuged up to 1000rpm.

Reactions were performed on an Applied Biosystems 7900HT Fast Real-Time PCR System using the standard thermal cycler protocol (50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15s and 60°C for 1 minute). All reactions were performed in triplicate. No template controls (water-only) and known positive controls were included on each plate.

### 2.11.3 qRT-PCR Analysis

Through the use of fluorescent reporter probes that interchelate with double-stranded DNA, the amplified PCR product is quantified as it accumulates in real time after each amplification cycle. During the exponential phase of the reaction the relative amount of DNA present can be calculated by plotting fluorescence against cycle number on a logarithmic scale. A threshold is set where fluorescence rises above background levels. Each sample in the reaction is then assigned a  $C_T$  (cycle threshold) value. This is the fractional cycle number at which fluorescence from that sample passed the threshold. In this analysis,  $C_T$  values for each sample were calculated using SDS 2.3 software. All subsequent analysis was done using Excel. The mean and standard deviation (SD) of  $C_T$  values for each triplicate were calculated. To ensure data accuracy, identical replicate samples should have a  $C_T$  standard deviation  $<0.3$ . The change in expression of each target gene was then calculated relative to the reference gene GAPDH using the  $2^{-\Delta C_T}$  method. This approach was validated by demonstrating that the efficiencies of the target and reference gene amplification were approximately equal. A  $\Delta C_T$  value was derived by subtracting the mean  $C_T$  value of the reference gene from the mean  $C_T$  value of the target gene. This is a normalisation step to adjust for possible variability in the amount of RNA added to the reverse transcription reactions. To allow comparison of data from separate plates, the  $\Delta C_T$  value for the calibrator (positive control) was subtracted from the  $\Delta C_T$  value for each sample ( $\Delta\Delta C_T$ ). Fold change compared with the positive control was calculated using the formula  $2^{-\Delta\Delta C_T}$ . To obtain error bars, the standard deviation of the  $\Delta\Delta C_T$  (s) value was calculated from the standard deviation of target gene  $C_T$  value ( $s_1$ ) and standard deviation of the reference gene  $C_T$  value ( $s_2$ ) using the formula,  $s = \sqrt{(s_1^2 + s_2^2)}$  (ABI User Bulletin #2, Applied Biosystems).

$\Delta C_T$ sample	Mean $C_T$ target gene – Mean $C_T$ reference gene
$\Delta\Delta C_T$	$\Delta C_T$ sample – $\Delta C_T$ calibrator (positive control)
Expression fold change	$2^{-\Delta\Delta C_T}$
SD of $\Delta\Delta C_T$ (s)	$\sqrt{s_1^2 + s_2^2}$

**Table 2.5: qRT-PCR Formulae**

$C_T$ : cycle threshold, SD: standard deviation, s1: SD of target gene  $C_T$  value and s2: SD of reference gene  $C_T$  value

To correlate gene expression changes seen by qRT-PCR and microarray, log base 2 of the  $2^{-\Delta\Delta C_T}$  value was plotted against log base 2 of the ratio of normalised microarray values for target gene to GAPDH. Correlation coefficients were calculated using Excel. The significance of the correlation coefficients was determined using the t-statistic  $[r/(\sqrt{1-r^2})/(N-2)]$  where r is the correlation coefficient and N, the degrees of freedom].

## 2.12 T Cell Receptor Gene Clonality Assay

### 2.12.1 Fluorescence Activated Cell Sorting of 3+4+, 3+8+ and 3+56+ Populations

AML PBMC samples were initially depleted of blasts using the StemSep™ cell separation protocol (StemCell Technologies) prior to cell sorting in order to reduce the time required on the cell sorter.

After thawing, cells were resuspended in 10mls of PBS+2% FCS. Cells were counted and then the samples were spun down at 1300rpm for 5 minutes at 4<sup>0</sup>C before being resuspended in 2% HAG at a concentration of  $5 \times 10^7$  cells/ml. Samples were then left for 20 minutes on ice. StemSep™ custom blast cocktail, CD11c and CD36 (Stem Cell Technologies) were added at concentrations of 100µl per ml of cells. Samples were left for 30 minutes at 4<sup>0</sup>C prior to the addition of colloid at a concentration of 100µl per ml of cells. After a further incubation at

4<sup>0</sup>C for 30 minutes with occasional agitation, blast separation was performed using 0.6” pump feed columns and a peristaltic pump. Column preparation and pump speeds were as defined in the manual. After cell separation, blasts were eluted from the column using 15mls of PBS + 2%FCS. All cell fractions were spun down at 1300 for 5 minutes at 4<sup>0</sup>C then resuspended in 5mls PBS+2%FCS. Cell counts were then performed. 1 x 10<sup>6</sup> cells from the blast fraction were removed for flow cytometric analysis of purity. Blast fractions were then pelleted and left at -20<sup>0</sup>C for future DNA extraction.

Cells for sorting were spun down at 1300rpm for 5 minutes at 4<sup>0</sup>C then resuspended at a concentration of 1.5 x 10<sup>6</sup> cells in 50µl PBS+2%FCS. Five µl of antibody per 50µl of cells were added and samples were left for 30 minutes at 4<sup>0</sup>C in the dark. Stained cells were then washed with 15X sample volume PBS+2%FCS. Cells were subsequently resuspended at a concentration of 10-20 x 10<sup>6</sup>/ml PBS+2%FCS + DAPI (1µl per 5mls). Debris was removed by passing the cells over 40µm cell strainers (BD Biosciences) into 5ml round bottomed polypropylene tubes (BD Biosciences). Labelled collection tubes containing 1ml PBS +2%FCS were also prepared.

Samples were sorted into three fractions: 3+4+56-, 3+8+56- and 3+56+ using a FACSAria cell sorter (BD Biosciences) in the flow cytometry laboratory at Cancer Research UK, 44 Lincoln’s Inn Fields. The purity of each sorted cell fraction was checked post-sort and the total number of sorted cells was noted.

Post sorting, cells were washed twice with PBS+2%FCS, pelleted and left at -20<sup>0</sup>C for future DNA extraction.

### **2.12.2 DNA Extraction (Qiagen DNAeasy)**

Cell pellets were thawed at RT. Two hundred µl PBS and 200µl AL lysis buffer were added to each sample. After vortexing for 15s, 40µl proteinase K (0.1mg/µl) were added and samples were transferred into labelled 1.5ml eppendorfs. Samples were briefly vortexed and then incubated at 70<sup>0</sup>C for 10 minutes in a heated block.

Two hundred and ten  $\mu\text{l}$  of 100% ethanol was then added to each. Samples were again vortexed and then applied to spin columns. These were then centrifuged at 8000rpm for 1 minute at RT following which the columns were transferred into fresh collection tubes. Five hundred  $\mu\text{l}$  of AW1 buffer was applied to each column and columns were again spun at 8000rpm for 1 minute at RT. After again transferring the columns to fresh collection tubes, 500 $\mu\text{l}$  of AW2 buffer was added to each. This time columns were spun for 3 minutes at 14000rpm and then transferred to labelled 1.5ml eppendorfs. Twenty  $\mu\text{l}$  warm AE buffer was added to each column and columns were then incubated at RT for 5 minutes. After centrifuging for 1 minute at RT at 8000rpm, a further 20 $\mu\text{l}$  AE buffer was added to each column. The last incubation and elution steps were repeated.

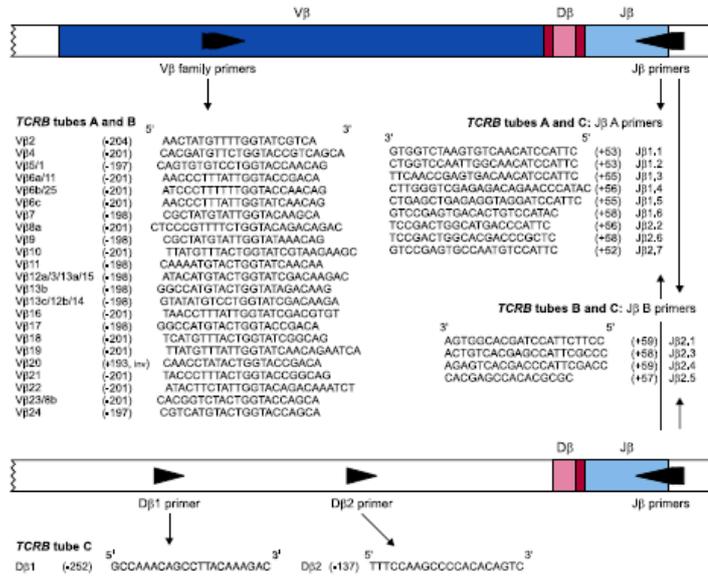
The quality and quantity of DNA obtained was assessed using a Nanodrop spectrophotometer.

DNA was stored at  $-20^{\circ}\text{C}$  until subsequent use.

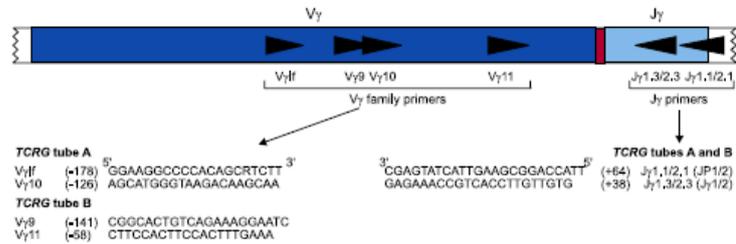
### **2.12.3 Multiplex PCR**

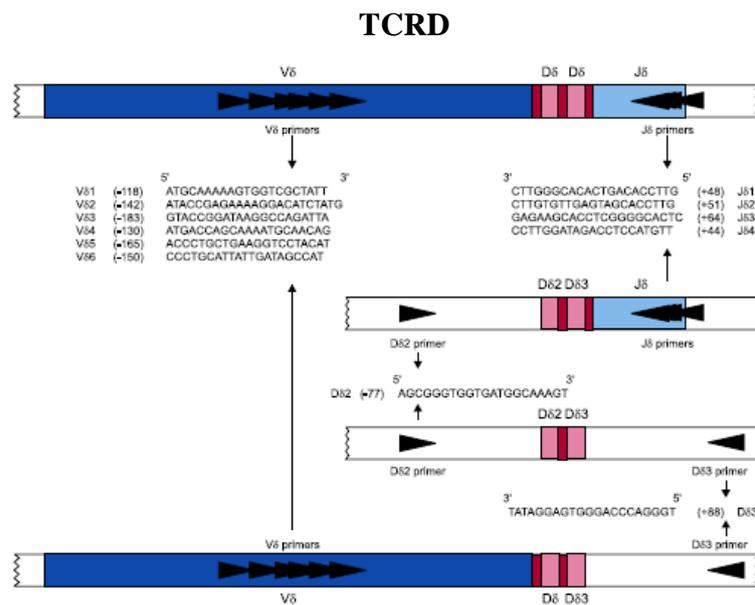
TCR gene rearrangements for beta, gamma and delta chains were assessed using multiplex PCR kits supplied by InVivoScribe Technologies (Gene Clonality Assays for ABI Fluorescence Detection) and used as per manufacturer's instructions. These assays were designed, standardised and validated in a collaborative study known as the BIOMED-2 Concerted Action (Bruggemann, *et al* 2007, van Dongen, *et al* 2003). The primers are listed in **Figure 2.9**.

### TCRB



### TCRG





**Figure 2.9: BIOMED-2 Primers**

Used in 6 tubes for identifying TCR gene rearrangements.

Kits were allowed to thaw and then gently vortexed to mix. Master mix tubes were labelled and placed on ice. Master mix was aliquoted in to the labelled tubes at a volume calculated as 45µl per sample plus 135µl for positive, negative and no template controls plus 20µl for pipetting errors. Amplitaq Gold polymerase (5U/µl) (Applied Biosystems) was then added to each master mix tube – for TCRB Tubes A and B: 0.45µl per 50µl total PCR reaction volume, for all other tubes: 0.25µl per 50µl total PCR reaction volume. Tubes were mixed by gently vortexing. Forty-five µl of master mix was aliquoted into the appropriate wells of a thermo-fast 96 well skirted PCR plate (Abgene UK). Five µl of DNA or water (Sigma Aldrich) was then added to relevant wells and mixed by pipetting up and down several times. DNA for positive and negative controls was provided with each kit. Plates were then sealed with MicroAmp optical adhesive film (Applied Biosystems) and briefly centrifuged up to 1000rpm. PCR reactions were performed in a PTC-225 thermocycler (MJ Research) using the following programme:

- Step 1: 95<sup>0</sup>C for 7 minutes (pre-activation)
- Step 2: 95<sup>0</sup>C for 45s (denaturation)
- Step 3: 60<sup>0</sup>C for 45s (annealing)
- Step 4: 72<sup>0</sup>C for 90s (extension)
- Step 5: Go to step 2, 34 more times
- Step 6: 72<sup>0</sup>C for 10 minutes (final extension)
- Step 7: Hold 15<sup>0</sup>C

Due to large quantity of primer-dimer formation in the initial practice run, it was felt appropriate to clean up all the PCR products. This substantially improved the quality of the reaction products.

The Qiagen PCR Purification Kit was used according to manufacturer's instructions. Samples were transferred from the plate to 1.5ml microfuge tubes. Five volumes (250µl) of DNA binding buffer PBI were added and the samples were transferred to spin columns. These were centrifuged at 13000rpm for 60s at RT. Flow-through from the columns was discarded and the columns replaced into the same collection tube. Seven hundred and fifty µl of wash buffer PE was then added and the columns re-spun at 13000rpm for 60s at RT. Once the flow-through was discarded, the columns were spun again at 13000rpm for 60s at RT. The columns were then transferred to clean 1.5ml microfuge tubes and the DNA eluted using 50µl EB buffer and spinning for 60s at 13000rpm at RT. The cleaned up DNA was then transferred to another 96 well plate.

#### **2.12.4 GeneScanning**

The PCR products were analysed by differential fluorescence detection using capillary electrophoresis (GeneScanning) on an ABI Prism 3100 Genetic Analyzer in the Equipment Park at Cancer Research UK, 44 Lincoln's Inn Fields. One µl of reaction product was used for analysis. No dilution was performed. To this was added 10µl of HI-deionised formamide containing LIZ 600 size standards. The reaction product mix was heated to 95<sup>0</sup>C for 2 minutes and then

snap chilled on ice for 5 minutes. A 50cm capillary was used for electrophoresis. Subsequent analysis was performed on Genotyper 3.7 software.

## 2.13 Cell Conjugation Assay

### 2.13.1 Cell Conjugation

Cell conjugation assays were performed as previously published (Ramsay, *et al* 2008a). In detail, coverslips were prepared on the day prior to the experiment by placing them in a 24 well tissue culture plate and adding 550 $\mu$ l of poly-L-lysine (Sigma Aldrich). These were left overnight at room temperature. The following day, T cells and APCs were thawed, washed in RPMI+10%FCS and aliquoted into eppendorfs - 1 x 10<sup>6</sup> T cells in RPMI+10%FCS or 2 x 10<sup>6</sup> APCs in RPMI alone. T cells were left at 37<sup>0</sup>C +5%CO<sub>2</sub> to revive until conjugation. APCs were stained with CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin) (Invitrogen Ltd.) by adding 1 $\mu$ l of a 10mM stock solution in DMSO to each eppendorf and then incubating at 37<sup>0</sup>C, 5% CO<sub>2</sub> for 30 minutes. Residual CMAC was then removed by centrifuging cells at 4000rpm for 1 minute then resuspending in RPMI+10%FCS. APCs were then either pulsed or not pulsed with 2 $\mu$ g/ml of a 'cocktail' of staphylococcal superantigens (staphylococcal enterotoxins A and B) (Sigma Aldrich) by adding 1 $\mu$ l of cocktail or media alone to the resuspended cells and incubating them at 37<sup>0</sup>C, 5% CO<sub>2</sub> for 30 minutes. Both APCs and T cells were then spun down at 4000rpm for 1 minute, washed with 1ml RPMI and then resuspended in 200 $\mu$ l RPMI. T cells were then added to APCs at a ratio of 1 to 2, spun at 1500rpm for 5 minutes and then incubated at 37<sup>0</sup>C, 5% CO<sub>2</sub> for 15 minutes. Next, the cell pellets were plated onto the poly-L-lysine coated coverslips by gently pipetting cells directly onto the coverslips within the 24 well plate then centrifuging the plate at 1000rpm for 5 minutes. Cells were subsequently fixed for 15 minutes at RT in 500 $\mu$ l 3% methanol-free formaldehyde (TAAB Laboratories Equipment Ltd) in PBS. To allow visualisation of intracellular proteins, cells were then permeabilised with 500 $\mu$ l 0.3% vol/vol triton x100 (Sigma Aldrich) in PBS for 5 minutes at RT. Non-specific staining was prevented by blocking with 500 $\mu$ l 0.1% BSA (Miltenyi

Biotec) in PBS for 10 minutes at RT. F-actin was visualised by staining with 400µl Rhodamine Phalloidin (Invitrogen Ltd.) 1:40 in blocking solution for 20 minutes at RT. Phosphotyrosine recruitment to the immunological synapse was visualised by firstly staining with 200µl phosphotyrosine (Millipore) 1:100 in 5% goat serum in PBS for 45 minutes at 4<sup>0</sup>C and secondly adding 200µl of goat anti-mouse Alexa 488 (Invitrogen) 1:500 in 5% goat serum in PBS and incubating at 4<sup>0</sup>C for a further 30 minutes. Between each step, coverslips were washed 3 times with PBS. Finally, coverslips were carefully mounted onto slides using fluorescent mounting medium (Dako) and left for 2 hours to dry prior to sealing the edges of the coverslips with nail hardener. Slides were left in the dark at 4<sup>0</sup>C until analysed.

### ***2.13.2 Immunofluorescence Confocal Microscopy***

Slides were examined using a Carl Zeiss 510 Meta confocal laser-scanning microscope using a x63 objective lens. Coverslips were examined consistently from top left top to bottom right. Images were acquired whenever a cell conjugate was observed with the aim of acquiring images of a minimum of 50 conjugates. Images to be compared from a single experiment were acquired during the same session using the same acquisition settings.

### ***2.13.3 Image Analysis***

Images were subsequently analysed for APC:T cell ratio, conjugate formation (% of total T cells forming conjugates with APCs), immunological synapse formation (% of cell conjugates that were immunological synapses) and phosphotyrosine recruitment to the T cell-APC interface (% of conjugates that demonstrated phosphotyrosine signalling molecule recruitment). An immunological synapse was defined as a conjugate that demonstrated polarisation of F-actin at the cell contact point. Conjugates that showed a distinct F-actin band were scored 1, conjugates without F-actin polarisation to the contact site were scored 0 and conjugates where there was weak F-actin polarisation were scored 0.5.

Recruitment of phosphotyrosine signalling molecules to the T cell-APC interface was scored in the same way.

## Chapter 3

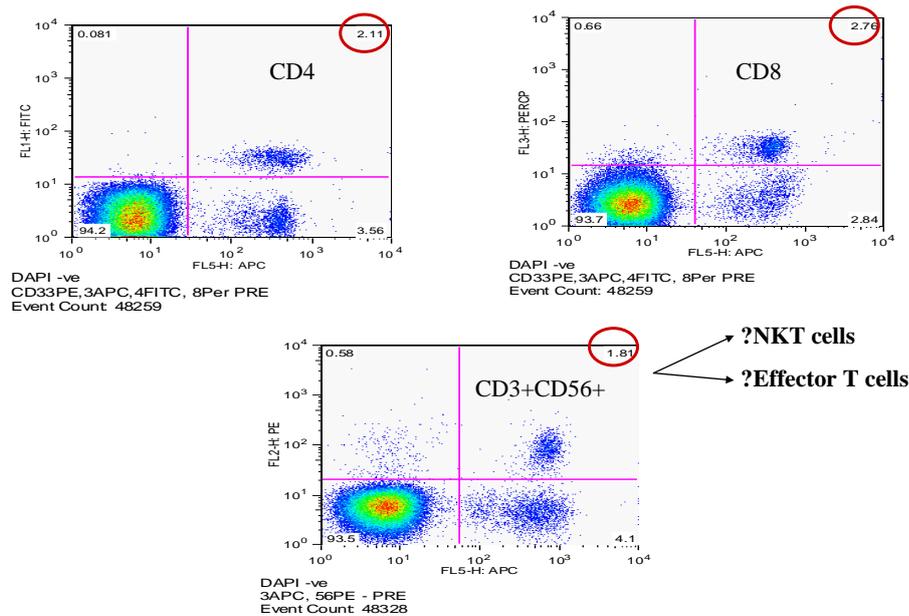
### Characterisation of T Cells from AML Patients

#### 3.1 Introduction

The description of the morphological appearance of the bone marrow in AML is the effacement of normal haematopoiesis with sheets of myeloblasts. This has resulted in a general perception that the numbers of T cells in the bone marrow and peripheral blood of patients presenting with AML is low. Certainly, relative to the number of circulating blasts, this is often the case but few studies have addressed the issue of absolute numbers of T cells in the peripheral blood of patients with AML or attempted to characterise them further. The existence of T cell precursors in the bone marrow of untreated AML patients has been demonstrated by the generation of T cell clones with cytotoxic efficacy against both autologous and allogeneic blasts (Jahn, *et al* 1995). Most of the available information on T cells in the peripheral blood of AML patients relates to immune reconstitution following chemotherapy or stem cell transplantation where the observation has been made that early absolute lymphocyte count recovery is predictive of superior survival (Behl, *et al* 2006, Porrata, *et al* 2002). Other reports have characterised treatment-induced T cell defects (Bruserud 1998b, Bruserud, *et al* 1998a, Wendelbo, *et al* 2004). A study of 45 AML patients at diagnosis compared to 9 healthy controls looked at lymphocyte subsets but only in terms of ratios of one cell subset to another; CD4/CD8 ratio, CD4+ CD45RA/RO ratio and CD8+CD45RA/RO ratio (Ersvaer, *et al* 2007). The distribution of these subsets in AML did not differ significantly from the controls with the exception of the CD8+ CD45RA/RO ratio which was significantly lower in the AML patients suggesting an increased pool of memory CD8 cells. There are two previously published manuscripts that have addressed the issue of absolute T cell numbers. Vidriales and colleagues undertook a dual labelling flow cytometric analysis of NK and T cell subsets in 66 patients with *de novo* AML compared with 30 healthy volunteers (Vidriales, *et al* 1993). This demonstrated that the total CD4 and CD8 cell counts are within the normal range in terms of both relative and absolute numbers. This finding was confirmed in a smaller study by Panoskaltsis *et al.* who

examined peripheral blood from 13 patients with AML and 8 healthy volunteers using a combination of CD45, CD3 and CD8 (Panoskaltzis, *et al* 2003). As a starting point for this project it was felt to be relevant to review this issue by enumerating and characterising T cells present in the peripheral blood of newly diagnosed patients with AML.

An observation made early during this study was that certain AML patients had large populations of CD3 cells that co-expressed CD56 (**Figure 3.1**). A significant increase in these cells in AML patients compared to healthy individuals had also been noted in the Vidriales paper, although less than 3% of the 45 patients in the Ersvaer study had T cells that were found to express CD56. The question arose as to the nature of these cells. Were they true natural killer T (NKT) cells or were they CD56 expressing T cells?



**Figure 3.1: A Large Population of CD3+CD56+ cells in AML Patients**

CD56 is the neural cell adhesion molecule (NCAM) (Thiery, *et al* 1977) also known as D2-CAM (Lyles, *et al* 1984), Leu19 and NKH-1 (Lanier, *et al* 1989). It was the first characterised of the immunoglobulin superfamily of cell adhesion molecules (Edelman and Crossin 1991) and it mediates calcium-independent

homophilic binding to CD56 on adjacent cells (Hoffman and Edelman 1983). It was initially identified in the nervous system where it was found to mediate cell-cell interactions of glial cells during embryogenesis (Lyles, *et al* 1984, Thiery, *et al* 1977). Within the haematopoietic lineage, CD56 is expressed on NK and T cells. NK cells are divided into CD56<sup>bright</sup> and CD56<sup>dim</sup> (Lanier, *et al* 1986): the CD56<sup>bright</sup> subset shows increased potential for cytokine production (Cooper, *et al* 2001) whereas the CD56<sup>dim</sup> subset, representing 90% of NK cells, has enhanced cytotoxicity associated with a mature differentiation state (Jacobs, *et al* 2001). Amongst T cells, CD56 has been reported to be expressed on a subset of effector T cells that makes up less than 5% of total peripheral blood lymphocytes (Lanier, *et al* 1986) as well as on classical NKT cells (Norris, *et al* 1999). True NKT cells and CD3+56+ cells continue to be confused within the literature (Peralta, *et al* 2008, Reis, *et al* 2008, Srivastava, *et al* 2008, Zhu, *et al* 2008). The major differences between them are listed in **Table 3.1**.

Although originally described by three groups in 1987 (Budd, *et al* 1987, Ceredig, *et al* 1987, Fowlkes, *et al* 1987), the term ‘NK T cell’ was first published in 1995 and was used to describe a distinct population of T lymphocytes in mice that shared some characteristics with NK cells including the expression of the C-lectin type NK receptor NK 1.1 (NKR-P1 or CD161c) (Makino, *et al* 1995). NKT cells are also found in humans (Dellabona, *et al* 1994, Porcelli, *et al* 1993) although no discriminatory cell surface marker has been identified. Characteristically, rather than the broad TCR chain usage found in healthy T cells, NKT cells only express V $\alpha$ 24-J $\alpha$ Q (now known as V $\alpha$ 24-J $\alpha$ 18) in humans (V $\alpha$ 14-J $\alpha$ 281 – now known as J $\alpha$ 18 - in mice) and most only use V $\beta$ 11 (V $\beta$ 8.2 in mice) (Lantz and Bendelac 1994). These cells are also unusual in that they recognise glycolipid presented by the monomorphic MHC class I-like molecule CD1d (Beckman, *et al* 1994, Bendelac, *et al* 1995). Their most potent ligand is the glycosphingolipid alpha-galactosylceramide ( $\alpha$ GalCer, also known as KRN7000) (Kawano, *et al* 1997) initially isolated from the marine sponge *Agelas mauritanicus* in a screen for compounds that could prevent tumour metastases to the liver in mice (Kobayashi, *et al* 1995). The reactivity of NKT cells for  $\alpha$ GalCer presented by CD1d is highly conserved between mice and humans suggesting a fundamental importance for

this T cell recognition system (Brossay, *et al* 1998). Natural glycolipid ligands have now been identified. These include the mammalian isoglobotrihexosyl ceramide (iGb3) (Zhou, *et al* 2004) and glycosphingolipids from the gram-negative bacteria *Sphingomonas* (Kinjo, *et al* 2005) and *Borrelia burgdorferi* (Kinjo, *et al* 2006). NKT cells have been recognised to be a potent source of immunoregulatory cytokines including IL-4, IFN $\gamma$  and TNF that are rapidly produced on activation (Zlotnik, *et al* 1992). Two subsets of NKT cells have been identified; the CD4 positive subset produces the anti-inflammatory T<sub>H</sub>2 cytokines IL-4 and IL-13 whereas the CD4 negative (also known as double-negative or DN) subset produces pro-inflammatory T<sub>H</sub>1 cytokines such as IFN $\gamma$  (Gumperz, *et al* 2002, Lee, *et al* 2002). NKT cells in humans can also express an unusual form of CD8, CD8 $\alpha^{\text{dim}}/\beta^-$  in contrast to healthy  $\alpha\beta$  T cells that are exclusively CD8 $\alpha^{\text{bright}}/\beta^{\text{bright}}$  (Prussin and Foster 1997). After activation, NKT cells are directly cytotoxic to cells expressing the cognate antigen using both perforin-dependent and FasL-dependent mechanisms (Metelitsa, *et al* 2001). This allows them to kill tumour cells *in vitro* (Metelitsa, *et al* 2003, Takahashi, *et al* 2003, van der Vliet, *et al* 2003). However, NKT cells can also activate NK cells, CD4 and CD8 T cells and DCs via cell-cell contact and the production of cytokines (Fujii, *et al* 2003b, Hermans, *et al* 2003, Metelitsa, *et al* 2001). NKT cells have been shown to have an extremely diverse range of actions from the prevention of autoimmunity to controlling responses to infection suggesting that an important natural function of these cells is the protection of self tissues from damaging inflammatory immune responses (Godfrey, *et al* 2000). The non-specific mechanism by which NKT cells recognise antigen has led to interest in these cells for cancer immunotherapy. Rather than having to design specific peptides for T cell recognition, NKT cells in all individuals recognise the same glycolipids and they do so using the same TCR. An anti-tumour effect has been demonstrated in tumour-bearing mice (Smyth, *et al* 2000) and injection of  $\alpha$ GalCer protects mice from experimentally-induced tumours (Kawano, *et al* 1998, Nakagawa, *et al* 1998). However, CD4<sup>+</sup> NKT cells have also been shown to suppress effector CD8 T cell function by the secretion of IL-13 and TGF $\beta$ , a mechanism hypothesized to down-regulate tumour immunosurveillance (Terabe, *et al* 2000). How the decision is made by NKT cells to promote or prevent cancer growth remains to be

elucidated but may involve the nature of the CD1d/glycolipid/TCR interaction, interaction with APCs and the prevailing cytokine milieu (Smyth, *et al* 2002). There have been several reports of reduced numbers of NKT cells or numbers of IFN $\gamma$ -secreting NKT cells in the peripheral blood of patients with cancer including haematopoietic malignancies (Dhodapkar, *et al* 2003a, Fujii, *et al* 2003a, Molling, *et al* 2005, Tahir, *et al* 2001, Yoneda, *et al* 2005).

Compared with NKT cells, CD3+CD56+ cells have a distinct morphology and function, have an unrestricted polymorphic TCR chain usage and are not CD1d-restricted (**Table 3.1**). These cells have been variously called NK-type T cells (Ohkawa, *et al* 2001), natural T (NT) cells (Doherty, *et al* 1999), NK-like T cells (Zoll, *et al* 2000) and NKT-like lymphocytes (Peralbo, *et al* 2007). CD56 expressing T cells were first described by two groups in 1986 (Lanier, *et al* 1986, Schmidt, *et al* 1986) and later further characterised by Ortaldo *et al.* (Ortaldo, *et al* 1991). In common with NK cells, CD3+56+ were morphologically described as 'large granular lymphocytes' and, unlike normal CTLs, found to be capable of killing the MHC Class I negative cell line K562. An anti-CD3 antibody inhibited this cytotoxicity suggesting that the TCR was involved in the interaction. The majority of CD3+56+ cells were found to express CD8. They are potent cytotoxic agents with enhanced cytotoxic granule expression, cytokine production and anti-tumour cytotoxicity compared with CD3+56- cells (Ohkawa, *et al* 2001, Pittet, *et al* 2000). These cells have been identified as an effector cell subset with a role in mucosal immunity in the intestine (Cohavy and Targan 2007), they make up a third of all hepatic CD3 cells (Doherty, *et al* 1999), have anti-viral effects (Kawarabayashi, *et al* 2000, Montoya, *et al* 2006) and may have a role in fertility (van den Heuvel, *et al* 2007). Numbers of these cells increase with aging (Peralbo, *et al* 2007). In addition, a case report has been described of a patient with AML who developed a polyclonal proliferation of CD3+CD8+CD56+ cells that killed autologous leukaemic blasts via engagement of the 2B4 activating receptor by the cell surface ligand CD48 (Costello, *et al* 2002a). CD3+CD56+ cells are generally seen as an *in vitro* phenomenon; 'cytokine-induced killer (CIK)' cells can be generated by culturing PBMCs with various combinations of cytokines but including IL-2 for 3-4 weeks (Schmidt-Wolf, *et al* 1993, Schmidt-Wolf, *et al*

1991). CIK cells display potent lytic activity and unlike, LAK cells, are not dependent on IL-2. They are therefore under investigation as consolidative immunotherapy in the minimal residual disease state and as an alternative to donor lymphocyte infusions in allogeneic stem cell transplantation (Linn and Hui 2003). Numbers of CD3+56+ cells have previously been shown to be increased in the peripheral blood of patients presenting with leukaemia (Vidriales, *et al* 1993) and are also increased in ascites from patients with ovarian carcinoma compared to blood and control ascites (Bamias, *et al* 2007). Another report has demonstrated that a large proportion of tumour-infiltrating lymphocytes in human primary and metastatic liver tumours are CD3+56+ cells (Shimizu, *et al* 1990).

	<b>NKT Cells</b>	<b>3+56+ Cells</b>
<b>Frequency</b>	0.01-1% of peripheral blood T cells	2-5% of PBMCs
<b>TCR chain use</b>	V $\alpha$ 24 V $\beta$ 11	Unrestricted
<b>Recognise</b>	Glycolipid	Peptide
<b>Molecular context of recognition</b>	CD1d	MHC

**Table 3.1: Differences between NKT cells and CD3+56+ Cells**

As both true NKT cells and CD3+56+ cells have a potential role in the immune response against tumours, the further characterisation of these CD56-expressing T cells in AML patients was relevant in the investigation into how the immune system sees AML.

### 3.2 Aims

The primary aim of this initial work was to quantify and classify T cells in patients presenting with AML in comparison to T cells in healthy volunteers. The secondary aim was to confirm the impression of an increased number of CD3+56+ cells in patients presenting with AML and to characterise the nature of this cell population.

### 3.3 Materials and Methods

#### 3.3.1 Samples

Frozen PBMCs from newly diagnosed patients with AML were used in this experiment. Historical patients were chosen on the basis of quantity of available material. In addition, PBMCs were frozen and subsequently used on every patient who underwent leucopheresis while this project was ongoing. The characteristics of these patients are listed in **Table 3.2**. The method for surface staining is described in section 2.7.1.

<b>Number</b>	36
<b>Median Age in years (Range)</b>	55 (17-81)
<b>Sex</b>	
Female	14
Male	22
<b>FAB Type</b>	
M0	1
M1	11
M2	7
M3	0
M4	13
M5	2
Others	2
<b>Prognostic Group</b>	
Favourable	10
Intermediate	23
Adverse	3
<b>Response to first line therapy</b>	
Complete Remission	22
Fail	4
Death	6
Not applicable	4
<b>Relapse</b>	
Yes	9
No	27
<b>Median Overall Survival in years (Range)</b>	0.8 (0.02-15)

**Table 3.2: Characteristics of AML Patients**

Blood was taken from healthy volunteers for the comparative analysis. Attempts were made to age-match the AML and healthy samples. Fresh blood was used in a whole blood lysis method described in section 2.7.2. A different method was used in order to obtain absolute cell counts on the healthy samples for which FBCs were not available. A comparison was made of the relative expression of CD56 on T cells in fresh samples undergoing the whole blood lysis method, fresh samples in which PBMCs were extracted using density gradient centrifugation and frozen samples. Minor tube-to-tube variation in CD56 expression was observed but no significant differences were seen (data not shown).

The characteristics of the healthy controls are listed in **Table 3.3**.

<b>Number</b>	17
<b>Median Age</b> in years (range)	35 (24-80)*
<b>Sex</b>	
Female	10
Male	7

**Table 3.3: Characteristics of Healthy Volunteers**

\*p=0.11 in t-test with age of AML patients.

### 3.3.2 Flow Cytometry

The initial staining protocol is shown in **Table 3.4**.

	<b>FITC</b>	<b>PE</b>	<b>APC</b>	<b>PerCP</b>	<b>PECy7</b>	<b>DAPI</b>
<i>Tube 1</i>	CD16	CD1d-T	CD56	CD4	CD3	√
<i>Tube 2</i>	CD16	CD1d-T	CD56	CD8	CD3	√
<i>Tube 3</i>		CD1d-C			CD3	√
<i>Tube 4</i>	V $\beta$ 11	V $\alpha$ 24	CD56		CD3	√
<i>Tube 5</i>	IgG1	IgG1	IgG1	IgG1	IgG1	√
<i>Tube 6</i>	IgG2a	IgG2b				√

**Table 3.4: Initial Staining Protocol**

Fluorochromes are listed on the top line. APC: allophycocyanin, PerCP: peridinin-chlorophyll protein, CD1d-T: CD1d tetramer loaded with  $\alpha$ GalCer analogue, CD1d-C: control unloaded CD1d tetramer (both provided by the NIH Tetramer Facility).

The gating strategy used for acquiring and analysing data and the method of calculating relative and absolute cell numbers is described in section 2.7.4. The maximum number of CD3 events possible was acquired. This was a mean of 74848 CD3 events for the healthy samples and a mean of 27505 CD3 events for the AML samples.

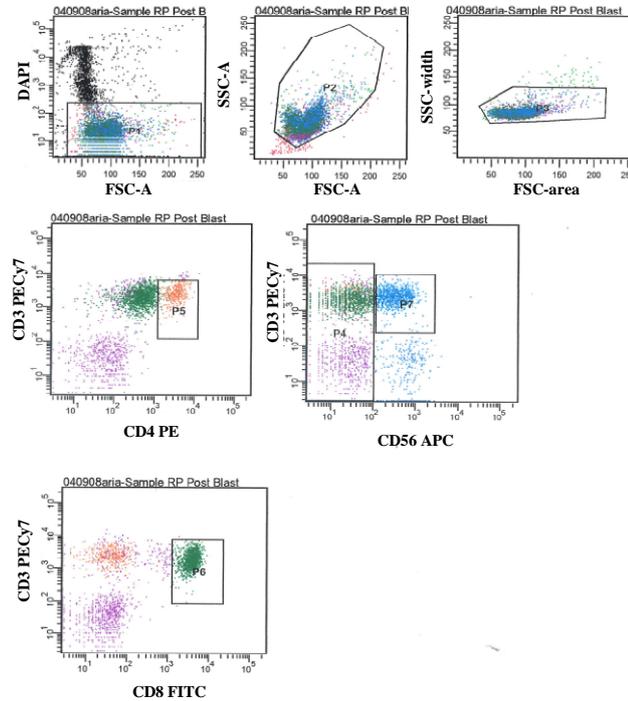
### 3.3.3 Statistics

All statistical analysis was performed using Excel software. T-tests were unpaired, 2-tailed with sample groups considered to have unequal variance. A p-value less than 0.05 was considered as the cut-off for statistical significance.

### 3.3.4 Assessment of TCR Gene Rearrangements

The clonality of T cell populations was assessed by multiplex PCR for TCR gene rearrangements using TCRB, TCRD and TCRG gene clonality assays provided by InVivoScribe Technologies. The methods used are described in section 2.12. CD3+4+, CD3+8+ and CD3+56+ populations were flow sorted from five patients with AML and two healthy individuals. The gating strategy is shown in **Figure**

**3.2.** Blasts were initially positively selected from AML PBMC samples prior to flow sorting and were included in the analysis.



**Figure 3.2: Gates Used for Flow Sorting Cell Populations**

P1: DAPI negative live cells, P2: mononuclear cells, P3: exclusion of doublets (aggregated cells) – area of the fluorescent light pulse is plotted against width. Doublets have a greater pulse width than a single cell as they take longer to pass through the laser beam and therefore can be excluded from the analysis, P4: CD56 negative, P5: CD4 positive, CD56 negative, P6: CD8 positive, CD56 negative, P7: CD56 positive.

Post flow sorting, cell counts and purities were recorded. DNA was extracted using the method detailed in section 2.12.2 and quality and quantity was checked on a Nanodrop spectrophotometer. All samples were made up to 40µl as 7 reactions were to be performed per sample with 5µl of DNA required in each. The clonality assay used is described as a robust assay system and that a wide range of DNA concentrations will yield a valid result. Details of all the samples tested are listed in **Tables 3.5-3.8**.

<b>CD3+4+56- Populations</b>							
	<b>Cell</b>			<b>DNA</b>			<b>Amount used in PCR</b>
	<b>Count post sort</b>	<b>Recovery</b>	<b>Purity</b>	<b>Quantity</b>	<b>A<sub>260</sub>/A<sub>280</sub></b>	<b>A<sub>260</sub>/A<sub>230</sub></b>	
<b>AML1</b>	6.9 x 10 <sup>6</sup>	31.5%	99.4%	3586.3ng	1.89	2.28	448ng
<b>AML2</b>	1.8 x 10 <sup>6</sup>	2.8%	99.0%	841.0ng	2.06	3.08	105ng
<b>AML3</b>	0.5 x 10 <sup>6</sup>	44.5%	99.8%	338.4ng	1.69	0.37	42.3ng
<b>AML4</b>	5.5 x 10 <sup>6</sup>	47.0%	99.2%	550.1ng	1.92	6.34	69ng
<b>AML5</b>	2.9 x 10 <sup>6</sup>	34.5%	98.1%	4403.2ng	1.97	2.51	550.4ng
<b>Healthy1</b>	1.8 x 10 <sup>6</sup>	51.4%	99.3%	5406.6ng	1.88	2.69	675.9ng
<b>Healthy2</b>	1.9 x 10 <sup>6</sup>	51.7%	99.8%	4230.7ng	1.90	2.86	528.8ng

**Table 3.5: Data for 3+4+56- Populations**

<b>CD3+8+56- Populations</b>							
	<b>Cell</b>			<b>DNA</b>			<b>Amount used in PCR</b>
	<b>Count post sort</b>	<b>Recovery</b>	<b>Purity</b>	<b>Quantity</b>	<b>A<sub>260</sub>/A<sub>280</sub></b>	<b>A<sub>260</sub>/A<sub>230</sub></b>	
<b>AML1</b>	5.3 x 10 <sup>6</sup>	52.0%	98.1%	3829.4ng	1.94	2.14	478.7ng
<b>AML2</b>	0.8 x 10 <sup>6</sup>	30.0%	96.7%	174.4ng	1.49	-3.18	22ng
<b>AML3</b>	1.7 x 10 <sup>6</sup>	39.5%	98.9%	1821.1ng	1.99	1.80	227.6ng
<b>AML4</b>	5.6 x 10 <sup>6</sup>	43.7%	99.5%	9644.9ng	1.91	2.37	1205.6ng
<b>AML5</b>	2.4 x 10 <sup>6</sup>	33.3%	96.6%	3477.3ng	1.90	2.43	434.7ng
<b>Healthy1</b>	0.7 x 10 <sup>6</sup>	46.7%	96.9%	1186.6ng	1.91	7.03	148.3ng
<b>Healthy2</b>	0.7 x 10 <sup>6</sup>	46.7%	96.4%	1390.0ng	1.79	2.91	173.8ng

**Table 3.6: Data for 3+8+56- Populations**

CD3+56+ Populations							
	Cell		DNA				Amount used in PCR
	Count	Recovery	Purity	Quantity	A <sub>260</sub> /A <sub>280</sub>	A <sub>260</sub> /A <sub>230</sub>	
	post sort						
<b>AML1</b>	0.6 x 10 <sup>6</sup>	67.8%	73.3%	116.28ng	5.33	-1.15	14.5ng
<b>AML2</b>	0.7 x 10 <sup>6</sup>	38.0%	84.2%	81.9ng	2.01	-0.98	10.2ng
<b>AML3</b>	0.9 x 10 <sup>6</sup>	44.6%	93.1%	2092.3ng	1.90	2.81	261.5ng
<b>AML4</b>	3.1 x 10 <sup>6</sup>	52.6%	90.5%	4111.1ng	1.90	2.52	513.9ng
<b>AML5</b>	0.3 x 10 <sup>6</sup>	45.0%	76.3%	303.2ng	1.31	2.63	37.9ng
<b>Healthy1</b>	0.1 x 10 <sup>6</sup>	44.8%	91.1%	346ng	1.73	11.15	43.25ng
<b>Healthy2</b>	0.2 x 10 <sup>6</sup>	43.2%	96.4%	423.7ng	1.50	2.73	53ng

**Table 3.7: Data for 3+56+ Populations**

Blast Populations						
	Cell		DNA			Amount used in PCR
	Count	Purity	Quantity	A <sub>260</sub> /A <sub>280</sub>	A <sub>260</sub> /A <sub>230</sub>	
	post sort					
<b>AML1</b>	51 x 10 <sup>6</sup>	91.4%	22.1ug	1.95	2.02	2762.5ng
<b>AML2</b>	78 x 10 <sup>6</sup>	92.3%	207.4ug	1.86	2.04	5761ng
<b>AML3</b>	230 x 10 <sup>6</sup>	93.3%	162.7ug	1.86	2.03	4519ng
<b>AML4</b>	375 x 10 <sup>6</sup>	93.2%	237.2ug	1.88	2.2	6589ng
<b>AML5</b>	149 x 10 <sup>6</sup>	96.2%	186.1ug	1.88	1.83	5169ng

**Table 3.8: Data for Blast Populations**

Multiplex PCR reactions were carried out as described in section 2.12.3 and analysed by GeneScanning (section 2.12.4). Data was subsequently reviewed using Genotyper 3.7 software.

### 3.3.5 Correlation with Clinical Data

Clinical data was obtained on all 36 AML patients and correlations with the absolute numbers of total T cells and CD3+56+ cells found in the peripheral blood were made using GraphPad Prism. Non-parametric tests of significance were performed as the datasets were demonstrated to be non-normally distributed by analysis of means using GraphPad InStat software.

## 3.4 Results

### 3.4.1 Total T Cells in AML

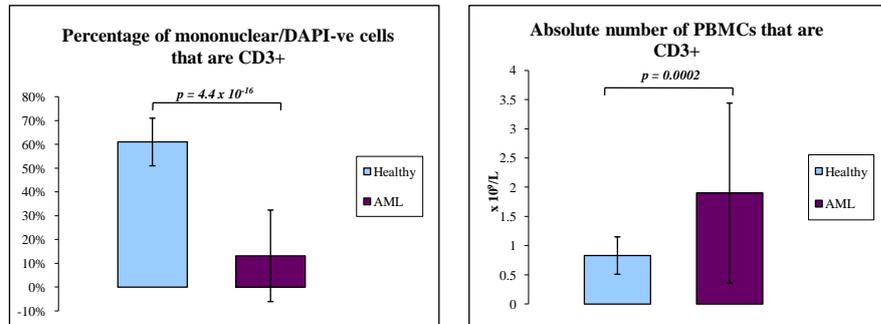
Initial phenotyping results are shown in **Table 3.9**. These indicate a significant increase in the absolute number of total T cells in the peripheral blood of patients presenting with AML compared with healthy controls. The absolute number of CD4 and CD8 cells was also greater in AML. The significantly increased number of CD3+CD56+ cells confirmed the previous observation that this population was increased in patients with AML. The fact that the population of CD3 cells that express CD16 is not significantly increased in terms of absolute numbers suggests that there is not a generalised proliferative response of T cells in AML.

	<b>Total CD3</b>	<b>CD3+4+</b>	<b>CD3+8+</b>	<b>CD3+16+</b>	<b>CD3+56+</b>
<b>Healthy</b>					
<b>Relative</b>	61.00%	64.44%	30.77%	3.14%	2.96%
	(+/- 10%)	(+/- 11.6%)	(+/-10.35%)	(+/- 1.83%)	(+/- 2.27%)
<b>Absolute</b>	0.8 x 10 <sup>9</sup> /L	536.2 x 10 <sup>6</sup> /L	263.4 x 10 <sup>6</sup> /L	24.7 x 10 <sup>6</sup> /L	27.6 x 10 <sup>6</sup> /L
	(+/- 0.32)	(+/- 224.8)	(+/- 170.0)	(+/- 20.2)	(+/- 32.8)
<b>AML</b>					
<b>Relative</b>	13.15%	51.64%	36.87%	1.52%	9.33%
	(+/- 19.24%)	(+/- 13.31%)	(+/- 12.77%)	(+/- 1.98%)	(+/- 6.95%)
	*	*		*	*
<b>Absolute</b>	1.9 x 10 <sup>9</sup> /L	1038.7 x	722.2 x 10 <sup>6</sup> /L	23.3 x 10 <sup>6</sup> /L	178.7 x 10 <sup>6</sup> /L
	(+/-1.54)	10 <sup>6</sup> /L	(+/- 650.7)	(+/- 27.1)	(+/- 254.2)
		(+/- 1031.2)			
	*	*	*		*

**Table 3.9: Total T Cells in AML**

Relative and absolute numbers of total CD3+, CD3+4+, CD3+8+, CD3+16+ and CD3+56+ cells in the peripheral blood of patients presenting with AML and healthy volunteers. \* indicates p<0.05 in a 2-tailed unpaired t-test. For total CD3, the relative number relates to CD3+ cells as a percentage of live mononuclear cells. For other subsets, the relative number relates to the cells as a percentage of total CD3+ cells. Figures listed are means with standard deviations in the following brackets.

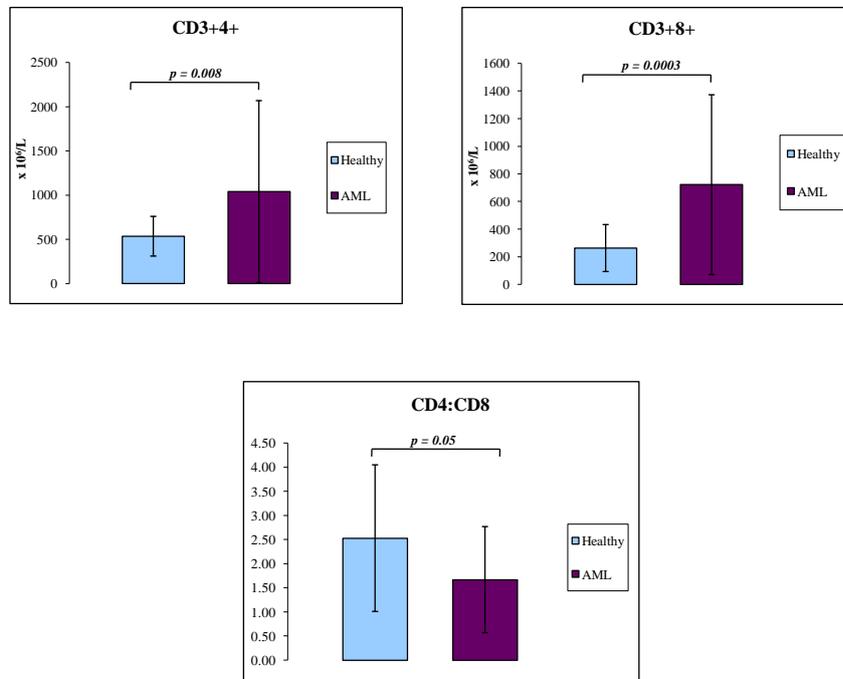
As expected, the percentage of T cells relative to live mononuclear cells was significantly lower in AML compared with healthy individuals. However, the absolute number of total T cells was significantly higher (**Figure 3.3**).



**Figure 3.3: Bar Charts Illustrating Total T Cells in AML**

The relative and absolute numbers of T cells in peripheral blood (PB) of patients presenting with AML compared with healthy controls.

The increase in total T cell numbers is accounted for by rises in both the CD4 and CD8 populations but, as illustrated by the CD4 to CD8 ratio, the CD8 population is increased more than the CD4 (**Figure 3.4**).

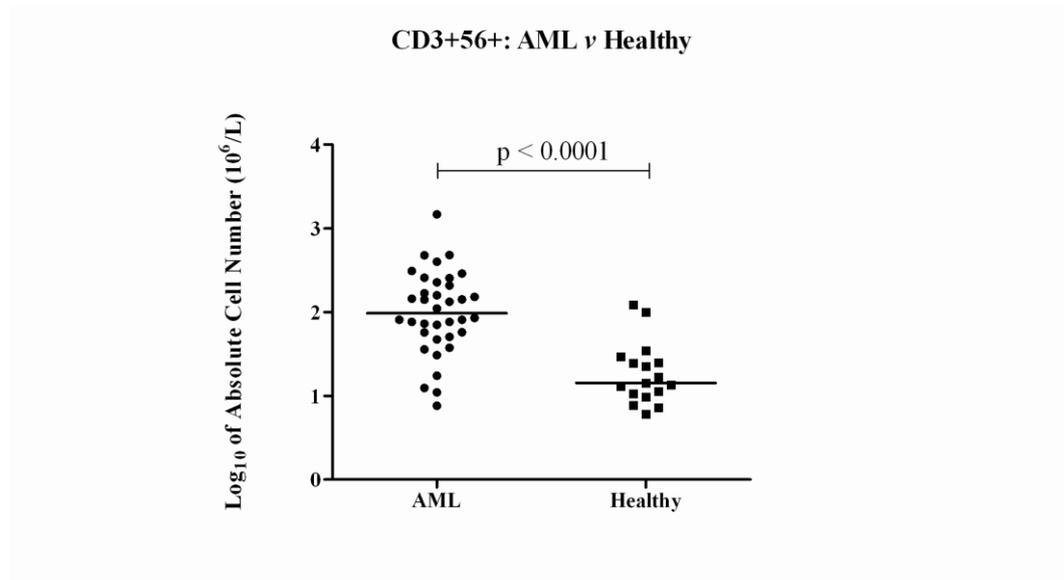


**Figure 3.4: CD4 and CD8 Cells in AML**

Bar charts illustrating the absolute numbers of CD4 and CD8 cells and the CD4:CD8 ratio in PB of patients presenting with AML compared with healthy controls.

### 3.4.2 CD56-expressing T Cells in AML

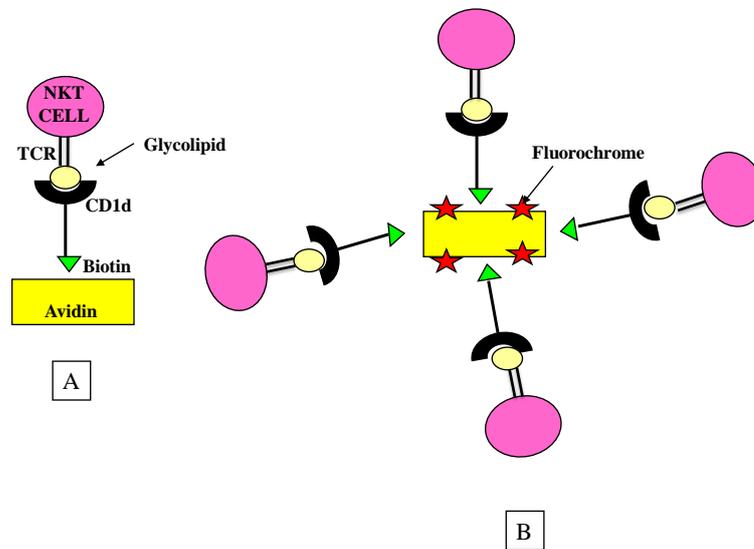
The absolute number of CD56-expressing T cells is significantly increased in the PB of patients presenting with AML compared with healthy volunteers (**Figure 3.5**)



**Figure 3.5: CD3+56+ Cells in AML**

Graph illustrating the significant increase in the absolute number of CD3+56+ cells in the PB of patients with AML compared with healthy controls. The p-value was obtained using a Mann Whitney unpaired, 2-tailed t test.

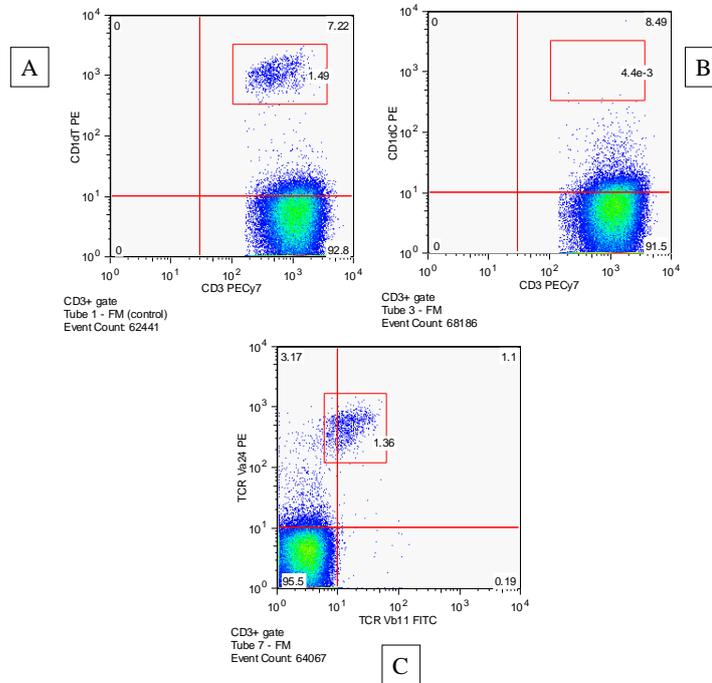
To identify if these cells were in fact true NKT cells, cells were stained with a CD1d tetramer loaded with a lipid analogue of  $\alpha$ GalCer (PBS57) and labelled with the fluorochrome phycoerythrin kindly provided by the NIH Tetramer Facility. Tetramers are a means of identifying T cells specific to an antigen of interest by flow cytometry. Their structure is illustrated in **Figure 3.6**.



**Figure 3.6: The Components of a Tetramer**

A CD1d molecule loaded with glycolipid is tagged with biotin and therefore binds to the protein avidin. NKT cells will recognise and bind to this complex (A). Avidin is a tetrameric protein and is therefore capable of binding four biotinylated CD1d molecules. It is labelled with a fluorochrome which allows the tetrameric complex to be detected by flow cytometry (B).

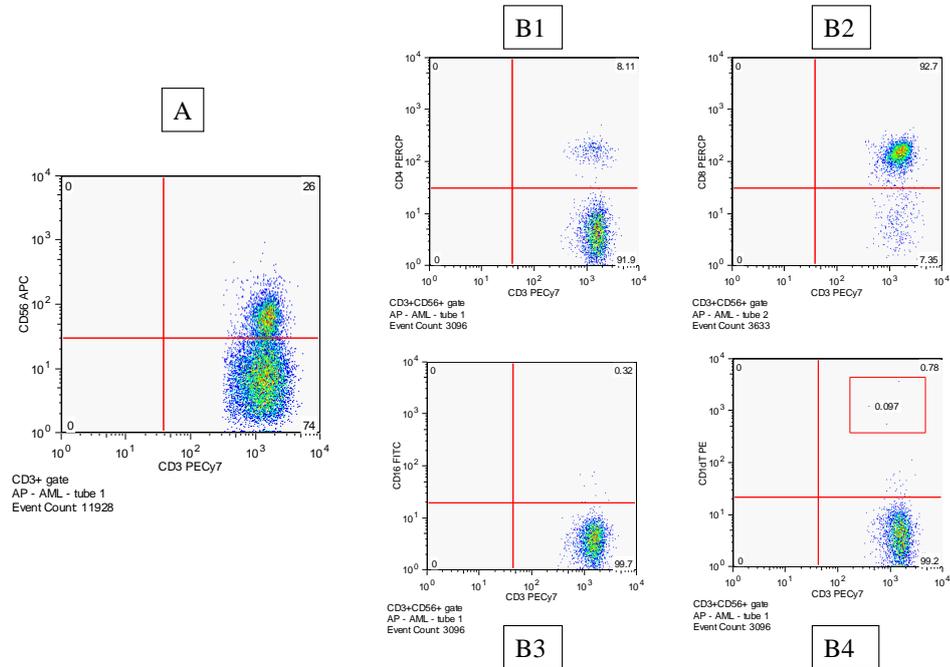
Non-specific background staining was excluded using an unloaded control tetramer. Results were confirmed by using antibodies directed towards V $\alpha$ 24 and V $\beta$ 11 (**Figure 3.7**).



**Figure 3.7: Scatter Plots of True NKT Cells**

Plot (A) demonstrates the appearance of true NKT cells labelled with CD3 and the glycolipid loaded tetramer (CD1dT). The minimal level of background staining seen with the control unloaded tetramer (CD1dC) is shown in (B). The size of the population is confirmed by staining for V $\alpha$ 24 and V $\beta$ 11 in a separate tube (C).

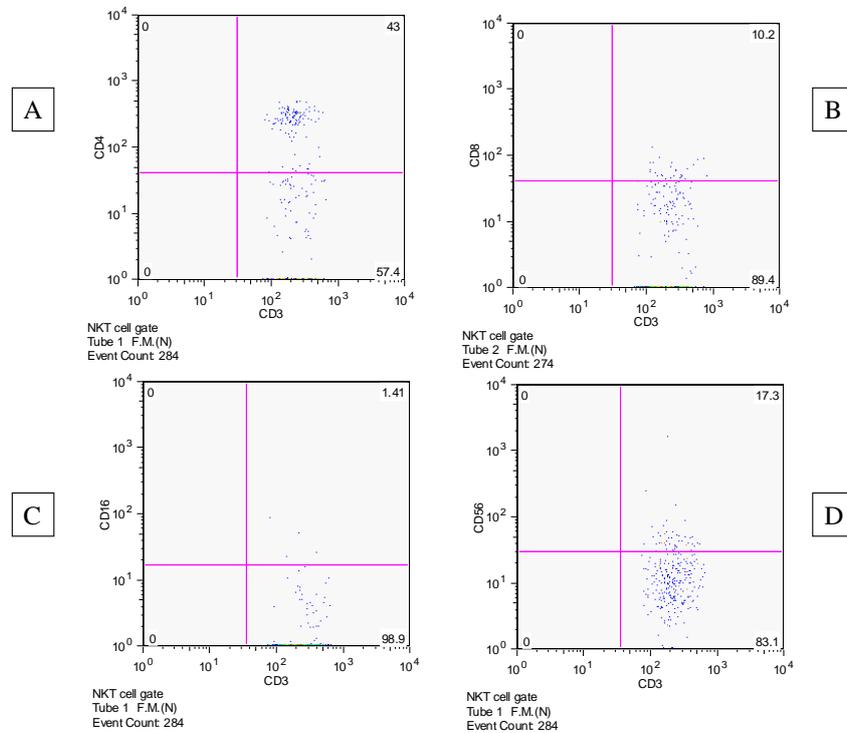
CD56-expressing CD3 cells were shown to be largely CD8 positive, CD16 negative and not true NKT cells on the basis of staining with a glycolipid loaded CD1d tetramer (Figure 3.8).



**Figure 3.8: Basic Phenotype of CD56-expressing CD3 Cells**

T cells expressing CD56 form a distinct population although expression of CD56 is at a low intensity (A). Only a small percentage of this population express CD4 (B1) with the majority being CD8 positive (B2). Minimal expression of CD16 was seen (B3) and this population failed to bind to the glycolipid-laden CD1d tetramer (CD1dT) (B4).

As true NKT cells are a rare cell population, due to the number of events acquired, the phenotype of these cells as identified by binding to the CD1d tetramer was only assessable in 12 healthy individuals and 22 AML patients (**Figure 3.9**).



**Figure 3.9: Surface Phenotype of True NKT Cells**

Plot (A) shows the expression of CD4; plot (B), the expression of CD8; plot (C), the expression of CD16 and plot (D), the expression of CD56.

A mean of 51% of NKT cells are CD4 positive in healthy individuals (SD 15%). A mean of 25% express CD8 (SD 17%) (the antibody used in this analysis is specific for alpha subunit). CD16 is only expressed on a mean of 5% of cells (SD 5%) and CD56 expression is also low at 14% (SD 14%).

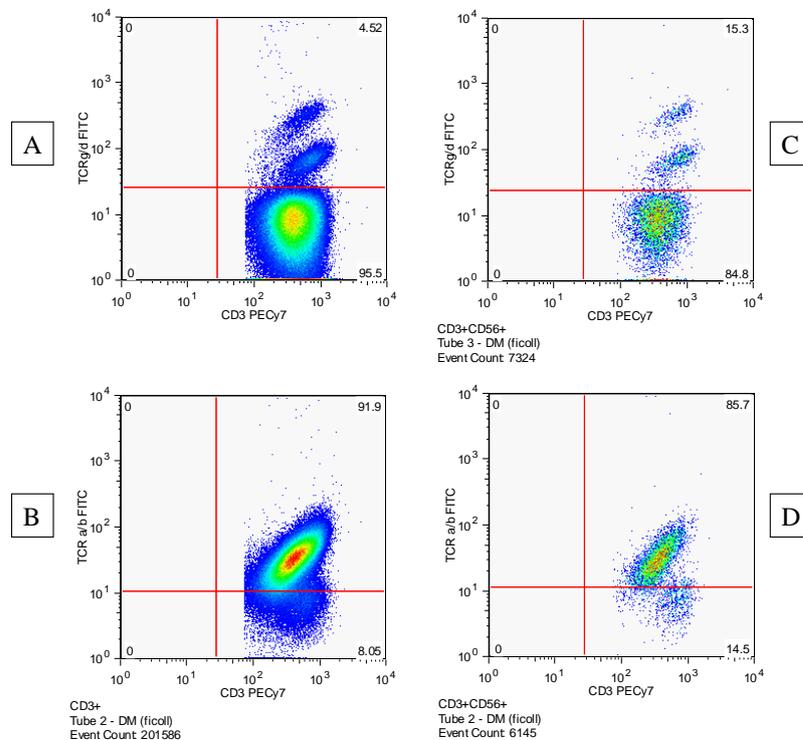
Using CD3 and the CD1d tetramer, the mean absolute number of NKT cells was  $0.95 \times 10^6/L$  with a standard deviation of  $2.3 \times 10^6/L$  in healthy individuals. Using antibodies to V $\alpha$ 24 and V $\beta$ 11, the mean absolute number was  $1 \times 10^6/L$  with a standard deviation of  $1.55 \times 10^6/L$ . There was no significant difference in the

absolute or relative number of NKT cells in patients with AML compared with healthy controls.

A significant difference was seen however in the relative numbers of NKT cells in the CD4 positive and CD4 negative subsets. In AML, NKT cells were more likely to be CD4 negative than in healthy individuals ( $p=0.01$ ).

### 3.4.3 CD3+56+ Cells are not $\gamma/\delta$ T Cells

Later experiments showed that CD3+56+ cells are not  $\gamma/\delta$  T cells (**Figure 3.10**).



**Figure 3.10: TCR Phenotype of CD3+56+ Cells**

Scatter plots of healthy T cells stained with CD3, CD56 and either TCR  $\gamma/\delta$  or TCR  $\alpha/\beta$ . Cells were gated on live mononuclear cells that were either CD3 positive (plots (A) and (B)) or double positive for CD3 and CD56 (plots (C) and (D)). (A) demonstrates that 4.5% of healthy T cells express the  $\gamma/\delta$  TCR whereas 92% express  $\alpha/\beta$  TCRs (B).  $\gamma/\delta$  T cells appear to be concentrated in the CD3+56+ population as shown by a percentage of 15% in (C) but the majority of this population still expresses the  $\alpha/\beta$  TCR (D) (N=2). The 2 populations of  $\gamma/\delta$  T cells observed are expected with the antibody used (BD Biosciences, cat. no. 559878 datasheet).

#### **3.4.4 Extended Phenotype of CD3+56+ Cells**

Extended phenotyping was performed on CD3+CD56+ cells on frozen PBMCs from five healthy volunteers and five AML patients to further characterise these cells. CD45RA and CD27 were used to distinguish effector, naïve and memory T cell subsets (Hamann, *et al* 1997, Hamann, *et al* 1999). The co-stimulatory T cell receptor CD28 and CD57, found on NK cells and a subset of T cells were also examined. Other cell surface markers examined were the IL-2 receptor  $\beta$  chain CD122, the NK receptors CD94, a KIR, and NKG2D, a C-type lectin type receptor. In view of the previous case report on a lymphoproliferation in an AML patient of CD3+56+ cells that expressed 2B4 (CD244) (Costello, *et al* 2002a), the expression of this high affinity receptor for CD48 was also investigated. The results are summarised in **Table 3.10**.

	CD3+56+ (Healthy) (n=5)	CD3+56+ (AML) (n=5)
<b>Effector:</b>		
CD45RA <sup>high</sup> /CD27-	12.7%	43.78% *
<b>Naïve:</b>		
CD45RA <sup>high</sup> /CD27+	23.61%	7.51% *
<b>Memory:</b>		
CD45RA <sup>low</sup> /CD27+	23.9%	11.29%
CD57+/CD28-	11.34%	35.36%
CD57+	33.92%	61.68% *
CD28+	84.6%	45.37% *
CD122+	67.85%	77.3%
NKG2D+	48.14%	65.05%
CD94+	15.3%	19.07%
CD244 (2B4)+	4.93%	14.27%

**Table 3.10: Extended Phenotype of CD3+56+ Cells**

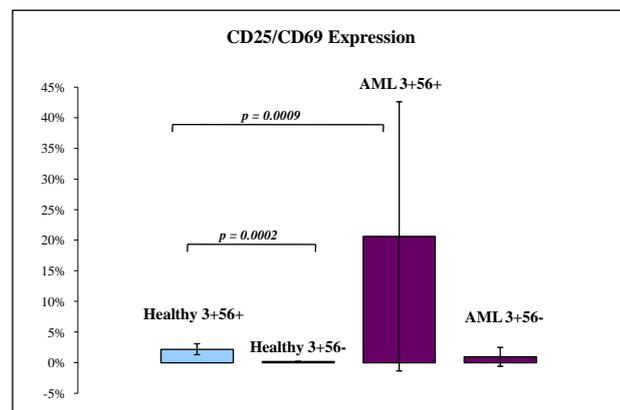
Phenotypic characteristics of CD3+56+ cells in healthy individuals compared with patients with AML. \* indicates a statistically significant difference in expression in AML compared to healthy (p<0.05).

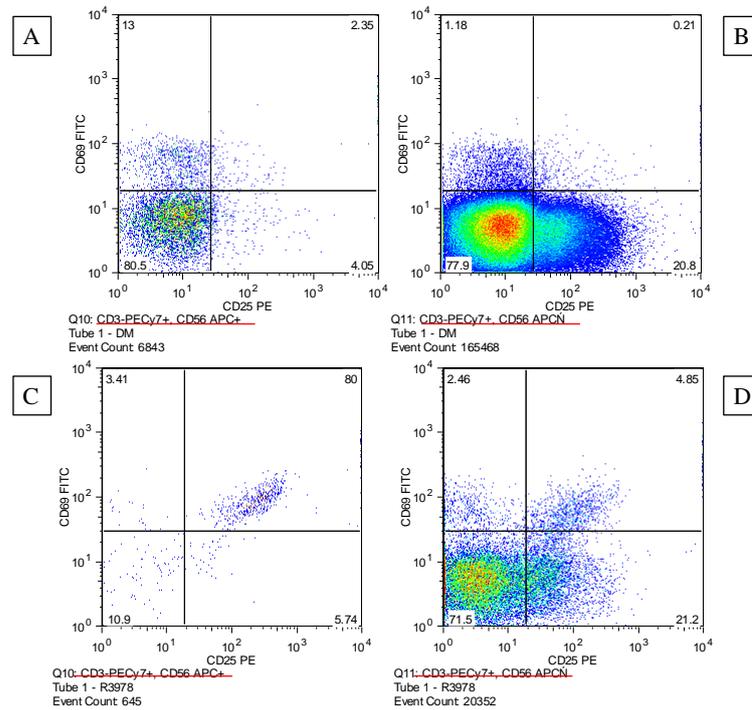
These results show that whereas in healthy individuals CD3+56+ cells have largely a naïve or memory phenotype, in AML this population is skewed towards an effector phenotype. This CD45RA<sup>high</sup>CD27- population has been associated

with a CD57+CD28- phenotype (Hamann, *et al* 1997) and these results demonstrate a significant difference in the expression of these surface markers in AML leading to a conclusion that CD3+56+ cells in AML patients have all the hallmarks of effector CTLs. CD3+56+ cells express the IL-2 receptor  $\beta$  at high level but there is no difference in its expression between the AML and healthy cell populations. Expression of NKG2D and CD94 is also seen but again no difference was observed between AML and healthy individuals. 2B4 was expressed at higher levels on CD3+56+ cells in AML patients but once again this did not meet statistical significance.

### 3.4.5 Expression of Activation Markers by CD3+56+ Cells

The percentage of CD3+56+ and CD3+56- cells that express the activation cell surface markers CD25 and CD69 was investigated by flow cytometry in frozen PBMCs from 11 healthy individuals and 21 AML patients. The results are shown in **Figure 3.11**.





**Figure 3.11: Activation Marker Expression on CD3+56+ Cells Compared with CD3+56- Cells**

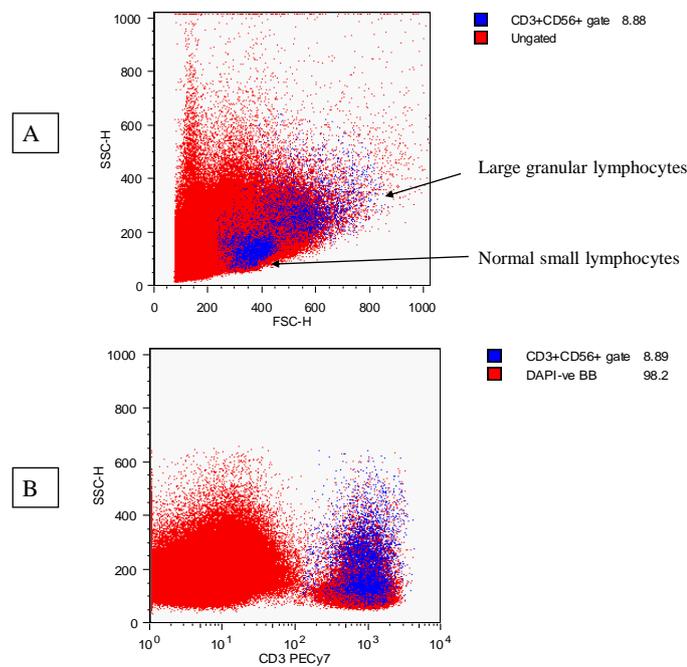
The bar graph shows the mean percentage of CD3+56+ and CD3+56- cells expressing CD25 and CD69 in patients with AML and healthy volunteers. The scatter plots below are gated on live mononuclear cells. Plot (A) shows the expression of CD25 and CD69 on CD3+CD56+ cells in a healthy volunteer. Plot (B) shows the same markers on CD3+CD56- cells in a healthy volunteer. Plot (C) shows expression of CD25 and CD69 on CD3+CD56+ cells in a patient with AML and plot (D) shows the same thing on CD3+CD56- cells in the same patient.

Significantly more CD3+56+ cells express the activation markers CD25 and CD69 than CD3+56- cells. Furthermore, CD3+56+ cells in AML show more expression of these markers than in healthy controls suggesting that these cells are in a higher state of activation in AML patients than in healthy individuals. If the subpopulation of large granular lymphocytes within the CD3+56+ subset is considered in the AML patients, the percentage of cells expressing both activation markers is even higher ( $p=0.004$  compared with total CD3+56+ cells).

### 3.4.6 CD3+56+ Cell Expression of Intracellular Cytotoxic Granules

The expression of perforin and granzyme B in CD3+56+ cells was investigated by intracellular staining and flow cytometry. Frozen PBMCs were used from five healthy individuals and 9 patients from the time of presentation with AML.

Forward and side scatter characteristics of CD3+56+ cells demonstrated these cells were made up of two separate populations with different scatter characteristics; one subset with the characteristics of normal small lymphocytes and one with the characteristics of large granular lymphocytes (**Figure 3.12**).



**Figure 3.12: Size and Granularity Characteristics of CD3+56+ Cells**

Scatter plot (A) shows CD3+56+ cells in blue backgated onto the FSC/SSC plot. Two populations of cells with different scatter characteristics are apparent. Plot (B) shows CD3 plotted against SSC with the CD3+56+ cells shown again blue.

Consistent with their granular morphology, a greater percentage of CD3+56+ cells express cytotoxic granules compared to CD3+56- cells in healthy individuals (**Table 3.11**) although this difference only reaches statistical significance when

considering perforin alone or the combined expression of perforin and granzyme B.

Healthy (n=5)	Mean % (SD) of cells expressing cytotoxic granules		
	Perforin+	GrB+	Perforin/GrB+
CD3+56+	64.6% (+/- 10.3%)	66.5% (+/- 25.9%)	51.9% (+/- 19.8%)
CD3+56-	28.5% (+/- 18.9%)	32.6% (+/- 27.9%)	21.4% (+/- 18.6%)
<i>p-value</i>	0.01	0.08	0.04

**Table 3.11: Expression of Cytotoxic Granules**

The expression of the cytotoxic granules perforin and granzyme B (GrB) was compared in CD3+56+ cells and CD3+56- cells in five healthy individuals. Figures shown are mean values with the standard deviation following in brackets.

There was no difference in the percentage of CD3+56+ cells expressing perforin and granzyme B in AML patients compared with healthy individuals. However, if the population of CD3+56+ cells with the highest side scatter (SSC<sup>High</sup>) was considered, that is, the large granular lymphocytes, differences did become apparent (Table 3.12).

Mean % (SD) of cells expressing perforin and granzyme B			
CD3+SSC <sup>High</sup> population		Total CD3+56+ population	
AML	Healthy	AML	Healthy
43.5% *	85.0%	53.3%	51.9%
(+/- 20.0%)	(+/- 8.8%)	(+/- 21.0%)	(+/- 19.8%)

**Table 3.12: Expression of Cytotoxic Granules in the CD3+SSC<sup>High</sup> Population**

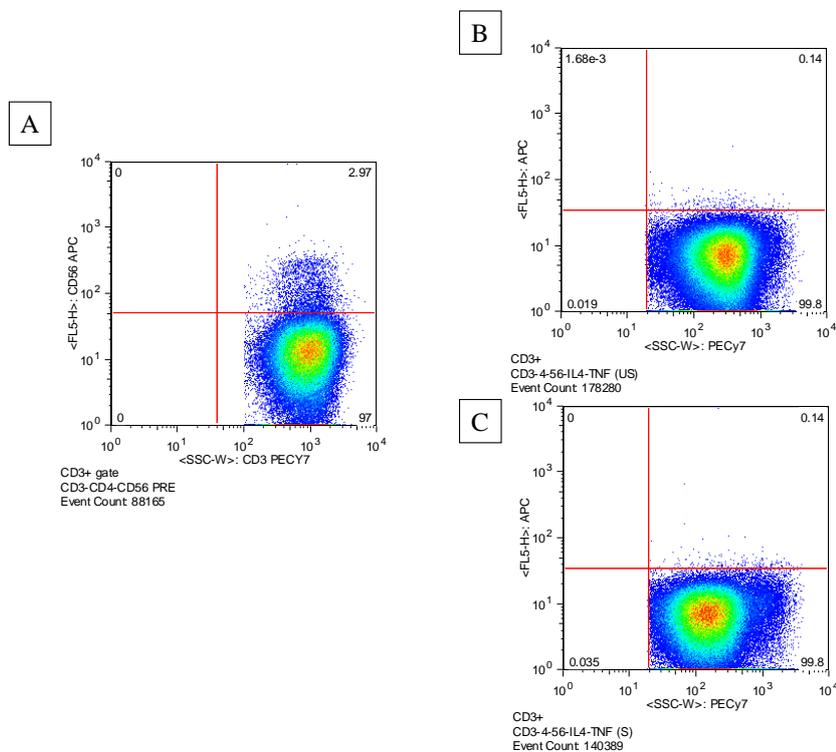
The expression of perforin and granzyme B in the CD3+SSC<sup>High</sup> and total CD3+56+ populations in AML patients was compared with healthy individuals. Figures shown are mean values with the standard deviation following in brackets. \* p-value 0.001 in a t-test comparing AML with healthy samples.

Therefore, although CD3+56+ cells in general express high levels of cytotoxic granules, the expression of these granules in the LGL population is lower in patients with AML than in healthy individuals.

### 3.4.7 Cytokine Production by CD3+56+ Cells

The production of IL-2, IFN $\gamma$ , IL-4 and TNF were examined by intracellular staining and flow cytometry with and without stimulation with PMA and ionomycin (for protocol see section 2.7.3) in frozen PBMCs from two healthy individuals and three AML patients.

Firstly it was observed that the expression of CD56 appeared to be altered by the protocol (**Figure 3.13**).

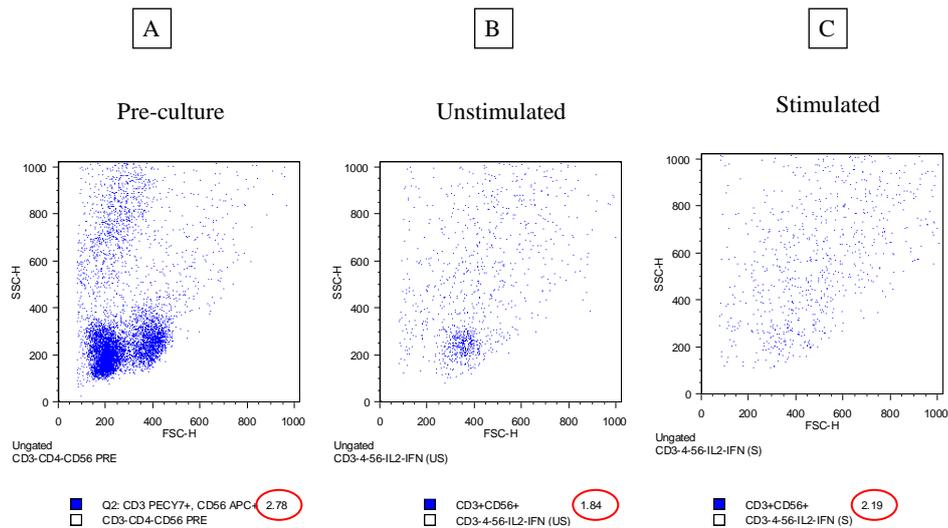


**Figure 3.13: Alteration in CD56 Expression During Cytokine Release Experiment**

The expression of CD56 on CD3+ T cells prior to five hours culture and without fixation (A), on fixed cells post culture but **without** stimulation with PMA and ionomycin (B), on fixed cells post culture **with** stimulation with PMA and ionomycin (C). PECy7: CD3, APC: CD56.

CD56 expression appeared to be lost on T cells during this experiment either due to the process of five hours culture at 37<sup>0</sup>C in 4% CO<sub>2</sub> or due to cellular fixation that was required for intracellular staining.

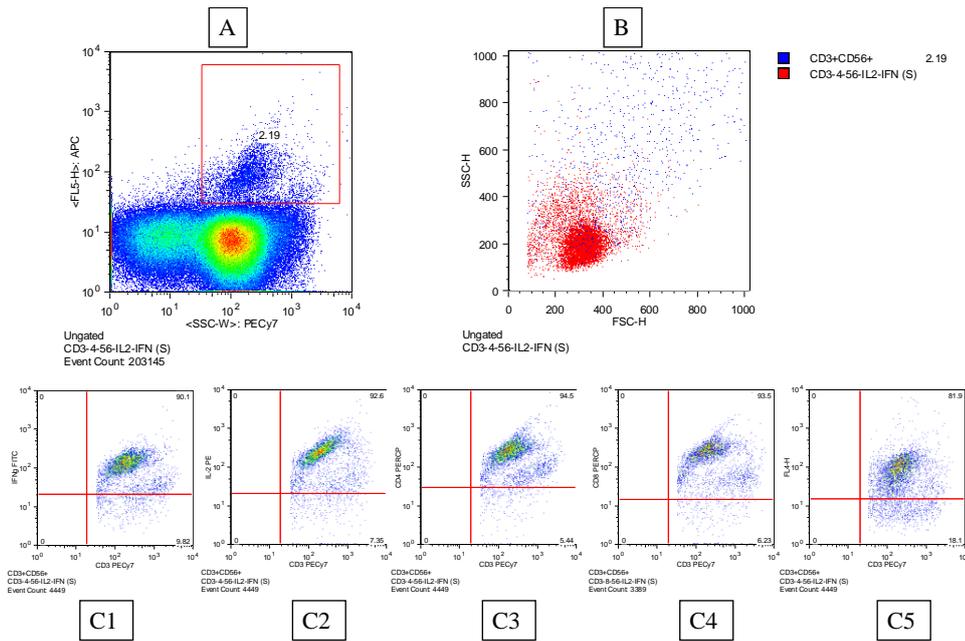
However, when the expression of CD56 was examined on CD3 cells without gating the mononuclear cell population first it was clear that the relative number of these CD3+56+ cells was not changing but instead a change was occurring in their scatter characteristics (**Figure 3.14**).



**Figure 3.14: Change in FSC/SSC Characteristics of CD3+56+ Cells During Cytokine Release Experiment**

The CD3+56+ population (blue) has been backgated onto a FSC/SSC plot. Prior to the five hour culture, this population shows the two subsets previously demonstrated (A). After five hours culture but without stimulation, the percentage of CD3+56+ cells remains similar but the cells show much broader scatter characteristics (B). With stimulation, this effect is even more marked such that the two original subsets can no longer be identified and all cells have greater forward and side scatter (C).

Therefore, culturing cells for five hours at 37<sup>0</sup>C in 4% CO<sub>2</sub> results in CD3+56+ cells becoming larger and more granular. This effect is magnified by stimulating cells with PMA and ionomycin. The unfortunate side effect of this is that these cells become autofluorescent which impacts on the assessment of their production of cytokines (**Figure 3.15**).

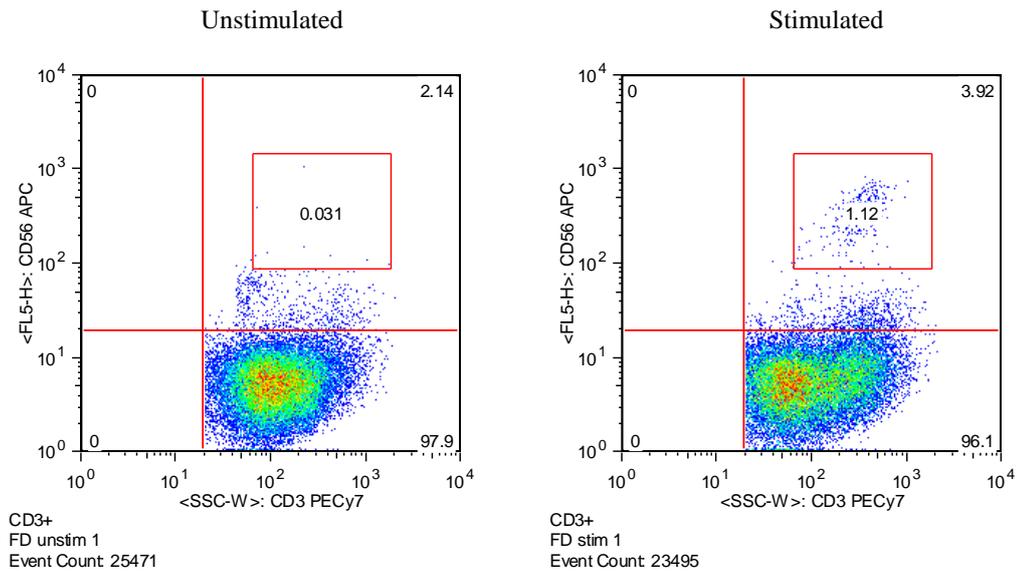


**Figure 3.15: Autofluorescence of CD3+56+ Cells After Culture.**

The CD3+56+ cells are clearly visible in (A) when no gating has been previously applied (PECy7: CD3, APC: CD56). Backgating this population onto the FSC/SSC plot of all cells confirms the broad scatter characteristics illustrated in figure 3.12 (B). These cells appear to strongly express IFN $\gamma$  (C1), IL-2 (C2), CD4 (C3) but also CD8 (C4). However, similar fluorescence is also seen in the FL4 channel (C5) in which no fluorochrome should be present.

Thus the change in scatter characteristics of CD3+56+ cells with culture and stimulation and consequent autofluorescence meant that accurate assessment of their production of cytokines by this method was not possible.

One noteworthy observation made was that on stimulation of PBMCs from AML patients with PMA and ionomycin, a marked increase in the number of CD3+56+ cells occurred suggesting that T cells from patients with AML start to express CD56 on activation (**Figure 3.16**). A similar observation was made on stimulation of healthy T cells but the effect was not as great as that seen in T cells from AML patients.



**Figure 3.16: Effect of Stimulation on CD56 Expression by T Cells**

Increased numbers of CD3 cells with high expression of CD56 were seen on stimulation of PBMCs from AML patients.

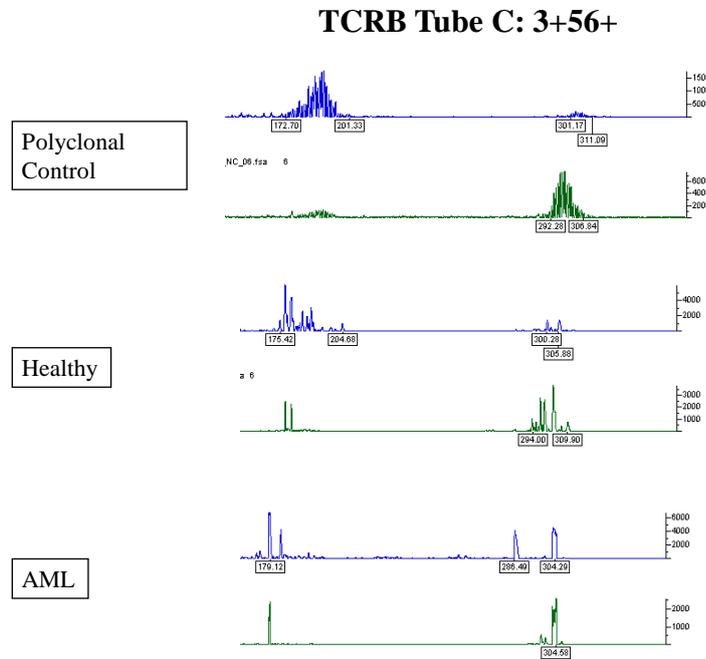
### 3.4.8 Assessment of Clonality of CD3+56+ Cells

The clonality of CD3+56+ cells was assessed in five of the previously studied AML patients chosen on the basis of available material (FAB types: 1 M1, 1 M2, 2 M4 and 1 M5a) and compared to that of two healthy individuals by examining TCR gene rearrangements of *TCRB*, *TCRG* and *TCRD*. The clonality of CD3+4+56- and CD3+8+56- populations was also investigated. Blasts were included in the analysis to ensure that any clonality observed was not due to contaminating blast cells which can also rearrange their TCR genes (Schmetzer, *et al* 2000, Yen, *et al* 1999).

All samples were tested with a Specimen Control Size ladder to ensure that no inhibitors of amplification were present and that there was DNA of sufficient

quality and quantity to generate a valid result. All samples passed this check. Polyclonal and clonal controls were included with each reaction.

**Figure 3.17** illustrates a typical trace seen for the CD3+56+ population.



**Figure 3.17: Typical GeneScanning Traces for CD3+56+ Cells**

Each sample has two traces as both 6FAM (blue) and HEX (green) were used to label the amplified product. The y-axis represents intensity and the x-axis represents size of product in base pairs (bp). The top trace demonstrates the typical picture seen for the polyclonal control for *TCRB* Tube C. A normal distribution type curve is seen around 170-210bp (6FAM product) and 285-325bp (HEX product). The middle trace represents healthy samples and a similar pattern to the polyclonal control is seen. However, the trace from the AML sample shows that the bell-shaped curve pattern has been lost and individual spikes are seen suggestive of an oligoclonal expansion.

Each trace was examined and subjectively graded as polyclonal or oligoclonal on the basis of adherence to the typical bell-shaped curve pattern. The results are illustrated in **Table 3.13**.

	<b>TCRB Tube A</b>	<b>TCRB Tube B</b>	<b>TCRB Tube C</b>	<b>TCRG Tube A</b>	<b>TCRG Tube B</b>	<b>TCRD</b>
	<i>Vβ+Jβ1/2</i>	<i>Vβ+Jβ2</i>	<i>Dβ+Jβ1/2</i>	<i>Vγ1-8, Vγ10 + Jγ1.3/2.3</i>	<i>Vγ9, Vγ11 +</i>	<i>Vδ+Dδ+Jδ</i>
<b>CD3+4+</b>						
AML1	oligoclonal	polyclonal	oligoclonal	polyclonal	polyclonal	polyclonal
AML2	polyclonal	polyclonal	oligoclonal	polyclonal	polyclonal	polyclonal
AML3	oligoclonal	polyclonal	polyclonal	oligoclonal	polyclonal	polyclonal
AML4	polyclonal	polyclonal	polyclonal	oligoclonal	oligoclonal	oligoclonal
AML5	N/A	N/A	polyclonal	oligoclonal	oligoclonal	oligoclonal
Healthy1	polyclonal	N/A	polyclonal	polyclonal	polyclonal	polyclonal
Healthy2	polyclonal	N/A	oligoclonal	N/A	oligoclonal	polyclonal
<b>CD3+8+</b>						
AML1	polyclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal
AML2	oligoclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal
AML3	oligoclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal
AML4	polyclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal
AML5	N/A	N/A	oligoclonal	oligoclonal	oligoclonal	oligoclonal
Healthy1	N/A	N/A	polyclonal	polyclonal	oligoclonal	polyclonal
Healthy2	polyclonal	N/A	polyclonal	oligoclonal	oligoclonal	polyclonal
<b>CD3+56+</b>						
AML1	oligoclonal	polyclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal
AML2	oligoclonal	polyclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal
AML3	oligoclonal	polyclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal
AML4	oligoclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal
AML5	N/A	N/A	oligoclonal	oligoclonal	oligoclonal	oligoclonal
Healthy1	polyclonal	N/A	polyclonal	oligoclonal	oligoclonal	polyclonal
Healthy2	polyclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal	polyclonal
<b>Blasts</b>						
AML1	polyclonal	polyclonal	oligoclonal	N/A	N/A	oligoclonal
AML2	monoclonal	polyclonal	oligoclonal	oligoclonal	oligoclonal	oligoclonal
AML3	monoclonal	polyclonal	oligoclonal	polyclonal	oligoclonal	oligoclonal
AML4	monoclonal	polyclonal	oligoclonal	polyclonal	oligoclonal	oligoclonal
AML5	N/A	N/A	oligoclonal	oligoclonal	oligoclonal	polyclonal

**Table 3.13: TCR Gene Clonality Results**

N/A indicates that no amplification occurred. Definition of polyclonal versus oligoclonal was subjective.

These results illustrate that oligoclonality is a common phenomenon amongst T cell populations in patients with AML. It is most frequently seen in the CD3+8+56- and CD3+56+ populations where there is a significant difference in the occurrence of oligoclonality in comparison with the CD3+4+56- population (**Table 3.14**).

AML Samples	p-value
3+4+ v 3+8+	$3.9 \times 10^{-5}$
3+4+ v 3+56+	$3.9 \times 10^{-5}$
3+8+ v 3+56+	1

**Table 3.14: Comparison of Clonality in Different T cell Populations in AML Samples**

Fishers' Exact Test (2-tailed). Figures in red represent a statistically significant difference in oligoclonality between different cell populations in AML.

Oligoclonality was also seen in the healthy T cell populations which meant that only rarely were significant differences seen in the occurrence of oligoclonality in AML compared with healthy samples (**Table 3.15**).

	TCRB Tube A	TCRB Tube B	TCRB Tube C	TCRG Tube A	TCRG Tube B	TCRD
CD3+4+56-	0.46	N/A	1	1	1	1
CD3+8+56-	1	N/A	0.047	0.285	1	0.047
CD3+56+	0.066	0.4	0.285	1	1	0.047

**Table 3.15: Comparison of Clonality of T Cell Populations Between AML and Healthy Samples**

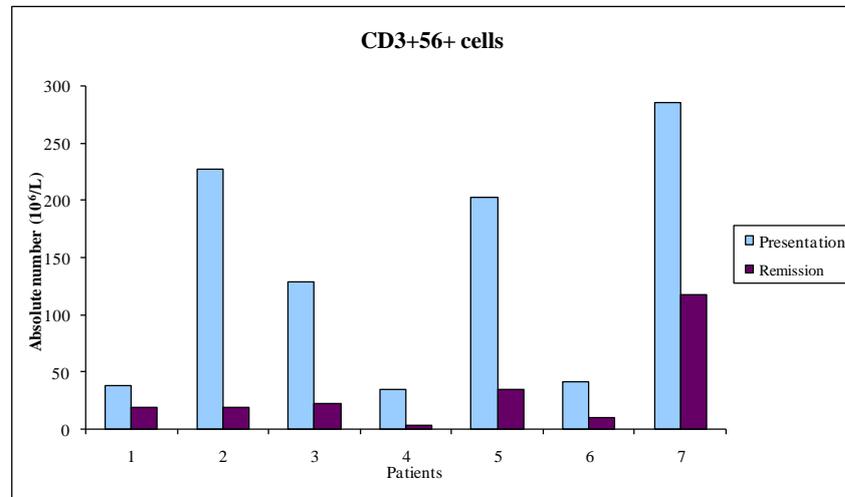
P-values obtained using Fishers' Exact Test (2-tailed) AML v Healthy. N/A indicates a result is not available. Figures in red represent a statistically significant difference in oligoclonality between AML and healthy cell populations.

Although generally considered to be polyclonal, the phenomenon of oligoclonality in healthy T cell populations has previously been described in CD8 cells where CDR3 length variation was noted in 72% of 56 individuals studied (Monteiro, *et*

*al* 1995). The same group found no evidence of clonal dominance in CD4 cells in 12 individuals examined consistent with their previous observations (Hingorani, *et al* 1993). However, other groups have reported skewing in the V $\beta$  repertoires in both CD4 and CD8 populations in healthy individuals when examined by flow cytometry (Davey, *et al* 1991, Grunewald, *et al* 1991). This probably represents the continuous nature of the adaptive immune response but means that T cell oligoclonality in the setting of disease must be interpreted with care. CD3+56+ cells in healthy individuals have previously been described as having oligoclonal expansions within the V $\beta$  repertoire by flow cytometry (Ohkawa, *et al* 2001). It was of interest to find that the blast populations often showed oligoclonal products. If blasts did rearrange their TCR genes it might be expected that the population would then show a clonal pattern. However, this was only seen in three samples. A more likely explanation for the oligoclonality seen in these samples is amplification of genes from contaminating T cells.

#### ***3.4.9 Comparison of Absolute Numbers of CD3+56+ Cells at Presentation and Remission of AML***

It was possible to obtain remission PB from 7 of the original AML patients used in this analysis. Using the whole blood lysis method for flow cytometry, absolute numbers of CD3+56+ cells were quantified (**Figure 3.18**).



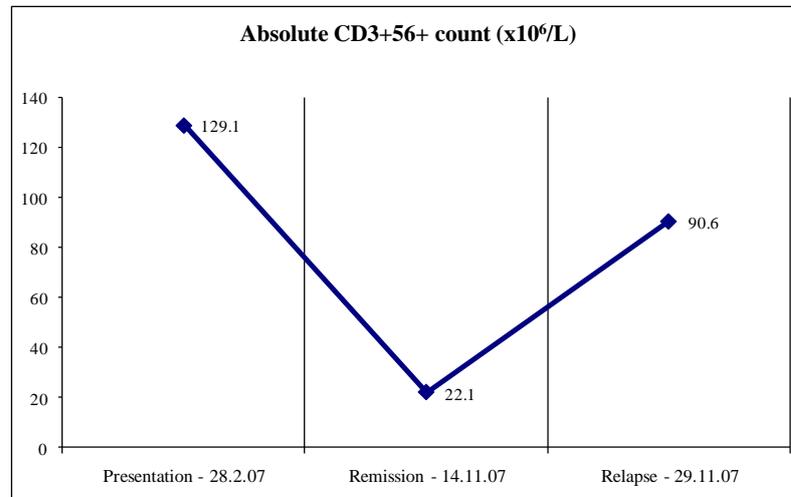
**Figure 3.18: Absolute Numbers of CD3+56+ Cells in Remission**

Absolute numbers of CD3+56+ cells were measured in the PB of 7 patients with AML at the time of presentation and at remission.

This shows that once patients with AML go into remission, the CD3+56+ population returns to the same level as in healthy individuals (mean of  $50.7 \times 10^6/L$  compared with a mean of  $27.6 \times 10^6/L$  in healthy controls).

#### ***3.4.10 Change in Absolute Numbers of CD3+56+ Cells in a Single Patient***

If CD3+56+ cells represented a failing mechanism of immunosurveillance, it might be expected that absolute numbers of these cells would increase prior to clinical evidence of disease. A peripheral blood sample was obtained from an AML patient whose presentation PBMCs had been examined when they were in a clinical remission with a normal FBC. One week later the patient returned to clinic in relapse. Another PB sample was obtained at this time. Absolute numbers of CD3+56+ cells were therefore available from presentation, one week prior to clinical relapse and at the time of clinical relapse (**Figure 3.19**).



**Figure 3.19: Absolute Number of CD3+56+ Cells at Presentation, Remission and Relapse**

Line graph of the absolute number of CD3+56+ cells in a single patient at the time of presentation, one week prior to relapse (when in remission) and at relapse.

Therefore, in this single example, CD3+56+ cells were within the normal range one week prior to relapse at a time when it was likely that tumour cells were escaping immune surveillance mechanisms and rapidly proliferating. The rise in the number of CD3+56+ cells is mirroring the rise in the blast count rather than preceding it as might have been predicted if these cells had a crucial role in control of tumour growth. These results might suggest that the changes in T cells occur secondarily to emergence of AML and the absence of such cells prior to relapse may explain why immunosurveillance failed.

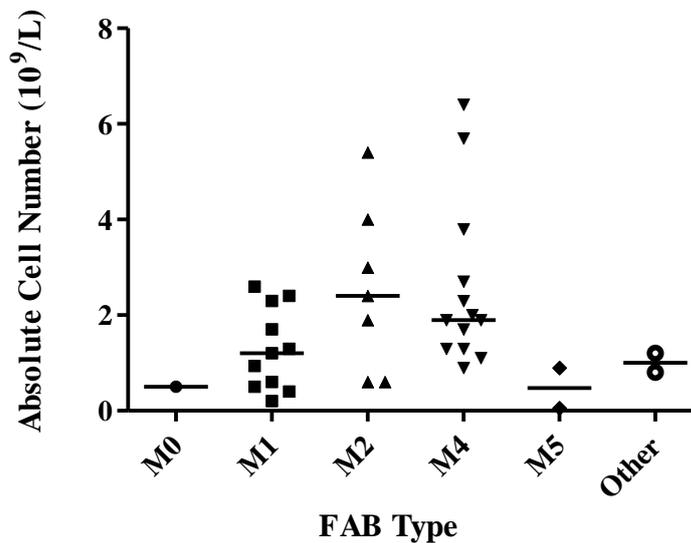
#### ***3.4.11 Correlation of Absolute Total CD3 and CD3+56+ Cell Numbers with Patient Characteristics***

Clinical notes were examined for 7 patients who had absolute CD3+56+ counts ranging from 7.6 x 10<sup>6</sup>/L to 1469.9 x 10<sup>6</sup>/L. No correlation could be found between the absolute CD3+56+ cell count and (i) whether the patient was

clinically ill on presentation, (ii) if the patient was on antibiotics, (iii) if the patient had a documented infection, (iv) albumin level and (v) significant past medical history.

Correlations were sought between absolute T cell number and FAB type, prognostic group, outcome of first therapy and relapse. There was a trend towards a higher T cell count in patients with FAB types M2 and M4 ( $p = 0.05$ ), an area which may be worth investigating further with larger numbers of patient samples (Figure 3.20).

### FAB Type $\nu$ Absolute T Cell Number

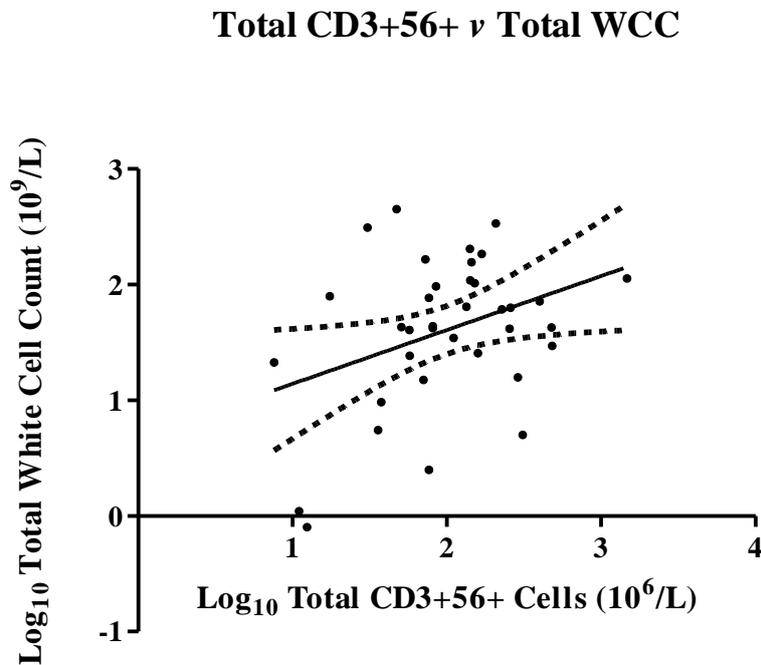


Kruskal-Wallis test	
P value	0.0503
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Do the medians vary signif. ( $P < 0.05$ )	No
Number of groups	6
Kruskal-Wallis statistic	11.05

**Figure 3.20: Correlation between FAB Type and Absolute T Cell Number.**

Lines represent median values.

There was no correlation between the absolute CD3+56+ count at presentation and the recorded total white cell count (**Figure 3.21**). The same was true for the absolute total T cell count compared with total white cell count (data not shown).

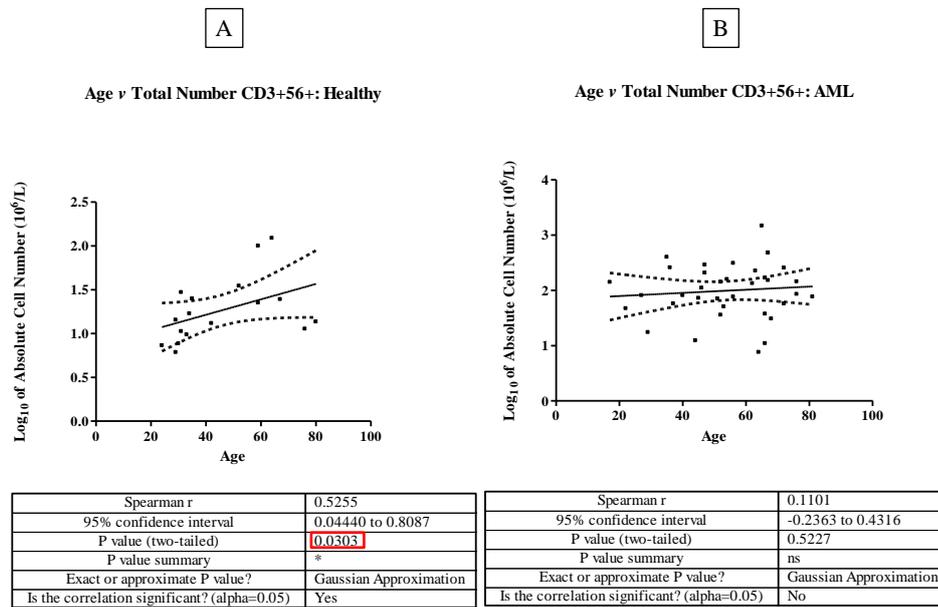


Number of XY Pairs	36
Spearman r	0.2186
95% confidence interval	-0.1285 to 0.5179
P value (two-tailed)	0.2003
P value summary	ns
Exact or approximate P value?	Gaussian Approximation
Is the correlation significant? (alpha=0.05)	No

**Figure 3.21: Correlation between Absolute CD3+56+ Cell Count in AML Patients with Presentation Total White Cell Count**

Lines represent a linear regression model and 95% confidence intervals.

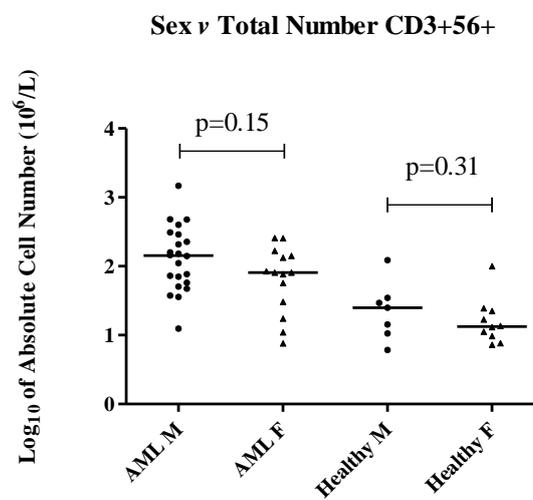
Although in the healthy controls the expected correlation between age and absolute number of CD3+56+ cells was seen ( $p = 0.03$ ) (Peralbo, *et al* 2007), this correlation was not seen in the AML patients (**Figure 3.22**).



**Figure 3.22: Correlation between Age and Total Number of CD3+56+ Cells**

Graph (A) demonstrates the significant correlation between age and CD3+56+ absolute cell number in healthy individuals. Graph (B) shows no significant correlation between age and absolute CD3+56+ cell count in patients presenting with AML. The lines on the graphs represent linear regression models with 95% confidence intervals.

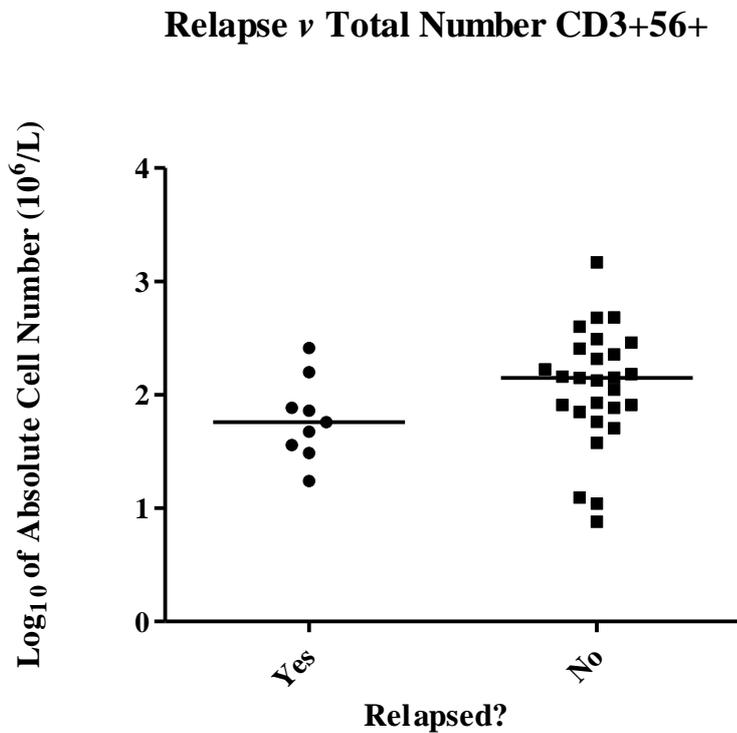
There was no correlation between sex and absolute CD3+56+ count in either healthy or AML patients (**Figure 3.23**).



**Figure 3.23: Correlation between Sex and Total Number of CD3+56+ Cells**

P-values were obtained using a 2-tailed Mann-Whitney test. M: male, F: female. Lines represent the median values.

No correlation was found between the absolute number of CD3+56+ cells and FAB type, prognostic group or outcome of first-line therapy. However, there was a trend towards a higher absolute number of CD3+56+ cells in the PB at the time of presentation with AML and a lower risk of relapse ( $p = 0.06$ ) (**Figure 3.24**).



Mann Whitney test	
P value	0.0650
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? ( $P < 0.05$ )	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	115.5 , 550.5
Mann-Whitney U	70.50

**Figure 3.24: Correlation between Risk of Relapse and Absolute Number of CD3+56+ Cells**

Line represents median values.

### 3.5 Discussion

Contrary to expectation, these results show that although the number of T cells relative to total live mononuclear cells is reduced in patients presenting with AML, the absolute number of T cells is higher than in healthy individuals. Both CD4 and CD8 subsets are increased but the greater rise occurs in the CD8 subset resulting in a significant reduction in the CD4:CD8 ratio in comparison to healthy volunteers. This does not appear to be the result of a general proliferative reaction of the bone marrow. These results differ from those published by Vidriales and Panoskaltsis (Panoskaltsis, *et al* 2003, Vidriales, *et al* 1993). Certainly, this study made use of different methods for the AML and healthy samples to enable calculation of absolute cell numbers. However, this approach was validated by a pilot experiment which showed no significant differences in relative cell number using the different approaches. Furthermore, the AML and healthy samples were not fully age-matched. Nonetheless, analysis showed no correlation between absolute cell numbers and age in the AML samples. Another issue is that leucopheresis specimens were the most frequently used form of AML sample. This process concentrates all white cells. Therefore the relative numbers of different subsets should remain the same. As the absolute number of cells was calculated using the recorded relative cell number in relation to the total white cell count on the day it should be accurate. Furthermore, when the whole blood lysis method was used on relapsed AML patients (for example figure 3.19), the elevated number of total CD3+56+ cells was confirmed.

CD56-expressing T cells were confirmed to be significantly increased in the peripheral blood of patients with AML compared with healthy volunteers. Although most AML samples were obtained by leucopheresis, CD56 expression was not thought to be directly altered as a result of this method of cell separation. It was shown that these cells are neither true NKT cells nor  $\gamma/\delta$  T cells and are largely CD8 positive and CD16 negative. However, they do express the NK receptors NKG2D and CD94. Extended phenotyping demonstrated that these cells more frequently express markers representative of effector CTLs (CD45RA<sup>High</sup> CD27-CD57+CD28-) in patients with AML compared with healthy individuals. In addition, the activation markers CD25 and CD69 are more frequently expressed

on these cells in patients with AML than in healthy controls. As such, CD3+56+ cells appear to be poised to kill. Analysis of their scatter characteristics illustrates that a large proportion of these cells are large granular lymphocytes. As a consequence, a significantly larger percentage of CD3+56+ cells express the cytotoxic granules perforin and granzyme B compared with CD3+56- cells. In AML compared with healthy CD3+56+ cells, the only difference observed is when the LGL population (CD3+SSC<sup>High</sup>) is considered in isolation. A significantly lower percentage of these cells express cytotoxic granules in AML compared with healthy controls. Thus although CD3+56+ cells in AML are more likely to be effector CTLs with high expression of activation markers they have a reduced cytotoxic potential. Unfortunately, the analysis of cytokine secretion by CD3+56+ cells was inhibited by development of autofluorescence by this cell population on culturing and stimulation. Assessment of TCR clonality demonstrated oligoclonal populations of both CD3+56+ cells and CD8+56- cells in AML patients suggestive of outgrowth of T cell populations responsive to particular antigens which or may not be tumour-derived. CD3+56+ cells return to normal levels when a patient goes in to remission but a rise in the level of this population is not observed prior to clinical relapse. Absolute numbers of CD3+56+ cells at presentation do not correlate with patients' FAB type, prognostic group or response to first-line therapy. This is perhaps not surprising; at the time a patient presents with AML the immunosurveillance process has failed. From that time onwards the chance of that individual going into CR depends on the susceptibility of the tumour cells to chemotherapy, an intrinsic property of the tumour cells themselves. Although there is now some evidence to suggest that traditional chemotherapeutic agents can induce an enhanced host immune response (Vereecque, *et al* 2004b). However, of marked interest is the trend towards higher numbers of CD3+56+ cells at presentation being associated with lower relapse risk. This observation requires confirmation with larger patient numbers. However, as illustrated by allogeneic transplantation, once bulk tumour is cleared by the direct toxicity of chemotherapy, minimal residual disease can be controlled by an operational immune system. Therefore, this data raises the possibility that these cells truly represent an immunosurveillance function of the immune system. Nonetheless, absolute cell number is a crude way of assessing

immunity. Functional assessment of the cytotoxic capability of CD3+56+ cells would be more relevant. This analysis was not performed here because of the difficulties in isolating sufficient numbers of pure CD3+56+ cells from presentation AML samples.

True NKT cells do not differ in terms of absolute numbers between AML and healthy individuals however the percentage of CD4 (DN) NKT cells is significantly higher in AML compared with normal. These cells represent the 'anti-cancer' T<sub>H</sub>1 subset and as such may have a role of their own in tumour control. It was of interest to note that NKT cells in humans do not in fact express CD56 at high levels. This observation is in keeping with another report suggesting that CD56 is generally not expressed (Metelitsa, *et al* 2003) but contrary to that made by several publications that have described V $\alpha$ 24V $\beta$ 11 cells in humans as expressing CD56 (Lee, *et al* 2002, Prussin and Foster 1997). The absolute number of NKT cells does not correlate with any patient characteristic.

Why are T cells, and more specifically CD3+56+ cells, increased in AML? This could represent failing immunosurveillance with the immune system desperately trying to control the uncontrollable. Most tumours escape immune recognition in ways such that T cells no longer see them and become unreactive. In AML, these immune cells appear to be proliferating, are activated, display effector phenotypes and have oligoclonal TCR gene rearrangements suggesting direction towards a particular antigen. Could immune recognition be occurring in this disease but effective cytotoxicity inhibited?

In order to find out more about T cells in AML it was now necessary to separate those T cells from the large bulk of myeloblasts. The following chapter describes how this was eventually achieved.

## Chapter 4

### Isolating T cells from AML presentation specimens

#### 4.1 Introduction

Work published on T cells in AML to date involves flow cytometric studies on whole PBMC specimens or functional work looking at the impact of AML blasts on normal or remission T cells (see section 1.4). In order to perform gene expression microarrays and functional studies on T cells from AML samples it was necessary to find a suitable method of obtaining highly pure and ideally untouched CD4 and CD8 T cells. This minimizes the theoretical chance of triggering signal transduction pathways within the cell by the binding of surface molecules and so potentially altering the gene expression pattern. Methods considered were Fluorescence Activated Cell Sorting (FACS) and immunomagnetic cell separation.

To separate the T cell populations, FACS was the obvious first choice. This method allows isolation at high purity of even very small cell populations. However, it requires sophisticated equipment and trained technical support. This was an available but highly stretched resource with a three week lead-time required for bookings. In general, the required cell population is labelled directly with an antibody. In order to obtain untouched cells, a cocktail of antibodies would need to have been used directed towards all other cells apart from the T cells. However, more importantly, even though the absolute number of T cells in the PB of patients with AML is increased, the number of T cells relative to the total white cell count is much reduced. Therefore, many more other cells have to be removed to get an equivalent number of T cells.  $5 \times 10^6$  cells CD4 or CD8 T cells were required to get sufficient quantity of RNA for gene expression profiling to avoid the necessity of template amplification. The amount of time that this would have entailed on a cell sorter was prohibitive. FACS was therefore excluded as a potential method at this stage.

Instead, immunomagnetic cell separation was explored. Two different systems were used; MACS® technology as developed by Miltenyi Biotec and StemSep™ cell separation method as marketed by StemCell Technologies. MACS® technology makes use of microbeads; superparamagnetic particles of approximately 50 nanometers in diameter to which antibodies are directly coupled. Cells are magnetically labelled by binding these microbeads. For untouched T cell isolation, a cocktail of biotin-conjugated monoclonal antibodies is incubated with the cells as a primary labelling reagent. Anti-biotin microbeads are then added as a secondary labelling reagent. Cell separation is achieved by passing the labelled cells through a MACS® column placed in a magnet (MACS Separator). The labelled cells are retained within the column and can then be eluted once the column is removed from the separator (Miltenyi, *et al* 1990). StemSep™ uses a slightly different labelling approach. Colloidal magnetic dextran iron particles are selectively bound to target cells by means of a bispecific tetrameric antibody complex that recognises both the dextran and the target cell surface antigen (Lansdorp, *et al* 1986). Cells are incubated first with an antibody cocktail and secondly with the magnetic colloid that magnetically labels the cells coated with antibody. Once again, labelled cells can be separated by passing the cell suspension over a column placed in a magnetic field. In a slight modification, StemCell Technologies supply a peristaltic pump that allows a controlled flow rate through the column rather than using gravity alone.

## **4.2 Aims**

The aim of this study was to find a means of successfully separating highly pure ideally untouched CD4 and CD8 T cells from presentation AML specimens for the purposes of RNA extraction for gene expression microarrays and for functional studies.

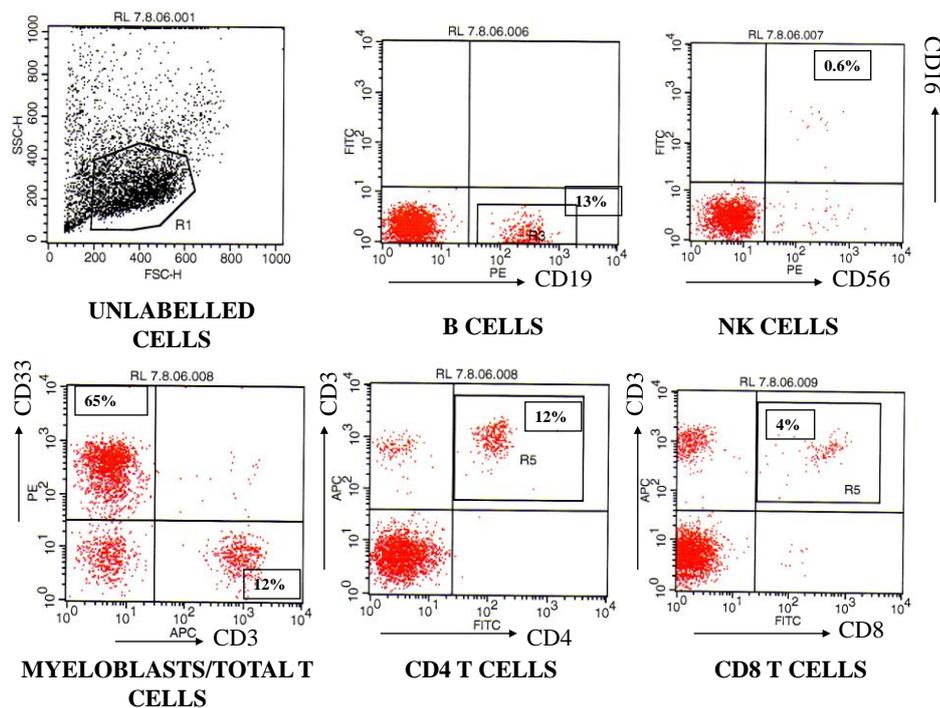
## **4.3 Materials and Methods**

Much of this initial optimisation work was made possible by the collection of a large leucopheresis sample from a newly diagnosed AML patient.

The methods used are described in detail in section 2.6.

#### 4.4 Results

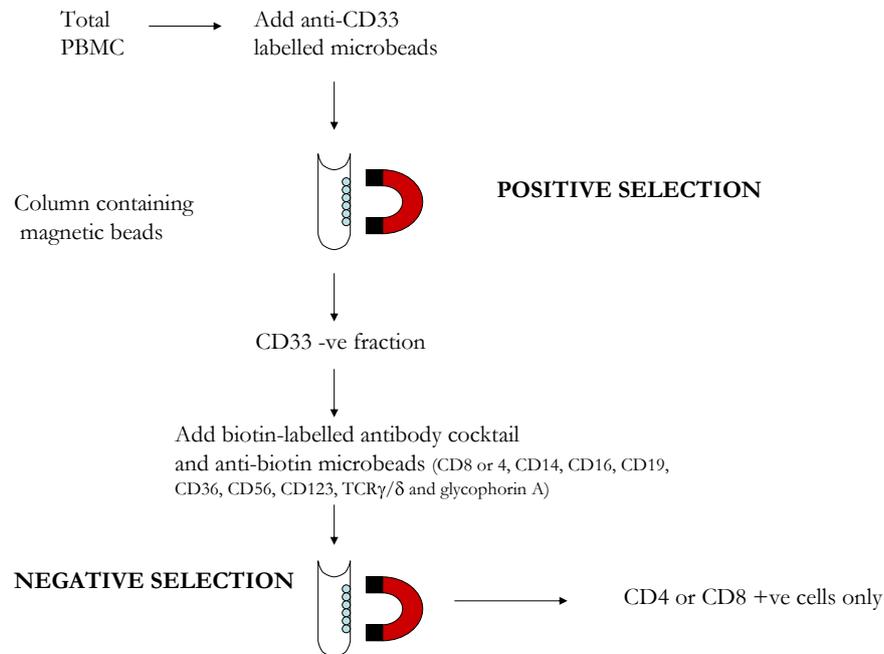
PBMCs from a patient with AML are made up of a large population of CD33 positive myeloblasts and markedly smaller numbers of B cells (CD19+), monocytes, CD4 (CD3+/CD4+) and CD8 (CD3+/CD8+) T cells and NK cells (CD16+/CD56+) as demonstrated by the flow cytometric analysis in **Figure 4.1**.



**Figure 4.1: Flow Cytometric Analysis of PBMCs from a Diagnostic AML PB Specimen**

The method previously used by our group for isolation of CD4 and CD8 T cells in CLL was immunomagnetic selection (Gorgun, *et al* 2005). A negative selection approach was employed: a cocktail of monoclonal antibodies was used to bind to all cells in the sample apart from the cell of interest which is therefore isolated untouched. CD4+ and CD8+ T Cell Isolation Kits II from Miltenyi Biotec were used in this part of the study.

The isolation kits used were designed for use with healthy peripheral blood. Separating T cells from leukaemia samples requires further consideration as the bulk of cells present do not express markers for which the antibodies within the cocktail are specific. In CLL the issue of removal of CLL B cells was addressed by adding x2 quantities of CD19 Microbeads to the CD4 or CD8 T cell negative isolation cocktail. Similarly, for AML samples, an extra step had to be included in order to remove circulating tumour cells. CD33 Microbeads were chosen as this antigen is highly expressed on myeloblasts. As myeloblasts often make up to 99% of total circulating cells it was felt a two-step procedure was necessary to ensure adequate myeloblast depletion (**Figure 4.2**).



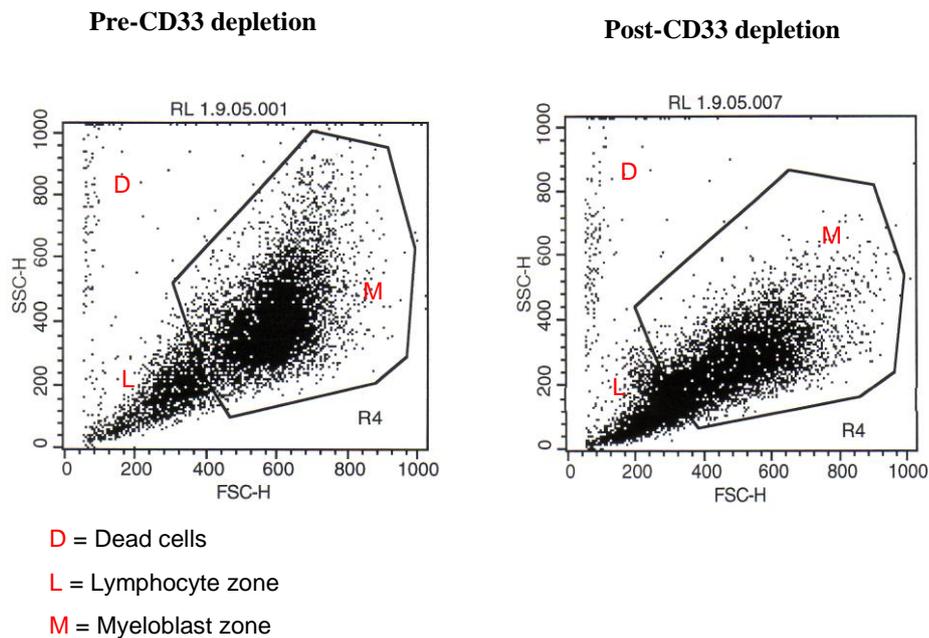
**Figure 4.2: Experimental Design for Isolation of Unlabelled CD4 or CD8 T Cells**

Supervised analysis of gene expression profiles of isolated CD4 or CD8 T cells from CLL patients and healthy donors demonstrated no significant difference in gene expression on the basis of a cell purity of between 70% and 85% or more than 85% (Gorgun, *et al* 2005). A cut-off of 70% was therefore set as an

appropriate purity to obtain for isolated CD4 and CD8 T cells in these experiments.

Using the recommended quantities of antibody for each step the quality of T cell purification was poor. As cell viability on thawing was between 50 and 75% and dead cells can bind non-specifically to the beads within the columns and inhibit adequate cell separation, a dead cell removal step was added. After dead cell separation by density gradient centrifugation, cell viability improved to between 75-100%. However, the addition of this step did not improve T cell purity.

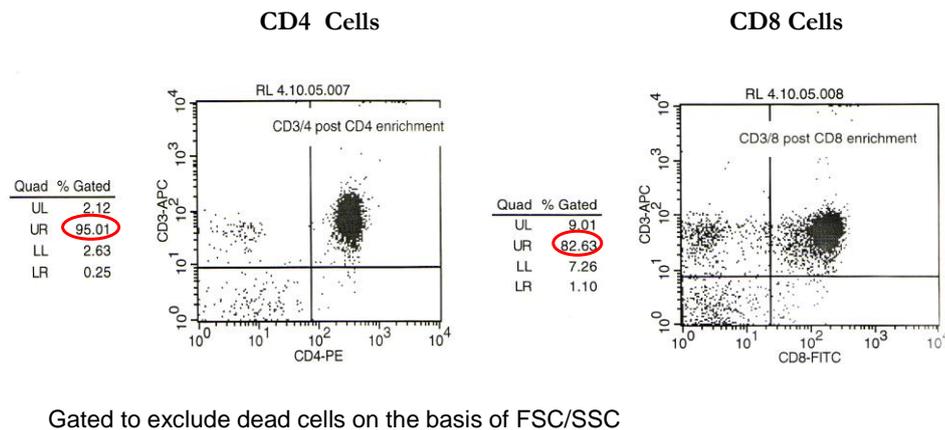
Flow cytometric analysis illustrated that the depletion of CD33+ve myeloblasts using the protocol outlined in **Figure 4.2** was ineffective (**Figure 4.3**).



**Figure 4.3: Depletion of Myeloblasts by CD33 Positive Selection**

Therefore to improve the quality of CD33 depletion the quantity of CD33 Microbeads was firstly doubled and then the CD33 labelled sample was passed through two columns prior to the negative selection step. Neither of these

modifications improved outcome. At this point it was felt to be important to ensure that the cell separation method worked well in my hands using the biological material it was designed for, that is, healthy blood. This worked well with good isolation achieved (**Figure 4.4**).



**Figure 4.4: Negative Selection of CD4 and CD8 T Cells from Healthy Peripheral Blood**

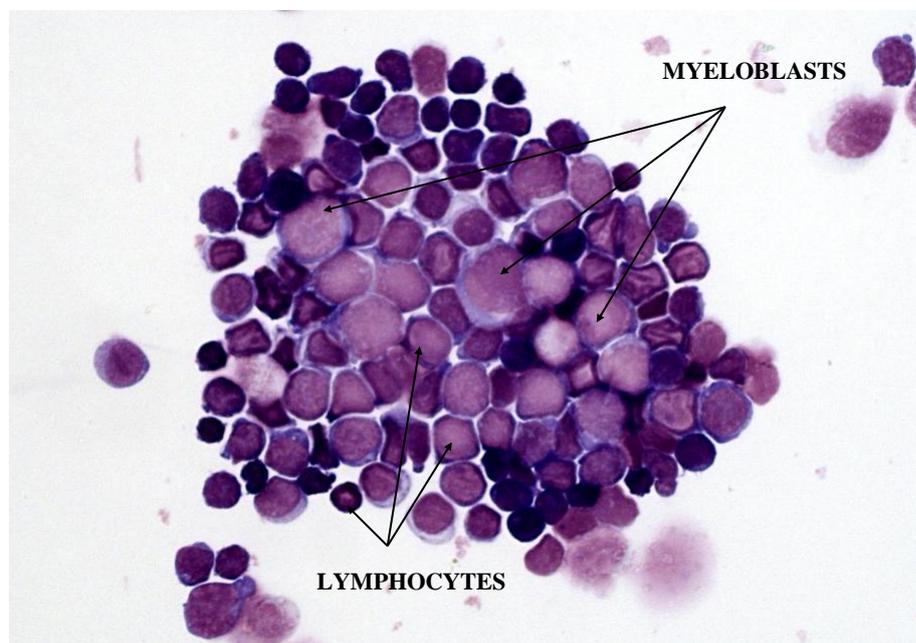
Next it was tested whether the problem was the result of using frozen rather than fresh samples. When a fresh AML sample was used with the x1 quantity of CD33 Microbeads, a relatively good isolation was achieved; 69% for CD4 and 51% for CD8. However, when repeated using a different patient sample, minimal CD4 or CD8 T cell isolation was achieved due to large numbers of residual myeloblasts. These experiments are summarized in **Table 4.1**.

Sample	PBMC Count	PBMC Viability	CD4		CD8		Modification
			Cell count	Purity	Cell count	Purity	
Frozen AML PBMCs	$8.6 \times 10^7$	50%	$1.5 \times 10^6$	<b>15%</b>	$2.25 \times 10^6$	<b>36%</b>	Protocol unchanged
Frozen AML PBMCs	$1 \times 10^8$	81%	$4.5 \times 10^6$	<b>5.5%</b>	$2 \times 10^6$	<b>3%</b>	Dead cell separation post thawing
Frozen AML PBMCs	$7.8 \times 10^7$	64%	$1 \times 10^6$	<b>10%</b>	$3.5 \times 10^5$	<b>6.7%</b>	2 x CD33 Microbeads
Frozen AML PBMCs	$5 \times 10^7$	76%	$1 \times 10^5$	<b>9%</b>	$2.5 \times 10^5$	<b>11%</b>	2 x CD33 Microbeads + 2 x LD columns
Fresh healthy PBMCs	$5.5 \times 10^7$	100%	$3.2 \times 10^6$	<b>95%</b>	$1.7 \times 10^6$	<b>82%</b>	Protocol unchanged
Fresh AML PBMCs	$4 \times 10^8$	92%	$2 \times 10^6$	<b>69%</b>	$1.6 \times 10^6$	<b>50%</b>	1 x CD33 Microbeads added to negative selection cocktail

Table 4.1: T cell Isolation Experiments

An experiment was then performed to discover the identity of the contaminating cells. An AML mix of antibodies was used – CD117, CD13, CD33 and CD123 all labelled with PE along with CD3. This confirmed that the final sample contains ‘AML mix’ positive blasts and T cells only.

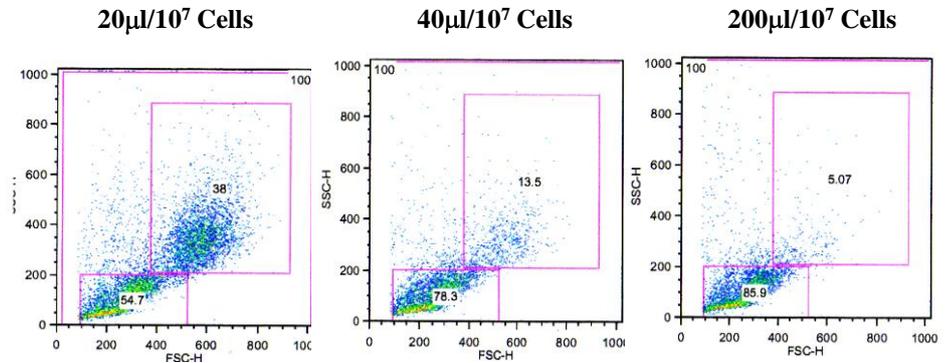
Cytospins were performed to visually assess the cells present in the sample after the isolation protocol. These confirmed that only blasts and lymphocytes were present (**Figure 4.5**).



**Figure 4.5: Cytospin of T Cell Positive Fraction**

A titration experiment was then performed to see if larger quantities of CD33 Microbeads would deplete more of the blasts – x1, x2 and x10 of CD33 Microbeads were used (**Figure 4.6**).

### CD33 MICROBEADS

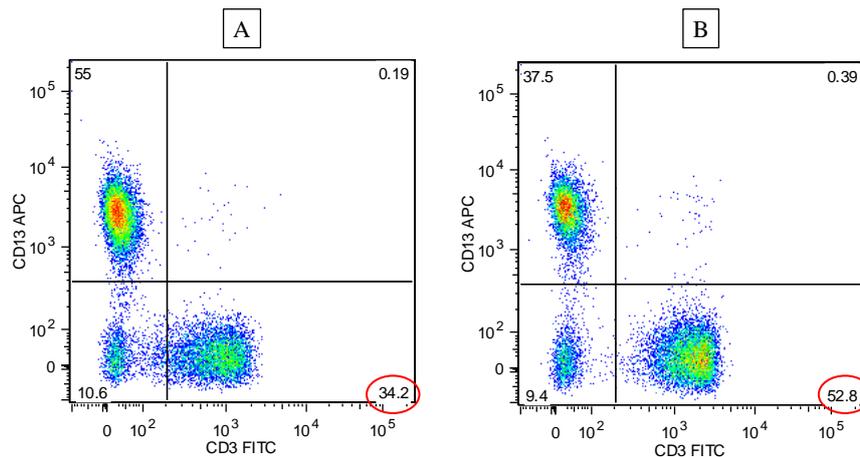


**Figure 4.6: Depletion of CD33+ve Myeloblasts using x1, x2 and x10 CD33 Microbeads**

This illustrated that x10 quantities of CD33 Microbeads resulted in a marked improvement in blast depletion.

x10 CD33 Microbeads were then used in the T cell separation protocol on two vials of frozen AML PBMCs, one for CD4 and one for CD8. On this occasion, the purity for CD8 was 49% and CD4 purity was only 6%. Therefore increasing the quantity of CD33 antibody used was only inconsistently successful.

On the advice of a colleague, at this stage another step was introduced. AML PBMCs were incubated with 2% HAG prior to incubation with CD33 Microbeads. This blocks Fc receptors on the cell surface that can non-specifically bind monoclonal antibodies. This is particularly the case for monocytes and macrophages but both healthy and malignant myeloblasts also express Fc receptors (Barrett, *et al* 1981, Ridway, *et al* 1976). The addition of this step did result in a small but significant improvement in isolated cell purity (**Figure 4.7**).



**Figure 4.7: The Impact of the Use of 2% HAG on Isolated Cell Purity**

These scatter plots illustrate the percentage of live mononuclear cells that are total T cells (x axis) and myeloblasts (y axis, on this occasion defined by labelling with CD13) in the isolated cell fraction after no pre-incubation with 2% HAG (A) and with pre-incubation with 2% HAG (B).

It was now apparent that CD33 alone was not sufficient for removing myeloblasts from PBMC samples in all AML patients. Further antibodies were required. However, AML samples are extremely heterogeneous and the expression of surface markers varies from patient to patient. This was noted by examining the surface expression of 7 potential blast markers in 36 AML patients by flow cytometry. These markers were chosen on the basis of which antibodies were potentially available for cell separation from StemCell Technologies.

	<b>CD11c</b>	<b>CD13</b>	<b>CD33</b>	<b>CD34</b>	<b>CD36</b>	<b>CD38</b>	<b>CD123</b>
<b>Mean</b>	47.4%	43.4%	31.6%	29.9%	22.3%	85.9%	76.4%
<b>Range</b>	3-99.7%	0.2-93.2%	0.04-81%	0.1-89%	0.4-79.0%	35.4-99.8%	9.4-98.8%

**Table 4.2: Expression of Cell Surface Markers on AML Blasts**

The expression of CD11c, CD13, CD33, CD34, CD36, CD38 and CD123 was examined on AML blasts in 36 patients. The mean percentage of blasts that expressed each marker along with the range of expression seen among the 36 samples is listed.

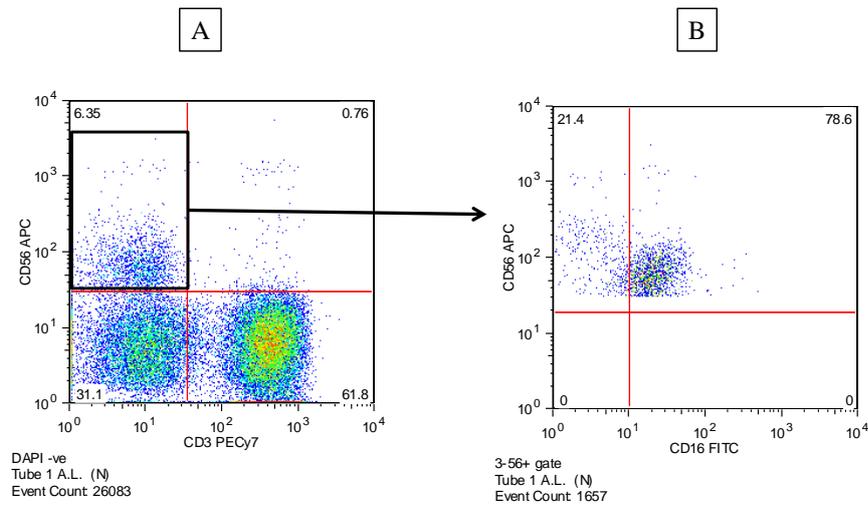
A cocktail of antibodies was required. An AML blast custom antibody cocktail was optimized, consisting of CD33, CD34, CD123, CD11c and CD36. CD33 was used at five times and CD123 three times their usual dose (**Table 4.3**). This was subsequently manufactured by StemCell Technologies as a custom StemSep antibody cocktail. The amount of magnetic colloid used was also increased from 60 $\mu$ l per ml of cells to 100 $\mu$ l per ml of cells in case this was a limiting factor.

Antibody					CD3 %		Conclusions
CD33	CD123	CD34	CD11c	CD36	PRE	POST	
1x	0	0	0	0	7.4	10.2	Use 5x CD33. 10x provides little advantage
5x	0	0	0	0	7.4	29.9	
10x	0	0	0	0	7.4	30.5	
0	1x	0	0	0	9.75	17.6	Use 3x CD123 5x provides little advantage
0	3x	0	0	0	9.75	28.3	
0	5x	0	0	0	9.75	29.1	
3x	3x	0	0	0	3	24.8	CD33 + CD123 insufficient alone
5x	3x	0	0	0	3	28.8	
5x	5x	0	0	0	0	30.8	
5x	3x	1x	0	0	8.4	92.1	5x CD33/3x CD123/1x CD34 very good for CD34+ve AMLs but not CD34-ve
5x	3x	1x	0	0	9.2	91	
5x	3x	1x	0	0	4.5	34	
5x	3x	1x	1x	0	1.6	50.4	Impact of addition of CD11c in a CD34-ve AML
5x	3x	1x	1x	1x	23	75	Full cocktail used on 3 different AML samples
5x	3x	1x	1x	1x	17.5	95.7	
5x	3x	1x	1x	1x	10.8	90	

**Table 4.3: Blast Antibody Cocktail Optimization Experiments**

Each line represents a single experiment.

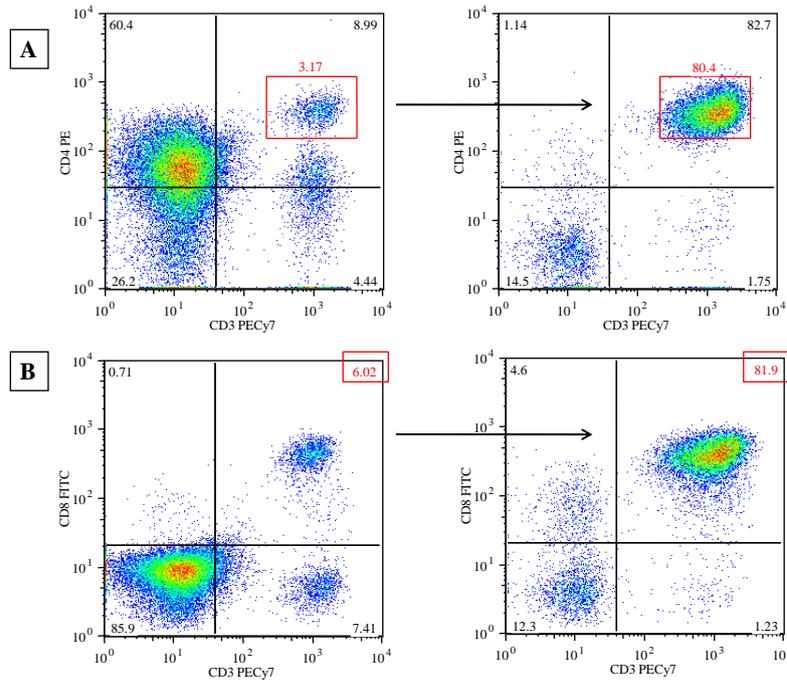
In addition, due to the findings in the previous chapter regarding CD56 expressing T cells it was felt to be important to include these T cells in any analysis of function. Therefore CD56 needed to be excluded from the negative selection cocktail. Analysis of previous flow cytometric data on healthy peripheral blood demonstrated that the majority of CD56-expressing NK cells also express CD16 (**Figure 4.8**)



**Figure 4.8: CD16 Expression on Healthy CD56-expressing NK Cells**

NK cells are CD3 negative CD56 positive as seen in (A). The majority of these cells are also CD16 positive as demonstrated in (B).

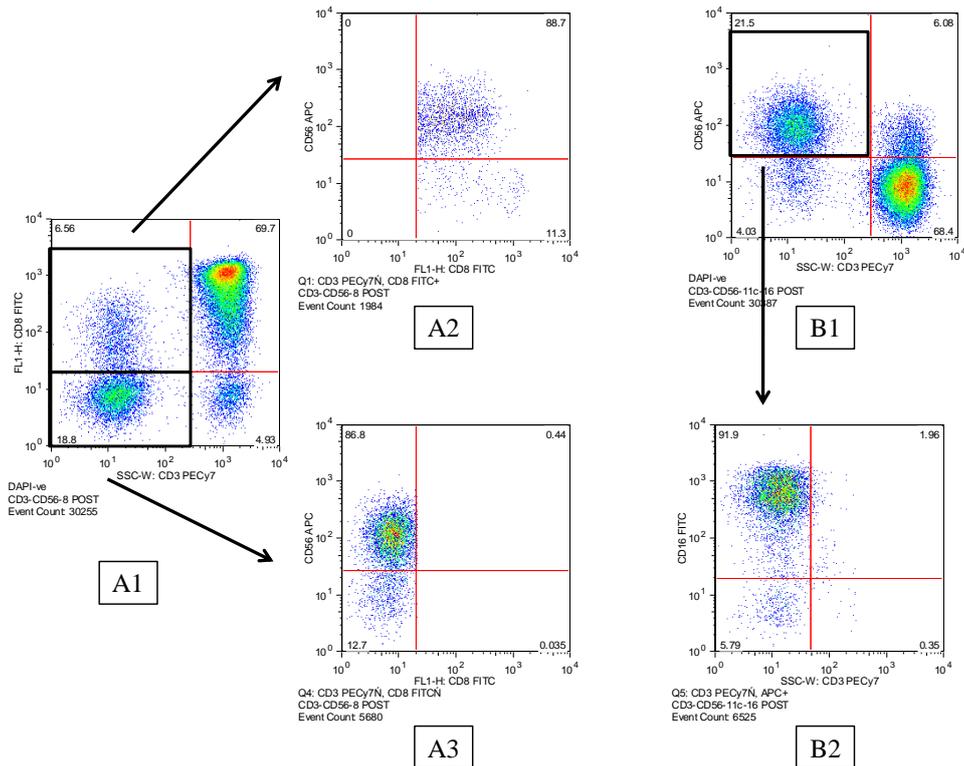
As the cocktail included CD16, it was concluded this would be sufficient to remove NK cells. StemCell Technologies therefore provided custom negative selection CD4 and CD8 T cell isolation kits composed of either CD4 or CD8, CD14, CD16, CD19, Glycophorin A and TCR  $\gamma/\delta$  antibodies. Finally, the combination of the blast depletion cocktail, T cell isolation custom cocktail, 2% HAG, increased magnetic colloid and the use of the peristaltic pump feed system resulted in isolation of highly pure untouched T cells from presentation AML PB samples (**Figure 4.9**).



**Figure 4.9: Successful T cell Isolation from AML Presentation PB by Negative Immunomagnetic Selection.**

CD4 T cell isolation is shown in (A), these cells made up 3.17% of live mononuclear cells pre-separation. The isolation protocol resulted in a purity of CD4 cells of 80.3%. CD8 T cell isolation is shown in (B). Initially, CD3+8+ cells made up 6.14% of total live mononuclear cells. Post separation, the purity of the isolated CD8 T cell fraction was 82%.

It was noted that the purity of the isolated CD8 cells although adequate was not always as good as for CD4. However, rather than the contaminating cells being myeloblasts as had previously been the case, they were now CD3-56+ NK cells. The majority of these cells were also CD8 positive explaining why the contamination was less of an issue in CD4 isolation where the cocktail contained CD8. They also expressed CD16 and therefore theoretically should have been removed by the CD16 antibody used in the isolation cocktail (**Figure 4.10**).

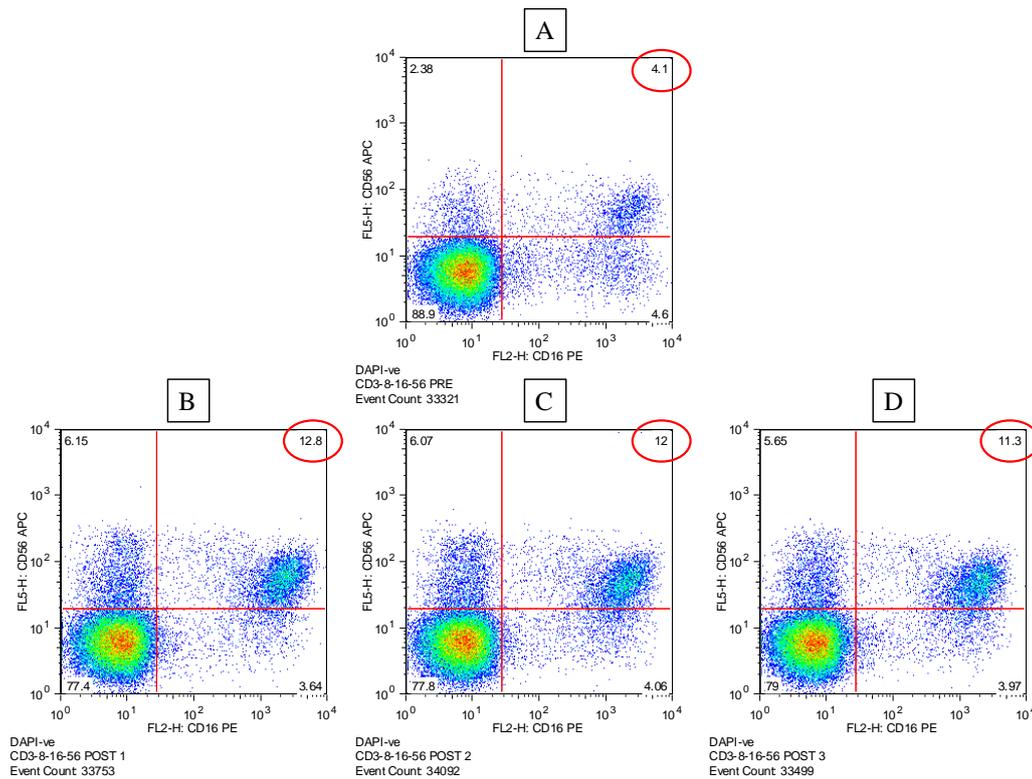


**Figure 4.10: Identity of Contaminating Cells in Isolated CD8 Specimens**

Isolated CD8 T cells had a purity of 70%. Contaminating cells fell into two subsets; CD3 negative CD8 positive (6.6%) and CD3 negative CD8 negative (19%) (A1). Both of these subsets were largely CD56 positive (A2 and A3). If CD3 negative CD56 positive cells were gated (B1), the majority were shown to be CD16 positive.

Using healthy PBMCs, altering pump speeds and cocktail quantities had no impact on the removal of this cell population. CD16 is an Fc receptor found on NK cells, neutrophils, monocytes and macrophages. It has two alternative forms; CD16a is found on NK cells and monocytes whereas CD16b occurs on neutrophils. It was confirmed with StemCell Technologies that the antibody used in the T cell isolation cocktail was directed towards CD16a and therefore should remove NK cells. A sample of CD16 antibody used in the custom T cell isolation cocktail was provided by StemCell Technologies and Miltenyi Biotec generously donated CD16 Microbeads. The addition of either of these CD16 antibodies did not improve the purity of CD8 cells isolated using the CD56 negative custom T cell cocktail (**Figure 4.11**). This demonstrated that CD16 had not been omitted in

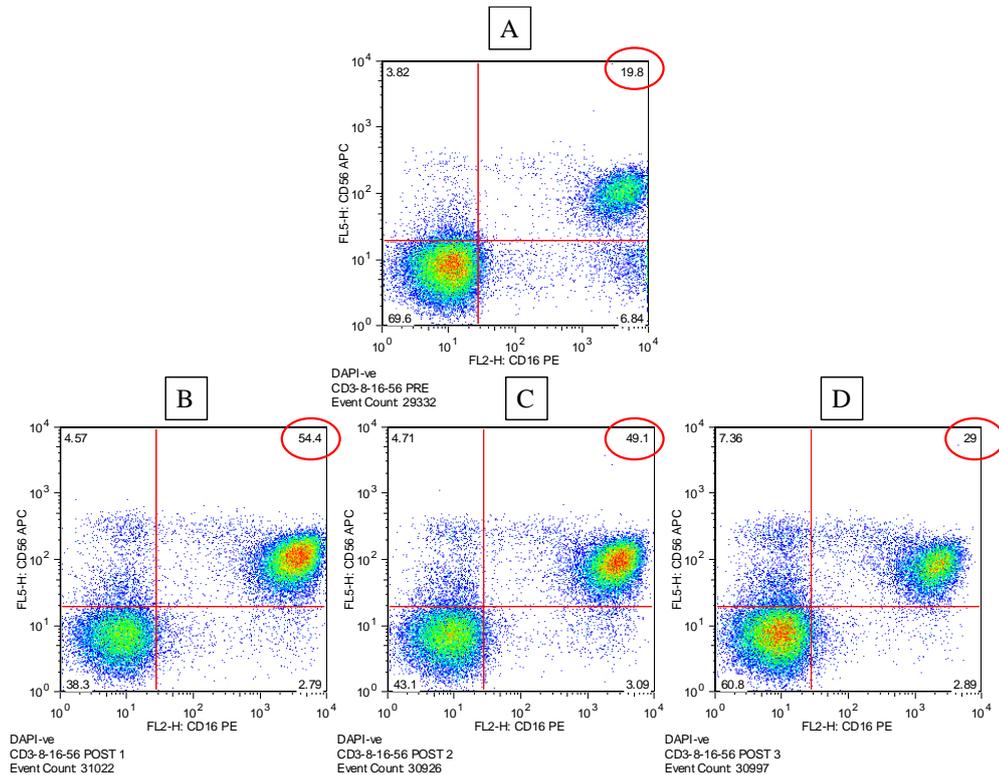
error from the StemCell Technologies T cell isolation custom cocktail and also that this antibody was not removing that CD16-expressing cells.



**Figure 4.11: Impact of the Addition of CD16 to the CD56 Negative Custom Cocktail**

Prior to cell separation, CD16+56+ cells make up 4.1% of total live mononuclear cells (A). After CD8 T cell isolation using the custom T cell isolation cocktail alone these cells make up 12.8% of the isolated cell fraction (B). With the addition of CD16 antibody from StemCell Technologies (C) or CD16 Microbeads from Miltenyi Biotec (D) the percentage of these cells does not alter.

The question then arose as to whether the human antiglobulin being used was blocking the CD16 antibody-binding site. However, omitting this incubation step did not significantly alter the removal of CD16-expressing cells. Addition of extra CD16 antibody in this situation made a small but not significant improvement (Figure 4.12).



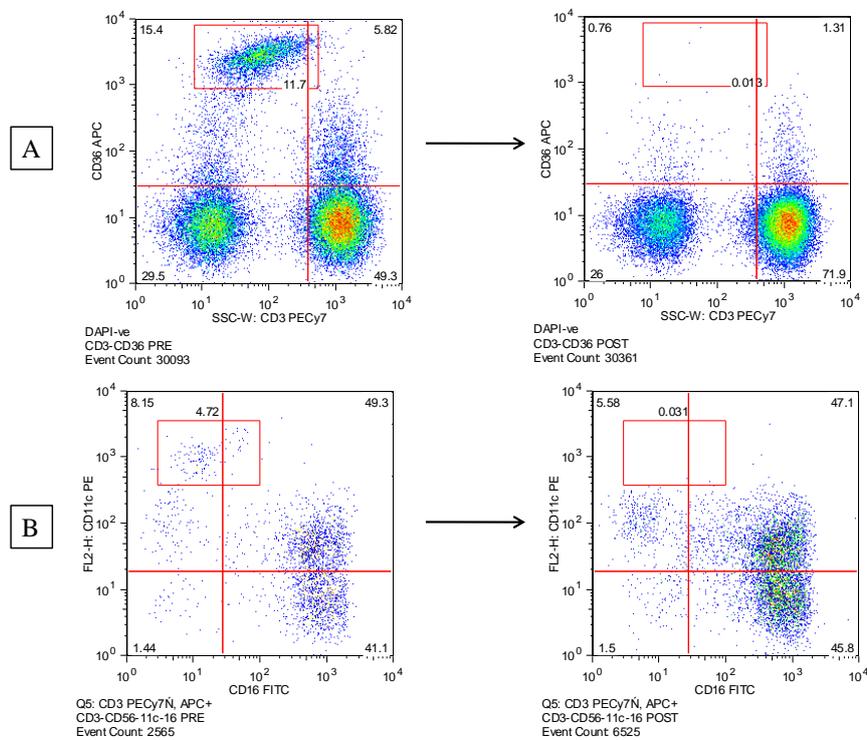
**Figure 4.12: Impact of Omission of 2% HAG Incubation step on Removal of CD16-expressing Cells**

In this individual, pre T cell separation, CD16+56+ cells made up almost 20% of live mononuclear cells (A). Post CD8 T cell isolation using the custom T cell isolation cocktail alone, these cells now make up 54% of the isolated cell fraction (B) although it is worth noting that the CD16+56- population has been removed. The addition of CD16 antibody from StemCell Technologies made little difference to the size of the CD16+56+ population (C). The additional use of CD16 Microbeads from Miltenyi Biotec had a minor effect but this population still makes up 29% of the isolated cell fraction (D).

It was concluded that the only way to adequately remove these CD3+56+16+ cells was to re-introduce CD56 back into the T cell isolation cocktail. However, having identified this population of potential biological relevance, it was deemed erroneous to exclude CD3+56+ cells from any analysis of T cells from AML patients (hereafter referred to as AML T cells) by removing them in the cell isolation method.

Of interest, contaminating CD3+16+56+ cells did not impact so significantly on the purity of the majority of AML samples tested. This was because the use of two

antibodies in the blast depletion cocktail helped to improve cell purity by removing other cells. CD36, the thrombospondin receptor, is found on platelets, red blood cells and monocytes and CD11c, integrin alpha X, is found on dendritic cells, monocytes, neutrophils and also NK cells. When tested on healthy PBMCs, CD36 was found to be present on 12% of PBMCs and the CD36 antibody effectively removed this population. With regards to CD11c, this surface marker was present on 57% of CD3-56+ cells. Three populations were visible; a CD16+11c<sup>Int</sup> subset, a CD16-11c<sup>Int</sup> subset and a CD16-11c<sup>High</sup> subset. Only the small CD16-CD11c<sup>High</sup> population was removed by the CD11c antibody (**Figure 4.13**).



**Figure 4.13: Effect of CD36 and CD11c Antibodies on Depletion of Unwanted Cells**

A CD36 positive population is clearly apparent in (A). Post T cell separation this has been effectively removed. (B) shows the CD16-11c<sup>High</sup> population pre T cell separation that is no longer visible post depletion. However, the CD11c<sup>Int</sup> populations are still present.

Therefore, the method of negative selection of T cells from presentation AML PB samples remained unchanged on the understanding that in certain individuals

contaminating CD3-16+56+ NK cells would compromise the purity of the CD3+8+ population. This variation in cellular populations would not be suitable for the molecular analyses of gene expression microarrays but may not compromise analysis of function provided flow cytometric data was taken into account.

This technique was subsequently used on PB from 6 AML patients to isolate T cells for the functional analyses detailed in chapter 7 (**Table 4.4**).

Sample	CD4% PRE	CD4% POST	Total CD4 Cell Count	CD8% PRE	CD8% POST	CD8% POST (inc CD56+)	Total CD8 Count
AML1	3.0%	76.0%	16 x 10 <sup>6</sup>	1.5%	36.0%	60.0%	10 x 10 <sup>6</sup>
AML2	14.0%	95.0%	18 x 10 <sup>6</sup>	4.0%	79.0%	94.0%	6 x 10 <sup>6</sup>
AML3	8.6%	89.0%	8 x 10 <sup>6</sup>	4.5%	73.0%	95.0%	2 x 10 <sup>6</sup>
AML4	3.2%	82.6%	2 x 10 <sup>6</sup>	6.1%	82.0%	92.8%	4 x 10 <sup>6</sup>
AML5	2.7%	96.9%	2 x 10 <sup>6</sup>	1.4%	91.9%	N/A	3.8 x 10 <sup>6</sup>
AML6	10.9%	85.5%	1.1 x 10 <sup>6</sup>	3.9%	72.0%	93.0%	3.2 x 10 <sup>6</sup>

**Table 4.4: Successful CD4 and CD8 T Cell Isolation from Presentation PB from 6 AML Patients**

Percentages listed are the percentage of cells relative to total live mononuclear cells. Cell counts were performed post isolation procedure. N/A indicates CD56 expression was not checked post-isolation.

## 4.5 Discussion

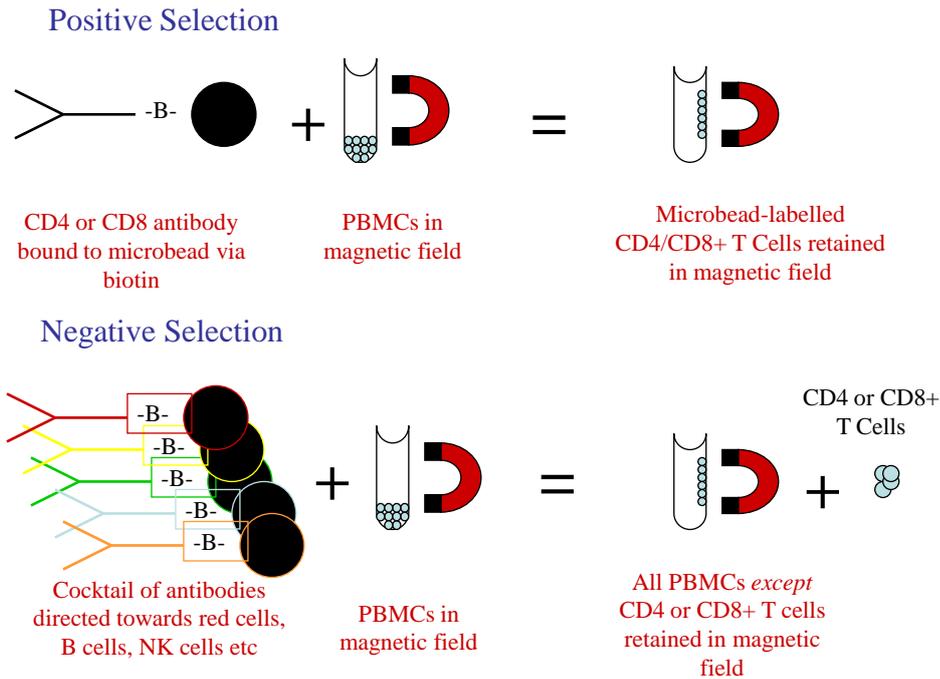
Finding a successful method of obtaining untouched CD4 and CD8 T cells from AML presentation PB specimens proved highly problematic because myeloblasts make up the majority of the PBMC population in these patients and have heterogeneous cell surface markers. Nonetheless a method was eventually optimised through the use of a combination of five antibodies directed towards AML cell surface markers and StemSep™ technology. This technique was subsequently used for isolating CD4 and CD8 T cells from AML patients for the functional assays described in chapter 7. As this method had taken some time to work out, in the meantime, focus had turned to alternative means of obtaining AML T cells for gene expression microarrays, namely, positive immunomagnetic selection. However, before this method could be used, the issue as to whether isolating cells by binding them through surface receptors resulted in the activation of transcription pathways and altered gene expression required resolution. This will be addressed in the next chapter.

## Chapter 5

# The Impact of the Method of Cell Selection on Gene Expression Profile

### 5.1 Introduction

Immunomagnetic selection is a popular and effective method of isolating cells. Companies such as Miltenyi Biotec and Stem Cell Technologies provide reagents that allow selection of particular cell populations within the lab without the requirement for FACS with its expensive and technologically complicated equipment. With positive selection, the required cells are isolated using an antibody bound directly to a magnetic bead. When the cell suspension is passed through a magnetic field, the labelled cells are retained and the unlabelled, unwanted cells pass through. With negative selection, an antibody cocktail is added that binds to all cells except the ones of interest. When this cell suspension is passed through a magnetic field, this time the unwanted cells are magnetically labelled and thus retained within the column and the desired cells are eluted effectively ‘untouched’ (**Figure 5.1**).



**Figure 5.1: Comparison of Positive *versus* Negative Selection for Cell Separation**

Negative selection has the disadvantage of reduced cell yield and purity however positive selection has the theoretical potential of activating the cell as a result of cross-linking surface antigens and therefore altering transcription. There are several reports of the impact of ligating antigens on the surface of T cells. Stanciu and colleagues demonstrated that positively selecting CD4 and CD8 T cells resulted in increased IL-4 production both at baseline and after 20 hours in culture compared with negatively selected cells (Stanciu, *et al* 1996). CD4 ligation has also been shown to induce cellular aggregates (Bernard, *et al* 2002), apoptosis (Berndt, *et al* 1998, Wang, *et al* 1994), TCR down-regulation (Chuck, *et al* 1993), and altered levels of downstream signalling molecules (Bernard, *et al* 2002, Milia, *et al* 1997). However, another report suggests no change in proliferative potential (as an indicator of activation) of positively selected T cells (Semple, *et al* 1993). For the purposes of gene expression profiling of lymphocytes, both immunological methods of separation have been used even within the same study (Palmer, *et al* 2006) yet at the time this work was initiated there were no published data directly comparing the impact of positive *versus* negative selection

on gene expression. Since that time, the results of a single study have been published that directly compared the gene expression profile of positively compared with negatively selected cells (Lyons, *et al* 2007). Three independent CD4 and CD8 separations were performed and the authors concluded that the few expression changes that were observed for CD4 cells could be attributed to contamination with monocytes.

## 5.2 Aims

Due to the potential problems associated with positively selecting T cells outlined and also to allow direct comparison with the CLL dataset in which T cells had been negatively selected, the initial aim had been to negatively select T cells from AML samples for gene expression microarray analysis. As described in Chapter 3, this method had proved difficult to optimise for AML samples, therefore attention turned to positive selection techniques. However, before performing the AML *versus* healthy T cell analysis, the question as to the impact of method of cell selection on the gene expression profile of healthy CD4 and CD8 T cells needed to be addressed. Therefore, in this study, the gene expression profile of CD4 and CD8 T cells isolated from healthy volunteers by positive and negative immunomagnetic selection was compared.

## 5.3 Materials and Methods

### 5.3.1 Samples

Peripheral blood was taken from healthy volunteers. CD4 and CD8 cells were separated by positive and negative selection using the methods detailed in Chapter 2, section 2.6. Cell purity was assessed by flow cytometry post separation. The characteristics of these samples are listed in **Table 5.1**.

	Number of Samples	Median Age	Male:Female	Mean Purity (range)
<b>CD4 Negative Selection</b>	5	44	2:3	92.9% (89-95.5%)
<b>CD4 Positive Selection</b>	5	44	1:4	94.9% (90-98.2%)
<b>CD8 Negative Selection</b>	5	35	4:1	85.0% (76-91%)
<b>CD8 Positive Selection</b>	6	33	5:1	89.9% (87-94%)

**Table 5.1: Characteristics of Healthy T Cell Samples**

### 5.3.2 RNA

RNA was extracted using the technique detailed in Chapter 2, section 2.9. Quality was assessed on both a Nanodrop spectrophotometer and Bioanalyzer. Quantity measurements were taken from Bioanalyzer values (**Table 5.2**).

	Total RNA ( $\mu\text{g}$ )	$A_{260}/A_{280}$
CO CD4_N	15	2.10
KS CD4_N	2.5	2.1
LG CD4_N	6.0	2.09
AD CD4_N	4.7	2.13
IK CD4_N	8.2	2.09
KS CD4_P	4.2	2.27
LG CD4_P	11.5	2.15
AD CD4_P	4.3	1.74
IK CD4_P	2.4	2.06
EN CD4_P	6.5	2.10
DM CD8_N	11.2	2.08
PR CD8_N	3.3	2.14
MR CD8_N	2.3	2.26
AD CD8_N	5.4	2.14
IK CD8_N	5.5	2.09
PR CD8_P	2.3	2.07
MR CD8_P	3.7	1.95
IK CD8_P	3.3	2.13
AD CD8_P	5.9	2.14
DW CD8_P	5.8	2.1
MG CD8_P	5.8	2.1

**Table 5.2: Quality and Quantity of T Cell RNA Obtained**

All 21 healthy samples included. \_P: positive selection, \_N: negative selection.

Depending on the amount of starting material available, between 2.3 and 5  $\mu\text{g}$  of RNA were used for subsequent synthesis of fragmented biotinylated cRNA using the protocol described in Chapter 2, section 2.10.

### 5.3.3 Quality Control (QC) Data for Affymetrix U133Plus2 GeneChips®

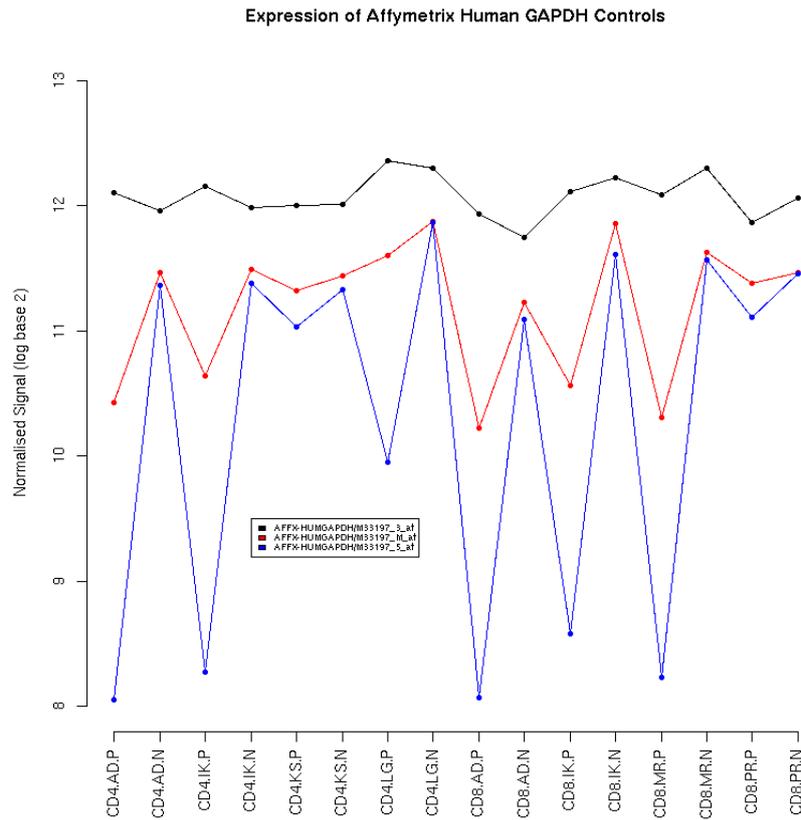
Fragmented biotinylated cRNA was hybridised onto Affymetrix U133Plus2 GeneChips®. Quality control data from the GeneChip runs is listed in **Table 5.3**.

Date Chip Run	Sample	Scale Factor (~1)	Background (<100)	No. Present (~50%)	No. Absent (~50%)	GAPDH 5'-3' Ratio (<3)	HSA 5'-3' Ratio (<3)
26.6.07	CO CD4_N	1.627	44.63	44.50%	54%	1.25	2.5
5.1.07	KS CD4_N	1.452	49.23	41.50%	56.80%	1.25	2.54
5.1.07	LGCD4_N	1.116	57.74	42.90%	55.40%	1.04	1.63
5.1.07	AD CD4_N	1.147	55.11	42.40%	55.90%	1.18	1.98
14.2.07	IK CD4_N	2.37	37.33	41.60%	56.80%	1.24	3.91
25.4.06	KS CD4_	1.168	47.44	42.50%	55.90%	1.53	2.26
25.4.06	LG CD4_P	1.025	61.56	43.10%	55.30%	<b>3.5</b>	<b>7.13</b>
25.4.06	AD CD4_P	1.119	56.24	41.70%	56.70%	<b>11.36</b>	<b>13.64</b>
25.4.06	IK CD4_P	0.989	92.61	38.20%	60.40%	<b>10.02</b>	<b>5.1</b>
26.6.07	EN CD4_P	1.219	62.94	43.90%	54.60%	1.25	1.53
26.6.07	DM CD8_N	1.724	45.31	43.50%	55.10%	1.35	2.26
5.1.07	PR CD8_N	1.104	50.61	43.30%	55.10%	1.26	1.98
5.1.07	MR CD8_N	1.182	53.39	43%	55.40%	1.29	2.22
14.2.07	AD CD8_N	2.28	40.18	41.60%	56.70%	1.35	4
14.2.07	IK CD8_N	2.678	37.16	40.90%	57.60%	1.22	3.42
1.6.06	PR CD8_P	2.13	57.14	39.20%	59.20%	1.46	1.98
17.5.06	MR CD8_P	1.104	54.5	42.3% %	56.1% %	<b>9.99</b>	<b>3.54</b>
17.5.06	IK CD8_P	0.983	52.04	44.30%	54%	<b>7.92</b>	<b>4.98</b>
17.5.06	AD CD8_P	1.114	52.19	42.30%	56%	<b>10.42</b>	<b>4.95</b>
26.6.07	DW CD8_	1.214	55.26	44.60%	53.80%	1.16	1.48
26.6.07	MG CD8_P	0.888	72.9	43.60%	54.90%	1.16	1.43

**Table 5.3: Quality Control Data for Affymetrix U133Plus2 GeneChips®**

All 21 healthy samples are included. \_P: positive selection, \_N: negative selection. Figures highlighted in bold failed QC criteria.

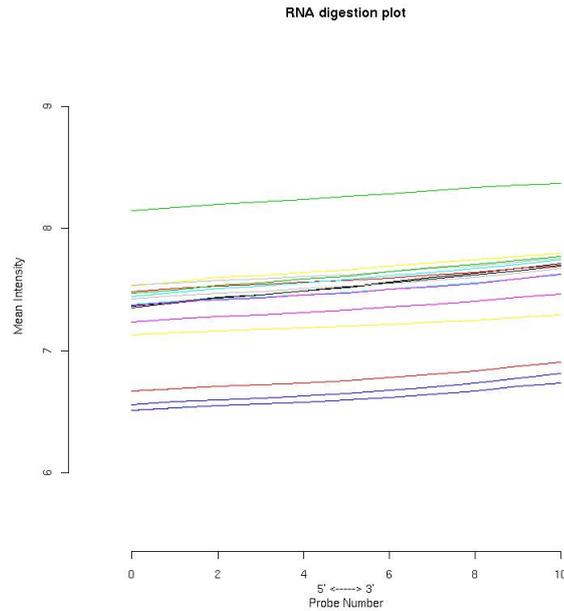
The six samples that failed the Affymetrix standard QC criteria for GAPDH and HAS 5'-3' ratios were not excluded from further analysis. This was because this measure is supposed to represent the presence of degraded RNA in the specimen with a ratio >3 indicating degradation. However, Bioanalyzer analysis had indicated good quality RNA at the start of the procedure and only samples that passed all QC checkpoints were hybridised to chips. Analysing the signals from the GAPDH probes alone revealed that only the probes from the 5' end showed marked variability (**Figure 5.2**).



**Figure 5.2: Expression of Affymetrix Human GAPDH Controls over 16 Samples**

This demonstrates good consistency for the 3' and mid-probesets with only the probeset that binds to the 5' end of the RNA transcript showing variability.

To examine if this was a consistent problem for all probesets binding to RNA 5' ends the following RNA digestion plot was drawn (**Figure 5.3**).



**Figure 5.3: RNA Digestion Plot**

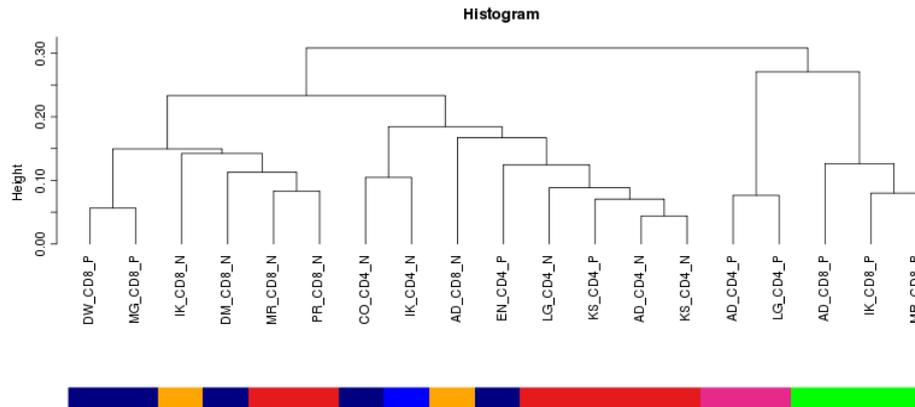
The mean fluorescence intensity of all 11 elements of every probeset on every chip across 16 samples is illustrated.

This plot identifies any trends in the data caused by RNA degradation (which typically targets the 5' end of the molecule) or 3'-biased amplification. If RNA degradation was present, probe intensities would be systematically lowered at the 5' end of a probeset. However, this plot shows that those elements that bind to the 5' end of RNA transcripts are binding equally well as those that bind to the 3' end (the lines are almost flat). Therefore the problem noted with the 5'-3' ratios in the QC check cannot indicate widespread truncated RNA. It is also of note that the majority of probes on the gene expression arrays target the 3' end of the RNA transcript.

### 5.3.4 Analysis of Microarray Data

Normalisation is the process whereby systematic or experimental error is removed from the dataset. In this analysis, microarray data was normalised using robust multi-array average (RMA) processing. Two samples, PR\_CD8\_P and IK\_CD4\_P, were removed from the analysis due to their discrepant normalised unscaled standard errors (NUSE). This quality control measure was calculated by

standardising RMA-estimated standard errors across the arrays. Unsupervised clustering was then performed (**Figure 5.4**).



**Figure 5.4: Unsupervised Clustering**

Dendrogram showing the results of clustering the 19 remaining arrays based on the signals of the 2099 probesets with a coefficient of variation  $> 0.1$ . Colour denotes the date on which the samples were run.

This clustering exercise demonstrated the presence within the samples of a batch effect; samples clustered according to the date on which they were processed rather than within their biological replicate groups. To accommodate for this, for each gene, a batch (date) and replicate group effect was modelled. It was then possible to look within the group effect, ignoring the batch effect, for genes differentially expressed using an unpaired t-test. This analysis was performed using Bioconductor software. The data was also examined on two alternative software packages, Genespring and Partek. In all cases the least stringent (default) multiple testing correction was applied to the analysis; a false discovery rate  $< 0.05$ . False discovery rate (FDR) is a form of multiple testing correction. This statistical correction adjusts the p-value to correct for the occurrence of false positives. This had to be applied as effectively 54,000 t-tests were being

performed. There are a number of different multiple testing corrections that can be applied. These vary from the most stringent Bonferroni to the least stringent Benjamini and Hochberg False Discovery Rate. The latter is usually the default setting on analysis programmes as it offers a good balance between discovery of statistically significant genes and limitation of false positive occurrences. A FDR of 0.01 means 1% of the genes found to be differentially expressed will be picked up due to chance and are therefore false positives.

### ***5.3.5 Validation of Microarray Data by qRT-PCR***

The expression of four genes found to be differentially regulated by microarray was validated by qRT-PCR (**Table 5.4**). These were chosen on the basis of which genes were most significantly altered in positively compared with negatively selected T cells (*NR4A2* and *MS4A3*) and genes which were of potential biological interest in T cells (*CXCR4* and *EEA1*).

Symbol	Name	Function	CD4 (log(2) fold change PvN)	CD8 (log(2) fold change PvN)
<b>NR4A2</b>	Nuclear receptor subfamily 4, group A, member 2	Probable nuclear receptor. May function as a general coactivator of gene transcription	NS	+2.7
<b>CXCR4</b>	Chemokine (C-X-C motif) receptor 4	Receptor for chemokine CXCL12/SDF-1	NS	+1.9
<b>MS4A3</b>	Membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	Haematopoietic modulator for the G1-S cell cycle transition	NS	-5.3
<b>EEA1</b>	Early endosome antigen 1	Binds phospholipid vesicles and participates in endosomal trafficking	NS	-1.6

**Table 5.4: Genes Validated by qRT-PCR**

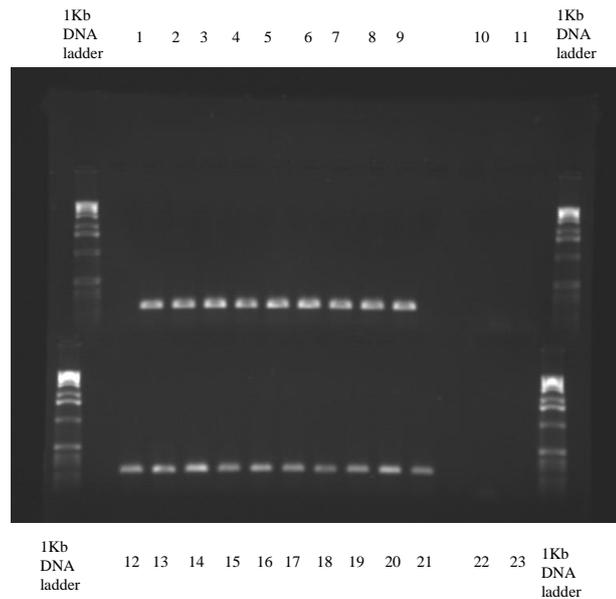
NS: not differentially regulated between positively and negatively selected cells.

qRT-PCR was performed using the same RNA samples that had been used for the microarrays. This meant that in many cases there was insufficient material available (**Table 5.5**).

	Samples for microarray	Samples for qRT-PCR
<b>CD4</b>	5	2
<b>Negative Selection</b>		
<b>CD4</b>	5	2
<b>Positive Selection</b>		
<b>CD8</b>	5	2
<b>Negative Selection</b>		
<b>CD8</b>	6	3
<b>Positive Selection</b>		

**Table 5.5: Availability of Microarray RNA Samples for qRT-PCR**

In all cases, 100ng of RNA were used for cDNA synthesis (**Figure 5.5**).

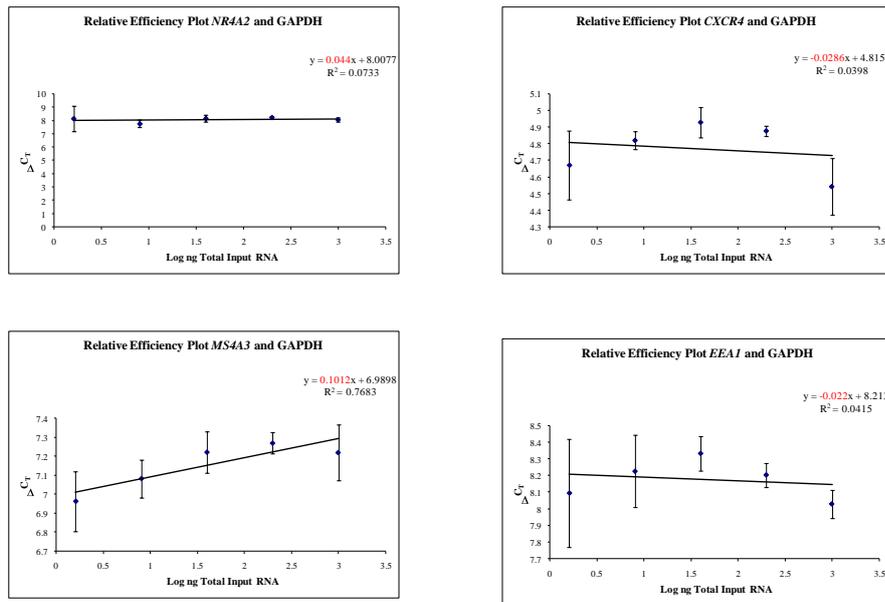


**Figure 5.5:  $\beta$ actin PCR for Confirmation of cDNA Synthesis**

CD4 negative selection: 1-2; CD4 positive selection: 3-4; CD8 negative selection: 5-6; CD8 positive selection: 7-9; negative control cDNA synthesis: 10 and 22; negative control  $\beta$ actin PCR: 11 and 23; Jurkat cell line: 12-16; K562 cell line: 17-21. The two rows look dissimilar due to the use of different size combs for forming wells.

Three cell lines, Jurkat, K562 and KE37, were checked initially to see which could be used as a positive control (calibrator) for each TaqMan® Assay. On the basis of which gave the lowest  $C_T$  values with each assay, K562 was chosen for *NR4A2*, *MS4A3* and *EEA1* and Jurkat was chosen for *CXCR4*.

As the  $2^{-\Delta\Delta C_T}$  method was used for the analysis of qRT-PCR data, a validation step was performed to demonstrate that the efficiencies of the target and reference gene (*GAPDH*) amplification were approximately equal. The positive control cell line chosen for each assay was used and input cDNA was 1000ng, 200ng, 40ng, 8ng and 1.6ng. The absolute value of the slope of log input amount *versus*  $\Delta C_T$  should be  $<0.1$  (**Figure 5.6**).



**Figure 5.6: TaqMan® Assay Relative Efficiency Plots**

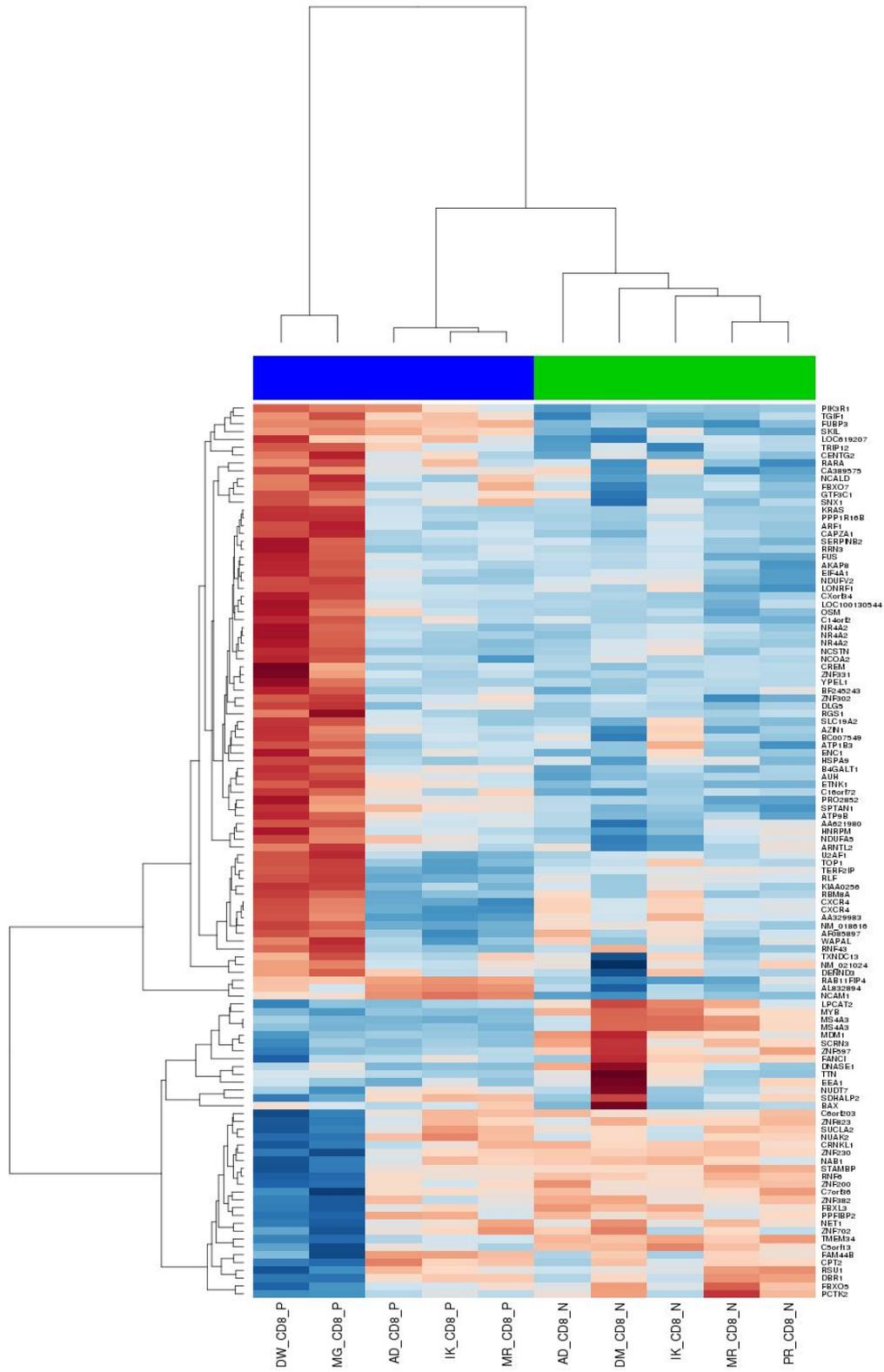
Plots of log input RNA *versus*  $\Delta C_T$  for *NR4A2*, *CXCR4*, *MS4A3* and *EEA1* with *GAPDH*. The absolute value of the slope is shown in red.

## 5.4 Results

### 5.4.1 Gene Expression Microarray

Using both Genespring and Partek software and the least stringent multiple testing correction ( $FDR < 0.05$ ), no genes were identified as being differentially expressed between positively and negatively selected cells for both CD4 and CD8.

However, using Bioconductor software and modelling for batch effect, 114 probesets were found to be differentially expressed (again using an  $FDR < 0.05$ ) in positively selected CD8 cells compared with negatively selected cells of which 76 were up-regulated and 38 down-regulated (**Figure 5.7**). Once again, no differentially expressed genes were found for CD4.



**Figure 5.7: Supervised Clustering of CD8 Samples**

Heatmap demonstrating how the expression of the 114 differentially expressed probesets varies across all CD8 samples.

To evaluate if these genes were of any biological significance in T cells, firstly, the gene-list was compared to that published by Wang and colleagues of genes differentially expressed in human T cells activated *ex vivo* with anti-CD3/anti-CD28 (Wang, *et al* 2008). 36 of the 114 genes (32%) were present in the list of 3793 genes differentially regulated in CD3+ activated cells. These are marked in yellow in the **Table 5.6** below. Only 9 genes were present in the list of 1258 genes differentially regulated in activated CD8 cells; *CXCR4*, *NDUFV2*, *CREM*, *SLC19A2*, *ZNF331*, *TGIF*, *MYB*, *FBX05* and *NET1*. A search of each of the 114 genes was then made on PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>) to look for associations with T cells within published literature. These are marked in green in **Table 5.6**.

Affymetrix Probe ID	Log(2) Fold Change	Symbol	Present in Wang Gene-list	Association with T cells	Description
204621_s_at	3.01	NR4A2	Yellow	Green	nuclear receptor subfamily 4, group A, member 2
216248_s_at	3.00	NR4A2	Yellow	Green	nuclear receptor subfamily 4, group A, member 2
204622_x_at	2.72	NR4A2	Yellow	Green	nuclear receptor subfamily 4, group A, member 2
202988_s_at	2.46	RGS1	Yellow	Green	regulator of G-protein signaling 1
1563473_at	2.32	PPP1R16B	Yellow	Green	protein phosphatase 1, regulatory (inhibitor) subunit 16B
242836_at	2.14	ATP1B3	Yellow	Green	ATPase, Na+/K+ transporting, beta 3 polypeptide
241740_at	2.06	CREM	Yellow	Green	cAMP responsive element modulator
209201_x_at	1.86	CXCR4	Yellow	Green	chemokine (C-X-C motif) receptor 4
211919_s_at	1.85	CXCR4	Yellow	Green	chemokine (C-X-C motif) receptor 4
1564248_at	1.85	LOC100130544	Yellow	Green	hypothetical protein LOC100130544
1559203_s_at	1.83	KRAS	Yellow	Green	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
1565716_at	1.80	FUS	Yellow	Green	fusion (involved in t(12;16) in malignant liposarcoma)
233127_at	1.76	ZNF331	Yellow	Green	zinc finger protein 331
238987_at	1.67	B4GALT1	Yellow	Green	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1
215850_s_at	1.48	NDUFA5	Yellow	Green	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa
240231_at	1.40	AZIN1	Yellow	Green	antizyme inhibitor 1
1566980_at	1.36		Yellow	Green	NA
241438_at	1.36	RLF	Yellow	Green	rearranged L-myc fusion
1566901_at	1.35	TGIF1	Yellow	Green	TGFB-induced factor homeobox 1
214805_at	1.34	EIF4A1	Yellow	Green	eukaryotic translation initiation factor 4A, isoform 1

242125_at	1.34	<b>CAPZA1</b>		capping protein (actin filament) muscle Z-line, alpha 1
230328_at	1.33	<b>RRN3</b>		RRN3 RNA polymerase I transcription factor homolog ( <i>S. cerevisiae</i> )
215322_at	1.33	<b>LONRF1</b>		LON peptidase N-terminal domain and ring finger 1
217591_at	1.25	<b>SKIL</b>		SKI-like oncogene
242298_x_at	1.20			NA
1559154_at	1.19	<b>SNX1</b>		sorting nexin 1
201341_at	1.15	<b>ENC1</b>		ectodermal-neural cortex (with BTB-like domain)
230170_at	1.14	<b>OSM</b>		oncostatin M
224454_at	1.14	<b>ETNK1</b>		ethanolamine kinase 1
227394_at	1.14	<b>NCAM1</b>		neural cell adhesion molecule 1
239476_at	1.07	<b>PIK3R1</b>		phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
243649_at	1.06	<b>FBXO7</b>		F-box protein 7
242113_at	1.05	<b>AKAP8</b>		A kinase (PRKA) anchor protein 8
231904_at	1.02	<b>U2AF1</b>		U2 small nuclear RNA auxiliary factor 1
239540_at	1.02	<b>GTF3C1</b>		general transcription factor IIIC, polypeptide 1, alpha 220kDa
242858_at	1.00	<b>C14orf2</b>		chromosome 14 open reading frame 2
1556952_at	1.00	<b>NCALD</b>		neurocalcin delta
1554602_at	0.98	<b>RBM8A</b>		RNA binding motif protein 8A
239404_at	0.98	<b>TOP1</b>		topoisomerase (DNA) I
216278_at	0.97	<b>KIAA0256</b>		KIAA0256 gene product
233834_at	0.97	<b>NCOA2</b>		nuclear receptor coactivator 2
238704_at	0.95			NA
232175_at	0.93	<b>ARF1</b>		ADP-ribosylation factor 1
232824_at	0.93	<b>ATP9B</b>		ATPase, class II, type 9B
244226_s_at	0.89	<b>RNF43</b>		ring finger protein 43
220702_at	0.86			NA
204614_at	0.86	<b>SERPINB2</b>		serpin peptidase inhibitor, clade B (ovalbumin), member 2
1556602_at	0.85	<b>SLC19A2</b>		solute carrier family 19 (thiamine transporter), member 2
210469_at	0.84	<b>DLG5</b>		discs, large homolog 5 ( <i>Drosophila</i> )
1565706_at	0.83			NA
214918_at	0.81	<b>HNRPM</b>		heterogeneous nuclear ribonucleoprotein M
233038_at	0.80	<b>CENTG2</b>		centaurin, gamma 2
239450_at	0.76	<b>NDUFV2</b>		NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa
240358_at	0.76	<b>DENND3</b>		DENN/MADD domain containing 3
228296_at	0.76	<b>YPEL1</b>		yippee-like 1 ( <i>Drosophila</i> )
239193_at	0.74	<b>FUBP3</b>		far upstream element (FUSE) binding protein 3
243872_at	0.72	<b>WAPAL</b>		wings apart-like homolog ( <i>Drosophila</i> )
241150_at	0.72	<b>SPTAN1</b>		spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)
208347_at	0.70			NA
1569212_at	0.69	<b>LOC619207</b>		scavenger receptor protein family member
237076_at	0.69	<b>NCSTN</b>		nicastrin

205238_at	0.69	<b>CXorf34</b>		chromosome X open reading frame 34
240094_at	0.69	<b>TXNDC13</b>		thioredoxin domain containing 13
223797_at	0.68	<b>PRO2852</b>		hypothetical protein PRO2852
232200_at	0.68	<b>HSPA9</b>		heat shock 70kDa protein 9 (mortalin)
225746_at	0.67	<b>RAB11FIP4</b>		RAB11 family interacting protein 4 (class II)
1565358_at	0.66	<b>RARA</b>		retinoic acid receptor, alpha
1566456_at	0.61			NA
240090_at	0.60	<b>AUH</b>		AU RNA binding protein/enoyl-Coenzyme A hydratase
225197_at	0.60	<b>C16orf72</b>		chromosome 16 open reading frame 72
244659_at	0.59	<b>TRIP12</b>		thyroid hormone receptor interactor 12
1564430_at	0.58			NA
223586_at	0.57	<b>ARNTL2</b>		aryl hydrocarbon receptor nuclear translocator-like 2
229136_s_at	0.57	<b>ZNF302</b>		zinc finger protein 302
1557279_at	0.53			NA
201174_s_at	0.44	<b>TERF2IP</b>		telomeric repeat binding factor 2, interacting protein
202811_at	-0.47	<b>STAMBP</b>		STAM binding protein
209272_at	-0.54	<b>NAB1</b>		NGFI-A binding protein 1 (EGR1 binding protein 1)
220397_at	-0.55	<b>MDM1</b>		Mdm4, transformed 3T3 cell double minute 1, p53 binding protein (mouse)
225132_at	-0.62	<b>FBXL3</b>		F-box and leucine-rich repeat protein 3
219074_at	-0.63	<b>TMEM34</b>		transmembrane protein 34
203403_s_at	-0.65	<b>RNF6</b>		ring finger protein (C3H2C3 type) 6
228855_at	-0.66	<b>NUDT7</b>		nudix (nucleoside diphosphate linked moiety X)-type motif 7
202930_s_at	-0.66	<b>SUCLA2</b>		succinate-CoA ligase, ADP-forming, beta subunit
239598_s_at	-0.71	<b>LPCAT2</b>		lysophosphatidylcholine acyltransferase 2
226693_at	-0.71	<b>SDHALP2</b>		succinate dehydrogenase complex, subunit A, flavoprotein pseudogene 2
206557_at	-0.72	<b>ZNF702</b>		zinc finger protein 702
219913_s_at	-0.74	<b>CRNKL1</b>		crooked neck pre-mRNA splicing factor-like 1 (Drosophila)
225030_at	-0.74	<b>FAM44B</b>		family with sequence similarity 44, member B
1557260_a_at	-0.75	<b>ZNF382</b>		zinc finger protein 382
1569543_at	-0.78	<b>RSU1</b>		Ras suppressor protein 1
230542_at	-0.78	<b>ZNF597</b>		zinc finger protein 597
208478_s_at	-0.78	<b>BAX</b>		BCL2-associated X protein
223576_at	-0.79	<b>C6orf203</b>		chromosome 6 open reading frame 203
205791_x_at	-0.79	<b>ZNF230</b>		zinc finger protein 230
243127_x_at	-0.80	<b>DNASE1</b>		deoxyribonuclease I
207338_s_at	-0.91	<b>ZNF200</b>		zinc finger protein 200
223433_at	-0.93	<b>C7orf36</b>		chromosome 7 open reading frame 36
219234_x_at	-0.93	<b>SCRN3</b>		secernin 3
218875_s_at	-0.94	<b>FBXO5</b>		F-box protein 5
241791_at	-0.95	<b>TTN</b>		titin
213007_at	-0.96	<b>FANCI</b>		Fanconi anemia, complementation group I
220987_s_at	-1.00	<b>NUAK2</b>		NUAK family, SNF1-like kinase, 2

204264_at	-1.00	<b>CPT2</b>		carnitine palmitoyltransferase II
237459_at	-1.02	<b>PCTK2</b>		PCTAIRE protein kinase 2
234295_at	-1.11	<b>DBR1</b>		debranching enzyme homolog 1 ( <i>S. cerevisiae</i> )
229732_at	-1.18	<b>ZNF823</b>		zinc finger protein 823
201829_at	-1.51	<b>NET1</b>		neuroepithelial cell transforming gene 1
204840_s_at	-1.57	<b>EEA1</b>		early endosome antigen 1
201310_s_at	-1.74	<b>C5orf13</b>		chromosome 5 open reading frame 13
204798_at	-1.82	<b>MYB</b>		v-myb myeloblastosis viral oncogene homolog (avian)
212841_s_at	-1.88	<b>PPF1BP2</b>		PTPRF interacting protein, binding protein 2 (liprin beta 2)
1554892_a_at	-4.53	<b>MS4A3</b>		membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)
210254_at	-5.30	<b>MS4A3</b>		membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)

**Table 5.6: List of 114 Differentially Expressed Genes in Positively Selected CD8 Cells**

Genes in red are up-regulated in positively selected cells compared with negatively selected. Genes in blue are down-regulated.

The most up-regulated gene *NR4A2*, a coactivator of general gene transcription, was also found to be up-regulated in the Wang list and the authors postulated that this gene is important for activation and proliferation of both CD4 and CD8 T cells (Wang, *et al* 2008). *CXCR4*, as well as being the co-receptor for HIV with CD4, is important for lymphocyte recruitment to the bone marrow (Peled, *et al* 1999). *CREM* binds to the IL-2 promoter and limits IL-2 production (Tenbrock, *et al* 2003). *AKAP8* is a key component of the signalling complex regulating T cell activation (Asirvatham, *et al* 2004). *RGS1* has a role in lymphocyte adhesion and chemotaxis (Rodriguez, *et al* 2004). Two genes, *TGIF1* and *SKIL* (SNO) are implicated in TGF $\beta$  signalling (Chen, *et al* 2003, Pearson-White and McDuffie 2003). The actin-capping protein *CAPZA1* recruits CD2 to the immune synapse (Hutchings, *et al* 2003). *OSM* is a cytokine that was noted to be up-regulated on T cell activation (Wang, *et al* 2008). *EEA1* is an early marker of T cell activation and is important for fusion of synaptic vesicles (Gasser, *et al* 2005). *MYB* is involved with proliferative responses of mature T cells (Nakata, *et al* 2007). Finally, *NCAM1* (CD56) has a role in cell adhesion allowing clustering of cell-cell contacts (Lanier, *et al* 1989).

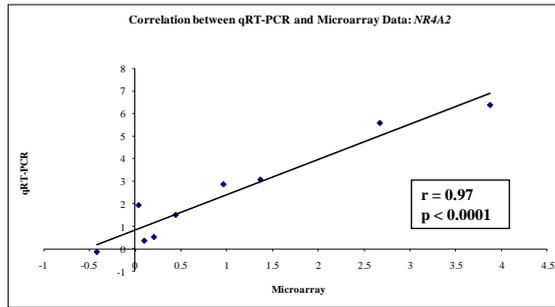
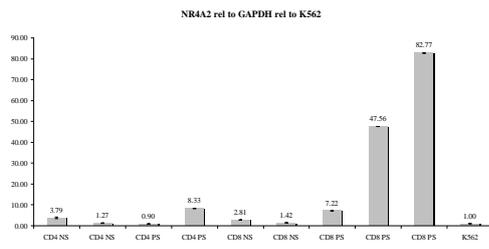
Analysis of the gene-list using Ingenuity software revealed no relevant predominant individual pathway or biological function.

As a means of validating this dataset, a comparison was made of CD4 samples compared with CD8 samples to see if the expected genes were present. All samples were loaded onto Genespring. Analysis of variance (ANOVA) testing was performed using an FDR <0.05 and genes were then filtered on the basis of two fold change. This gave a list of 364 genes. This validation list was then compared to a published list of signature genes for CD4 and CD8 cells (Palmer, *et al* 2006). Of the four signature genes for CD4, three were present in the list; *CD4*, *ANK3* and *CTSB*. For CD8, plasma membrane receptors included in the list were *CD8A* and *CD8B*, killer cell lectin-type receptor and *IL2RB*. Common cytotoxicity associated genes were granzyme K, granzyme A, granzyme B, perforin 1 and granulysin. Of the three genes in the category ‘other’, one, *CCL5*, was present in the validation gene-list.

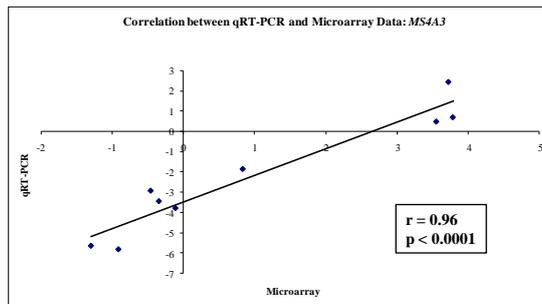
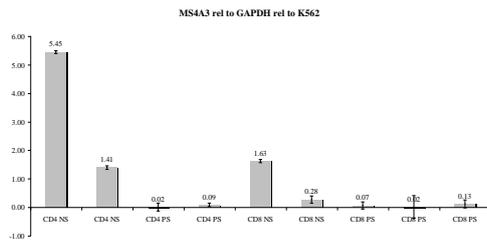
#### **5.4.2 qRT-PCR Validation**

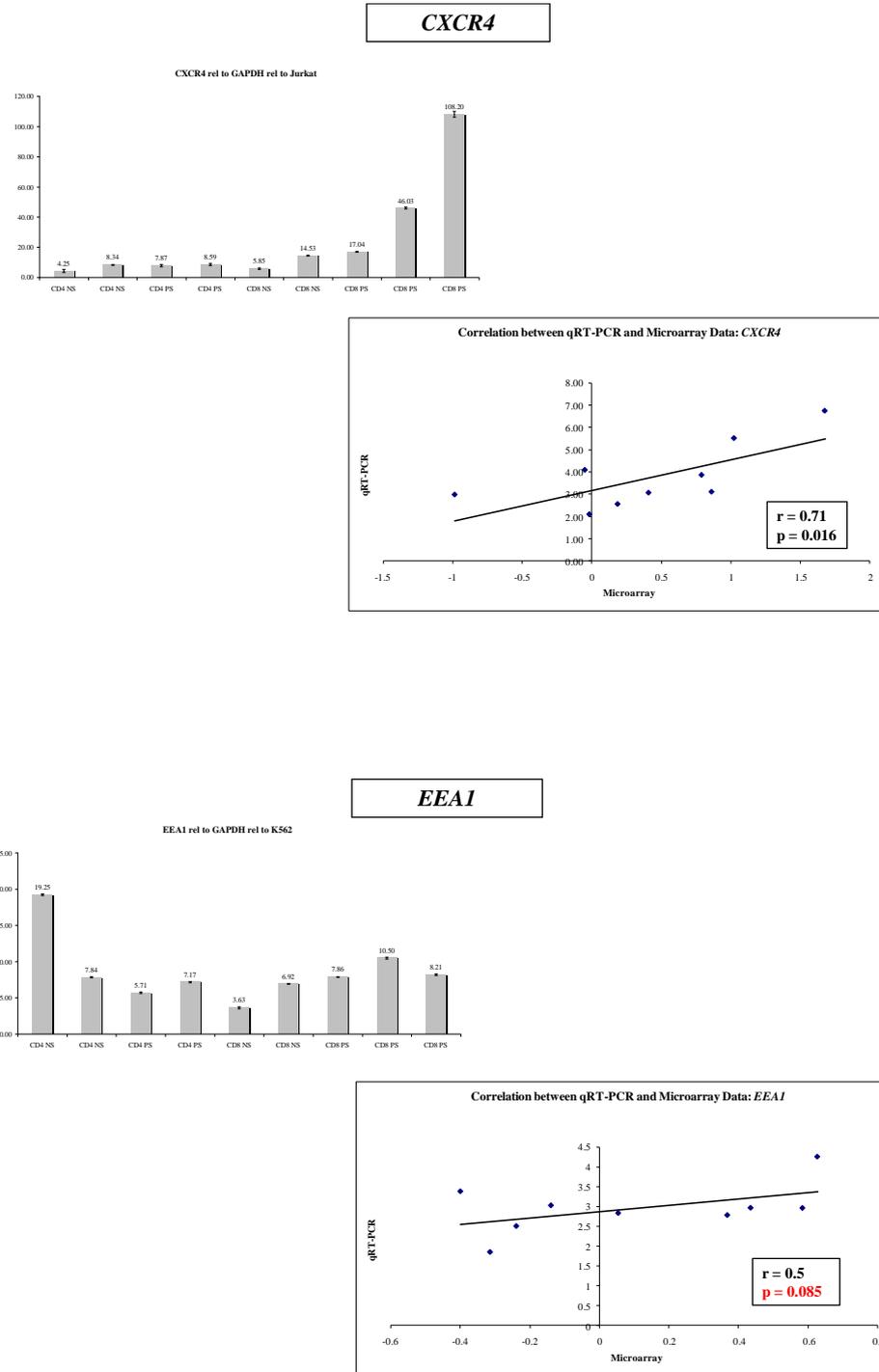
Only one out of the four genes validated failed to show a significant correlation between the microarray and the qRT-PCR data. This was *EEA1* which was shown to have a  $-1.6 \log(2)$  fold change in the microarray results (**Figure 5.8**).

**NR4A2**



**MS4A3**





**Figure 5.8: qRT-PCR Validation**

qRT-PCR data and correlations with microarray data for *NR4A2*, *MS4A3*, *CXCR4* and *EEA1*.

## 5.5 Discussion

This study was initiated to answer the question - does the method of immunomagnetic cell selection have an impact on the gene expression profile of CD4 and CD8 T cells? The data presented here suggest that any effect it does have is minimal. The initial analysis performed on Genespring and Partek software packages demonstrated no differential gene expression using the least stringent multiple testing correction. This agrees with the conclusion published after this analysis by Lyons and colleagues and with unpublished data from Miltenyi Biotec. Using a third software package, Bioconductor, the same multiple testing correction but on this occasion modelling for the observed batch effect, 114 genes were found to be differentially expressed in CD8 cells with none in CD4. This represents a mere 0.2% of the probesets on the Affymetrix U133Plus2 GeneChip®. Nonetheless, that list of 114 genes does contain within it several genes previously reported to be involved in T cell activation or to have associations with T cells in the published literature. Although only a small number of genes were validated, three out of four genes had their differential expression in positively and negatively selected CD8 cells confirmed by qRT-PCR. However, it still remains only a handful of genes the significance of which should not be over-interpreted. It can therefore be concluded that positive immunomagnetic selection has no effect on the gene expression profile of CD4 cells and minimal effect on CD8 cells.

Several problems were encountered in the course of this study. The phenomenon of batch effect was noted within the gene expression data. This means that samples clustered according to the date of processing rather than according to any biological category. In retrospect, this should have been part of the experimental design since this is a recognised problem and could have been accommodated for by careful random processing of samples over a period of a few days. Statistical modelling was therefore performed to remove the batch effect from the analysis. Another potential explanation for the batch effect observed is that there is so little other difference between the samples that date of processing becomes the over-riding difference. However, due to concern about the dataset, an additional step was performed that confirmed its validity: if the CD4 samples were compared

with the CD8 samples, the expected genes were found to be present. The second issue that arose was the elevated 5'-3' ratios for GAPDH and HAS for 6 samples in the Affymetrix quality control checks. These samples were not removed from the analysis as the RNA digestion plot illustrated that there was not global evidence of RNA degradation within the samples. Finally, one out of the four genes chosen for assessment by qRT-PCR did not corroborate the microarray data. This was the gene that had the lowest fold change within the group (-1.6 log(2) fold change) and therefore as such was less likely to be validated by the more sensitive technique of qRT-PCR (Dallas, *et al* 2005).

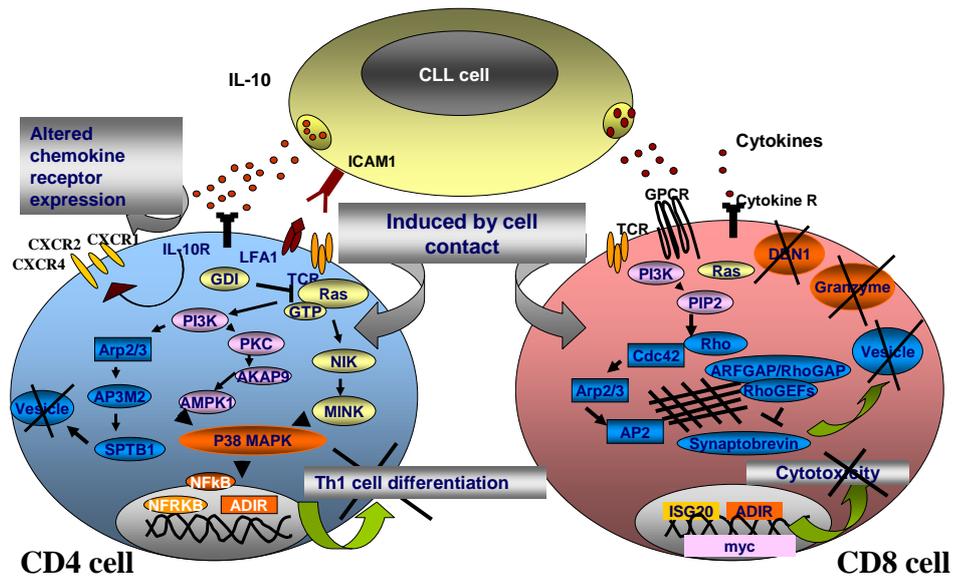
Having established the minimal impact of positive selection on cellular transcription in CD4 and CD8 T cells, a positive selection technique was optimised for the separation of T cells from AML samples. The gene expression profile of these cells could then be compared to the data from healthy T cells generated in this part of the project. In view of the findings presented here, all positively and negatively selected healthy T cells were used in the comparison with positively selected AML T cells. These results will be described in the next chapter.

## Chapter 6

### Gene Expression Profile of AML T Cells

#### 6.1 Introduction

The previous work in this project had now made it possible to achieve the initial objective of this work; to examine the gene expression profile of T cells in patients with newly diagnosed AML compared to that of healthy T cells. Our group had previously examined the same question in CLL (Gorgun, *et al* 2005). In that study, CD4 cells were negatively selected from 22 patients with CLL and 12 healthy donors and CD8 cells were negatively selected from 20 CLL patients and 12 healthy donors. 3-15µg of total RNA were used for gene expression microarrays. Fragmented biotinylated cRNA was hybridised to the Affymetrix U133A chip and biostatistical analysis performed using Permax 2.1 and dChip. Using a P value <0.05, 90 genes were found to be differentially expressed in CD4 cells from CLL patients with 22 found to be up-regulated and 68 down-regulated. Many of these genes were involved in the Ras-dependent JNK and p38 MAPK pathways both of which play a major role in T cell differentiation into T<sub>H1</sub> or T<sub>H2</sub> cells. The patterns of expression seen in these pathways could result in impaired differentiation into T<sub>H1</sub> cells. For CD8, 273 genes were differentially expressed in CLL patients with 105 down-regulated and 168 up-regulated. For these, the major pathways affected were cytoskeletal formation, vesicle trafficking and cytotoxicity. These changes would be expected to result in impaired cytotoxicity. From the gene expression data, a representative protein expression panel was developed to demonstrate by Western blotting that the changes seen in CLL T cells could be induced in T cells from healthy allogeneic donors in co-culture experiments with CLL cells. Direct contact was required for these changes to occur and they did not appear to be cytokine-mediated (**Figure 6.1**). More recently these results have been confirmed in a T cell leukaemia-1 (TCL-1) transgenic mouse model of CLL (Gorgun 2009). We now questioned whether similar T cell defects were apparent in another haematologic malignancy, AML.



**Figure 6.1: Gene Expression Changes in CD4 and CD8 T Cells in CLL**

(From (Gorgun, *et al* 2005))

## 6.2 Aims

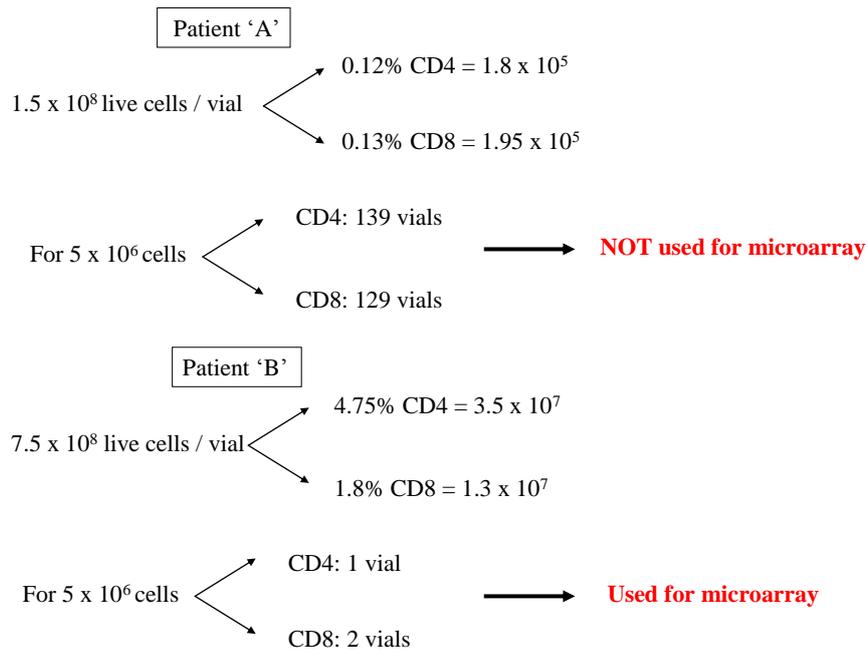
As the previous study had documented potential defects in T cells in patients with CLL compared to those from healthy individuals, it was hypothesized that similar T cell defects may also occur in other malignancies. Therefore, the aim of this study was to test if gene expression changes were also present in the T cells of patients with AML compared with healthy individuals and if so, to compare those changes to those seen in the T cells from CLL patients.

## 6.3 Materials and Methods

### 6.3.1 AML Samples

Frozen cryovials of PBMCs from patients at the time of diagnosis of AML were used. Patients were initially chosen on the basis of the number of cryovials available in the Tissue Bank with preference given to those for whom large numbers were available. The previous flow cytometric data were used to estimate

the number of T cells available in each patient vial. As  $5 \times 10^6$  T cells were required to obtain sufficient RNA for microarray analysis and a yield of 20% was predicted on cell separation, patients were selected for whom it was feasible to get the required number of T cells (**Figure 6.2**). It is appreciated that there is therefore an inherent selection bias but this was necessitated by the need for sufficient T cells to make the experiments feasible.



**Figure 6.2: Worked Example of the Method for Determining Samples for Microarray Analysis**

The characteristics of the 10 AML patients from whom CD4 and CD8 cells were separated are shown in **Table 6.1**.

	<b>FAB Type</b>	<b>Prognostic Group</b>	<b>Cytogenetics</b>	<b>% Blasts in PB at presentation</b>	<b>Response to first line therapy</b>	<b>Relapse?</b>
<b>AML 1</b>	M2	Intermediate	Normal	92%	Fail	No
<b>AML 2</b>	M4	Favourable	Inv(16)	61%	CR	Yes
<b>AML 3</b>	M4	Favourable	-Y, inv(16)	58%	CR	No
<b>AML 4</b>	M4	Intermediate	Normal	61%	CR	Yes
<b>AML 5</b>	M1	Intermediate	Failed	100%	CR	No
<b>AML 6</b>	M4	Favourable	Inv(16)	83%	CR	No
<b>AML 7</b>	M1	Intermediate	+13	66%	CR	No
<b>AML 8</b>	M2	Favourable	t(8;21)	80%	Fail	No
<b>AML 9</b>	M4	Intermediate	Normal	40%	Fail	No
<b>AML 10</b>	M1	Intermediate	Normal	96%	Fail	No

**Table 6.1: Characteristics of AML Patients**

### 6.3.2 Cell Selection

In view of the previous data showing that positive selection has minimal effect on gene expression profile, T cells were positively immunomagnetically selected from diagnostic AML samples using the modifications detailed in Chapter 2, Section 2.6.2. The mean purity of CD4 samples was 85.9% (range 71.5 – 97.5%) with a mean yield of 33% (range 14 – 54%). The mean purity of CD8 samples was 92.3% (range 83.5 – 97.1%) with a mean yield of 40.5% (range 17.5 – 62%).

### 6.3.3 RNA

RNA was extracted as detailed in Chapter 2, section 2.9 (**Tables 6.2 and 6.3**).

	<b>Total RNA</b>	$A_{260}/A_{280}$	$A_{260}/A_{230}$	<b>RIN</b>
<b>AML1</b>	5.73µl in 9µl	1.73	2.52	9.7
<b>AML2</b>	8.14µg in 9µl	1.79	2.46	9.4
<b>AML3</b>	8.58µg in 9µl	1.8	2.43	9.5
<b>AML4</b>	10.92µg in 9µl	1.82	2.43	9.2
<b>AML5</b>	4.04µg in 9µl	1.66	2.6	8.9
<b>AML6</b>	7.96µg in 9µl	1.8	2.48	9.4
<b>AML7</b>	16.28µg in 9µl	1.88	2.37	9.1
<b>AML8</b>	6.69µg in 9µl	1.75	2.58	9.3
<b>AML9</b>	4.76µg in 9µl	1.59	2.70	9.4
<b>AML10</b>	5.4µg in 9µl	1.68	2.63	9.5

**Table 6.2: Quality and Quantity of RNA for CD4 T Cells**

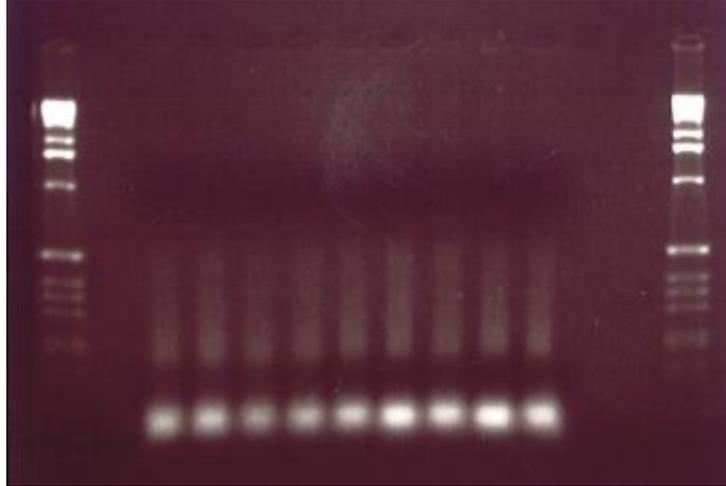
RIN: RNA Integrity Number.

	<b>Total RNA</b>	$A_{260}/A_{280}$	$A_{260}/A_{230}$	<b>RIN</b>
<b>AML1</b>	5.6µl in 9µl	1.75	2.55	9.5
<b>AML2</b>	4.37µg in 9µl	1.61	2.76	9.3
<b>AML3</b>	7.62µg in 9µl	1.77	2.49	9.6
<b>AML4</b>	6.78µg in 9µl	1.75	2.49	9.3
<b>AML5</b>	2.69µg in 9µl	1.57	2.87	8.9
<b>AML6</b>	14.4µg in 9µl	1.86	2.42	9.3
<b>AML7</b>	17.28µg in 9µl	1.87	2.35	9.1
<b>AML8</b>	6.52µg in 9µl	1.70	2.51	9.4
<b>AML9</b>	3.77µg in 9µl	1.62	2.77	9.5
<b>AML10</b>	5.4µg in 9µl	1.65	2.59	9.5

**Table 6.3: Quality and Quantity of RNA for CD8 T Cells**

RIN: RNA Integrity Number.

Fragmented biotinylated cRNA was synthesized using the protocol detailed in Chapter 2, Section 2.10 in all cases using 3 $\mu$ g of RNA as starting material (**Figure 6.3**).



**Figure 6.3: Fragmented Biotinylated cRNA with 1Kb Ladder**

#### **6.3.4 Quality Control Data for Affymetrix U133Plus2 GeneChips®**

Fragmented biotinylated cRNA was hybridised onto Affymetrix U133Plus2 GeneChips®. Quality control data from the GeneChip runs is listed in **Tables 6.4 and 6.5**.

<b>Sample</b>	<b>Noise (&lt;4)</b>	<b>Scale Factor (~1)</b>	<b>Background (&lt;100)</b>	<b>No. Present (~50%)</b>	<b>No. Absent (~50%)</b>	<b>GAPDH 5'-3' Ratio (&lt;3)</b>	<b>HSA 5'- 3' Ratio (&lt;3)</b>
<b>AML 1</b>	1.71	1.802	39.51	44.3%	54.0%	1.62	2.49
<b>AML 2</b>	1.52	1.322	45.49	44.6%	53.6%	1.82	2.08
<b>AML 3</b>	1.57	1.044	45.74	44.3%	54.0%	1.50	1.91
<b>AML 4</b>	1.60	1.152	46.44	43.2%	55.0%	1.57	1.84
<b>AML 5</b>	1.50	1.341	45.33	42.6%	55.6%	1.72	2.11
<b>AML 6</b>	1.45	1.442	43.73	43.2%	55.1%	1.49	2.21
<b>AML 7</b>	1.75	2.211	38.07	41.0%	57.3%	1.67	2.16
<b>AML 8</b>	2.24	1.515	47.09	43.5%	54.7%	1.58	2.04
<b>AML 9</b>	1.85	1.819	40.97	44.5%	54.0%	1.51	2.47
<b>AML 10</b>	2.19	1.298	45.39	46.0%	52.2%	1.60	2.12

**Table 6.4: Quality Control Data for CD4 T Cells**

<b>Sample</b>	<b>Noise (&lt;4)</b>	<b>Scale Factor (~1)</b>	<b>Background (&lt;100)</b>	<b>No. Present (~50%)</b>	<b>No. Absent (~50%)</b>	<b>GAPDH 5'-3' Ratio (&lt;3)</b>	<b>HSA 5'- 3' Ratio (&lt;3)</b>
<b>AML 1</b>	1.45	1.500	44.30	44.8%	53.5%	1.60	2.31
<b>AML 2</b>	1.28	1.302	39.09	45.1%	53.0%	1.43	1.98
<b>AML 3</b>	1.36	1.515	40.79	43.3%	55.0%	1.32	1.89
<b>AML 4</b>	1.32	1.750	40.70	43.6%	54.7%	1.67	2.18
<b>AML 5</b>	1.4	1.671	43.90	42.2%	56.0%	1.44	2.09
<b>AML 6</b>	1.21	2.068	38.18	42.2%	56.0%	1.42	2.47
<b>AML 7</b>	1.24	1.681	38.01	43.1%	55.3%	1.47	1.73
<b>AML 8</b>	1.28	1.972	39.87	41.1%	57.2%	1.37	2.13
<b>AML 9</b>	1.21	1.606	36.93	43.2%	55.1%	1.28	2.05
<b>AML 10</b>	1.27	2.350	38.85	41.6%	56.7%	1.64	2.38

**Table 6.5: Quality Control Data for CD8 T Cells**

### 6.3.5 Healthy Samples

Data from these chips was compared to the data from healthy CD4 and CD8 T cells previously generated (see chapter 5). Both positively and negatively selected healthy T cells were included in the analysis as it had previously been demonstrated that the method of cell selection had minimal impact on gene expression profile. The sample groups are compared in **Table 6.6**.

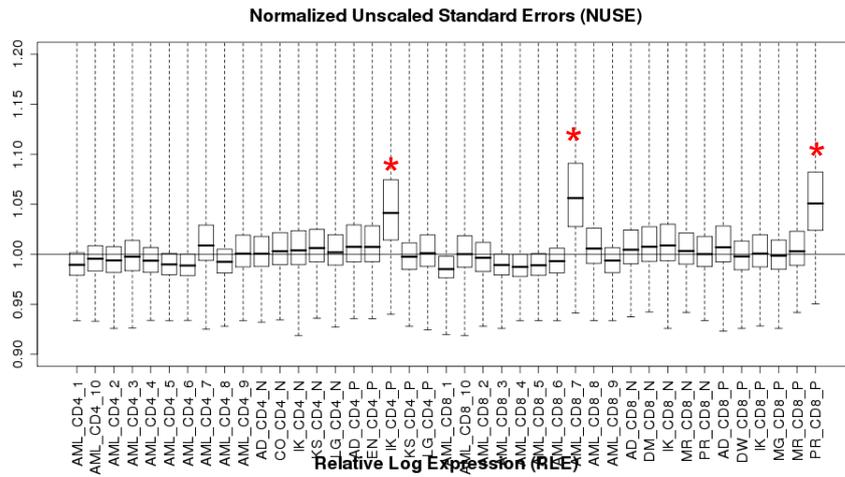
	Number of Samples	Median Age	Male:Female	Purity of selected cells (range)
AML CD4	10	62	8:2	85.9% (71.5 - 97.5%)
Healthy CD4	10	44	3:8	94.3% (89 - 98.2%)
AML CD8	10	62	8:2	92.3% (83.5 - 97.1%)
Healthy CD8	11	33	9:2	87% (76 - 94.1%)

**Table 6.6: Characteristics of AML and Healthy Donor T Cell Datasets**

### 6.3.6 Analysis of Microarray Data

Initial analysis was performed using Bioconductor software.

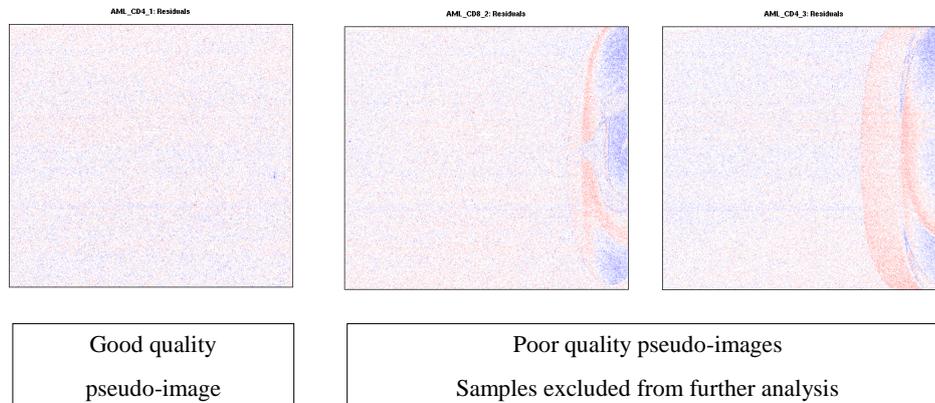
Samples were at first normalised using RMA and five samples were then excluded on the basis of QC checks. Three samples were excluded due to discrepant NUSE. Using this quality control measure the differences in variability between probesets could be adjusted and the boxplot of these standardised values used to compare chip qualities. Ideally, distributions should be tight and centred around one (**Figure 6.4**).



**Figure 6.4: NUSE Analysis of All Samples**

\*: sample excluded on the basis of analysis.

Two samples were excluded on the basis of poor chip pseudo-images. These were built on the residuals from the RMA model fitting procedures and high-lighted artefacts that could cause problems.



**Figure 6.5: Pseudo-images of GeneChip® Data**

Normalisation is the process whereby systematic or experimental error is removed from the dataset. Initial analysis made it clear that there were marked differences in the gene expression profile of T cells in AML patients compared with healthy individuals. So different were these groups that it was felt that using RMA to normalise the samples was inappropriate as this form of normalisation makes the assumption that the vast majority of genes will be expressed across all the samples in an equivalent fashion. This was not true for the current dataset. An alternative form of normalisation, Microarray Suite Version 5.0 (MAS5), was therefore applied. This considers data on a per-array basis.

After normalisation, probesets were removed that were not reliably detected above background i.e. not called “present” by MAS5 in at least one of the replicate groups at a frequency of  $\geq \text{GroupSize}/2$ . The remaining data was then scale normalised such that the arrays were aligned along their median signal intensities.

A gene-list was generated using a FDR of  $<0.01$  and a fold change  $>2$ .

Pathway analysis was subsequently performed using Ingenuity software and PubMed.

### 6.3.7 Validation of Microarray Data by qRT-PCR

The genes listed in **Table 6.7** were chosen for validation of expression levels by qRT-PCR either because they were the most significantly differentially regulated genes in AML compared with healthy T cells (*FOSB* and *JUN*) or because they were of potential biological significance in T cells (*ATM*, *CD48* and *ACTN1*).

<b>CD4</b>	<b>CD4</b>	<b>CD8</b>	<b>CD8</b>
<b>UP-regulated</b>	<b>DOWN-regulated</b>	<b>UP-regulated</b>	<b>DOWN-regulated</b>
AML $\nu$ Healthy	AML $\nu$ Healthy	AML $\nu$ Healthy	AML $\nu$ Healthy
(log(2) fold change)	(log(2) fold change)	(log(2) fold change)	(log(2) fold change)
FOSB (x 6)	ATM (x 1.5)	FOSB (x 5.3)	ACTN1 (x 2.2)
JUN (x 5)	CD48 (x 1.5)	JUN (x 4.7)	ATM (x 1.5)

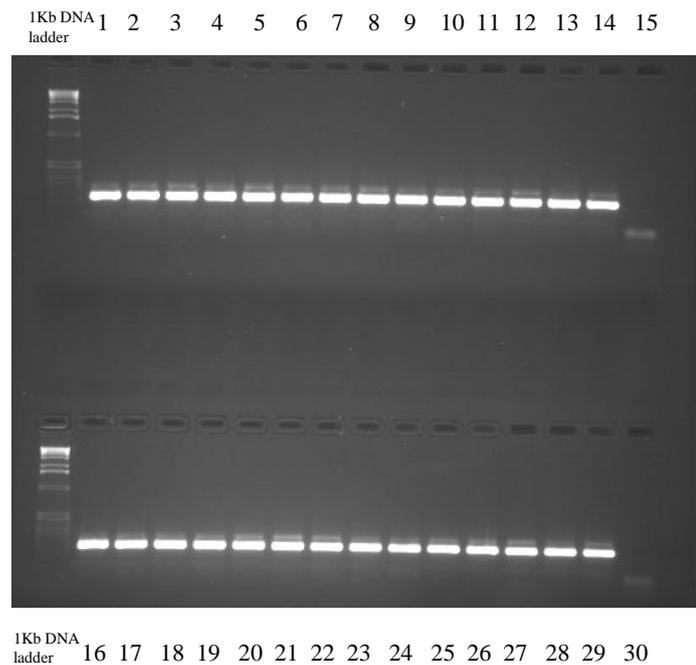
**Table 6.7: Genes Chosen for Validation of Microarray Data**

qRT-PCR was performed using the same RNA samples that had been used for the microarrays. This meant that in some cases there was insufficient material available (**Table 6.8**).

	<b>Samples for microarray</b>	<b>Samples for qRT-PCR</b>
<b>AML CD4</b>	10	8
<b>Healthy CD4</b>	10	4
<b>AML CD8</b>	10	7
<b>Healthy CD8</b>	11	5

**Table 6.8: Samples Available for qRT-PCR**

In all cases, 100ng of RNA were used for cDNA synthesis (**Figure 6.6**).



**Figure 6.6:  $\beta$ actin PCR for Confirmation of cDNA Synthesis**

AML CD4: 1-8; Healthy CD4: 9-12; Jurkat cell line: 13; K562 cell line: 14; -ve control cDNA PCR: 15; AML CD8: 16- 22; Healthy CD8: 23-27; Jurkat cell line: 28; K562 cell line: 29; -ve control  $\beta$ actin PCR: 30.

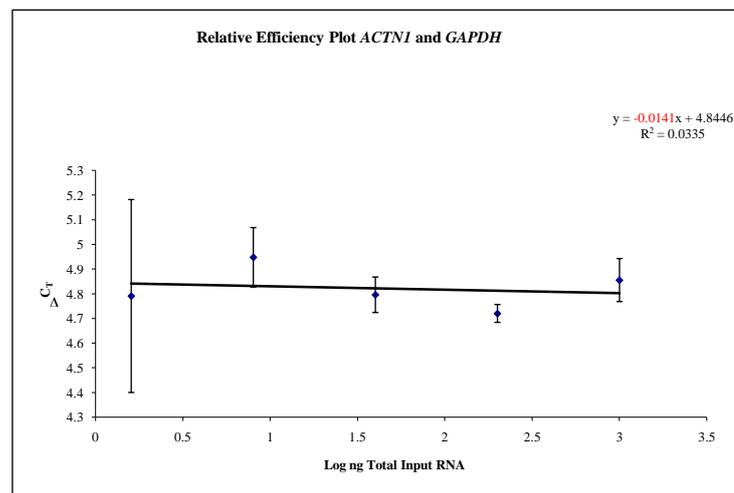
All five TaqMan® Gene Expression Assays were initially tested on Jurkat and K562 T cell lines to determine which cell line could be used as a positive control (calibrator) for each gene (**Table 6.9**).

PlateID	Sample	Detector	Task	Ct	d
13	NTC	GAPDH	Endogenous Control	Undetermined	.
14	NTC	GAPDH	Endogenous Control	Undetermined	.
15	NTC	GAPDH	Endogenous Control	Undetermined	.
16	Jurkat 100ng	GAPDH	Endogenous Control	23.2761	.
17	Jurkat 100ng	GAPDH	Endogenous Control	23.58798	.
18	Jurkat 100ng	GAPDH	Endogenous Control	23.456646	.
19	K562 100ng	GAPDH	Endogenous Control	23.961023	.
20	K562 100ng	GAPDH	Endogenous Control	24.027622	.
21	K562 100ng	GAPDH	Endogenous Control	23.900625	.
25	NTC	ACTN1	Target	Undetermined	.
26	NTC	ACTN1	Target	Undetermined	.
27	NTC	ACTN1	Target	Undetermined	.
28	Jurkat 100ng	ACTN1	Target	26.148853	.
29	Jurkat 100ng	ACTN1	Target	26.089544	.
30	Jurkat 100ng	ACTN1	Target	26.277779	.
31	K562 100ng	ACTN1	Target	27.33762	.
32	K562 100ng	ACTN1	Target	26.79669	.
33	K562 100ng	ACTN1	Target	27.3511	.
37	NTC	ATM	Target	Undetermined	.
38	NTC	ATM	Target	Undetermined	.
39	NTC	ATM	Target	Undetermined	.
40	Jurkat 100ng	ATM	Target	29.50393	.
41	Jurkat 100ng	ATM	Target	29.42611	.
42	Jurkat 100ng	ATM	Target	29.504808	.
43	K562 100ng	ATM	Target	30.983465	.
44	K562 100ng	ATM	Target	30.946243	.
45	K562 100ng	ATM	Target	31.350554	.
49	NTC	FOSB	Target	Undetermined	.
50	NTC	FOSB	Target	Undetermined	.
51	NTC	FOSB	Target	Undetermined	.
52	Jurkat 100ng	FOSB	Target	36.20867	.
53	Jurkat 100ng	FOSB	Target	35.81131	.
54	Jurkat 100ng	FOSB	Target	36.148712	.
55	K562 100ng	FOSB	Target	33.890285	.
56	K562 100ng	FOSB	Target	33.59959	.
57	K562 100ng	FOSB	Target	33.9467	.
61	NTC	JUN	Target	Undetermined	.
62	NTC	JUN	Target	Undetermined	.
63	NTC	JUN	Target	Undetermined	.
64	Jurkat 100ng	JUN	Target	34.018536	.
65	Jurkat 100ng	JUN	Target	33.996746	.
66	Jurkat 100ng	JUN	Target	34.103077	.
67	K562 100ng	JUN	Target	33.037357	.
68	K562 100ng	JUN	Target	32.95192	.
69	K562 100ng	JUN	Target	33.553886	.
73	NTC	CD48	Target	Undetermined	.
74	NTC	CD48	Target	Undetermined	.
75	NTC	CD48	Target	Undetermined	.
76	Jurkat 100ng	CD48	Target	26.38322	.
77	Jurkat 100ng	CD48	Target	26.59288	.
78	Jurkat 100ng	CD48	Target	26.666067	.
79	K562 100ng	CD48	Target	37.474052	.
80	K562 100ng	CD48	Target	36.51134	.
81	K562 100ng	CD48	Target	38.268417	.

### Table 6.9: Choice of Positive Control for each TaqMan® Assay

The highlighted cell line was the one chosen to be the positive control for that TaqMan® assay on the basis of the lowest  $C_T$  values.

The  $2^{-\Delta\Delta C_T}$  method was used for analysis of qRT-PCR data. This approach was validated by demonstrating that the efficiencies of the target and reference gene (*GAPDH*) amplification were approximately equal. The positive control cell line chosen for each assay was used and input cDNA was 1000ng, 200ng, 40ng, 8ng and 1.6ng. The absolute value of the slope of log input amount *versus*  $\Delta C_T$  should be  $<0.1$ . A representative plot is shown in **Figure 6.7**.



**Figure 6.7:** Plot of Log Input RNA *versus*  $\Delta C_T$  for *ACT1* and *GAPDH*

The absolute value of the slope is shown in red.

### 6.3.8 Protein Validation of Gene Expression Changes

As numerous cell surface markers featured in the gene-lists generated, protein validation was undertaken by flow cytometry rather than western blotting. The markers examined are listed in **Table 6.10**.

Cell Surface Molecule	Function	CD4 (log(2) fold change AML $\nu$ Healthy)	CD8 (log(2) fold change AML $\nu$ Healthy)
<b>CD48</b>	Adhesion molecule crucial for T cell activation	-1.58	-1.2
<b>CD69</b>	Earliest inducible T cell activation marker	+1.75	+2.44
<b>CD94</b>	Killer cell lectin-like receptor subfamily D1. Pairs with NKG2A	-1.97	NS
<b>CD86</b>	T cell costimulatory receptor	+1.24	NS
<b>CD21 (CR2)</b>	Complement component that binds to C3d	NS	-2
<b>CCR7</b>	Activates T cells Ligand = CCL19	NS	-1.25
<b>CD119</b>	IFN $\gamma$ receptor I	+1.7	+1.4
<b>Dectin1</b>	Pattern recognition receptor for beta- glucans	+2.7	NS

**Table 6.10: Cell Surface Molecules used for Protein Validation of Gene Expression Changes**

NS: not significantly differentially expressed between AML and healthy T cells.

This experiment was performed on five AML samples and five samples from healthy donors. As live cells were required, cryovials from AML patients stored on the same day as the original vial used for microarray analysis were used. For healthy controls, no cells had been frozen at the time of initial cell separation for microarray work so fresh blood was taken from five of the original individuals used for the microarray studies. Once PBMCs had been separated, they were frozen for future use to ensure that the flow cytometric assessment was done on samples treated equivalently. The cell staining protocol is listed in **Table 6.11**.

Tube number	APC	PE	FITC
1	CD3 (IgG <sub>1</sub> )	CD4 (IgG <sub>1</sub> )	CD48 (IgM)
2	CD3 (IgG <sub>1</sub> )	CD8 (IgG <sub>1</sub> )	CD48 (IgM)
3	CD3 (IgG <sub>1</sub> )	CD4 (IgG <sub>1</sub> )	CD69 (IgG <sub>1</sub> )
4	CD3 (IgG <sub>1</sub> )	CD8 (IgG <sub>1</sub> )	CD69 (IgG <sub>1</sub> )
5	CD3 (IgG <sub>1</sub> )	CD4 (IgG <sub>1</sub> )	CD94 (IgG <sub>1</sub> )
6	CD3 (IgG <sub>1</sub> )	CD4 (IgG <sub>1</sub> )	CD86 (IgG <sub>1</sub> )
7	CD3 (IgG <sub>1</sub> )	Dectin 1 (IgG <sub>2b</sub> )	CD4 (IgG <sub>1</sub> )
8	CD3 (IgG <sub>1</sub> )	CD21 (IgG <sub>1</sub> )	CD8 (IgG <sub>1</sub> )
9	CD3 (IgG <sub>1</sub> )	CCR7 (Rat IgG <sub>2a</sub> )	CD8 (IgG <sub>1</sub> )
10	CD3 (IgG <sub>1</sub> )	CD119 (IgG <sub>1</sub> )	CD8 (IgG <sub>1</sub> )
11	IgG <sub>1</sub>	IgG <sub>1</sub>	IgG <sub>1</sub>
12		IgG <sub>2b</sub>	IgM
13		Rat IgG <sub>2a</sub>	

**Table 6.11: Staining Protocol for Flow Cytometric Validation of Microarray Data**

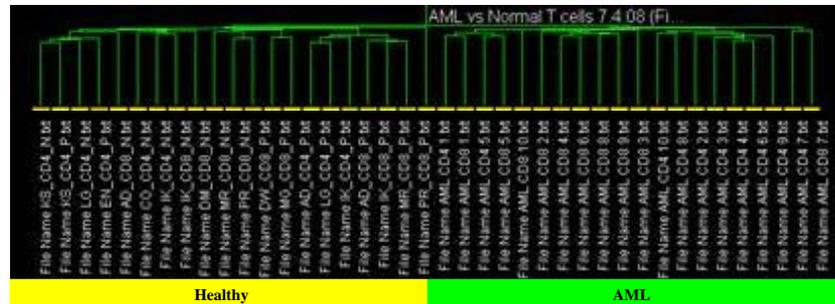
The fluorochromes APC, PE and FITC are listed at the top. The isotype of each antibody is listed in brackets. Tubes 11-13 represent isotype control tubes.

## 6.4 Results

### 6.4.1 Gene Expression Microarray

#### 6.4.1.1 Unsupervised Clustering

Unsupervised clustering of all samples using all probesets resulted in a clear separation of healthy and AML T cells illustrating the global differences in gene expression profile between T cells from patients with AML and healthy individuals (**Figure 6.8**). AML T cells did not cluster according to FAB type or prognostic group. Therefore, using gene expression profiling, it is possible to differentiate AML from healthy individuals by analysis of T cells that are not part of the malignant clone.



**Figure 6.8: Unsupervised Clustering of All samples and All Probesets**

#### 6.4.1.2 Generation of Gene-lists

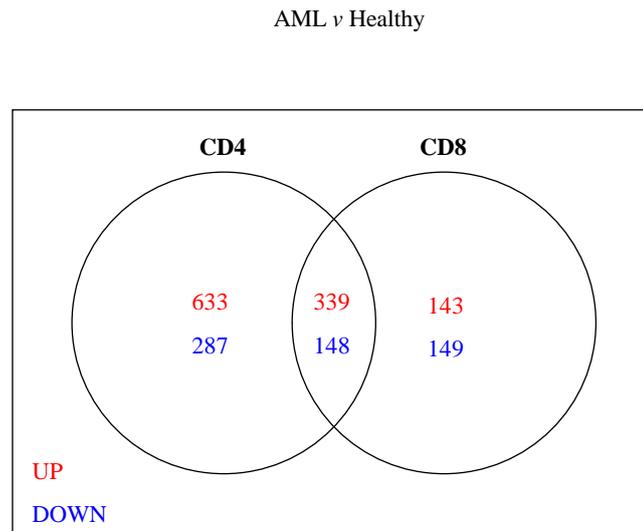
The broad gene expression differences between AML and healthy T cells are also demonstrated by the fact that out of 54675 probesets analysed, using standard filtering criteria of  $FDR < 0.05$ , 16% are differentially expressed in AML compared with healthy CD4 cells and 10% in CD8 cells. As this was far too great a number of genes to work with, more stringent filtering criteria were applied;  $FDR < 0.01$  and fold change  $> 2$ . This resulted in 1407 differentially expressed genes for CD4 and 779 for CD8 (**Table 6.12**). It was felt to be inappropriate to apply yet more stringent filters as clearly there are broad differences between the two groups. Manipulating the gene-lists to result in much smaller numbers of genes may have been more workable but would not have made biological sense.

Differentially Expressed Probesets AML v Healthy		
Analysis	CD4	CD8
FDR < 0.05	8745	5648
FDR < 0.01	1407	779
FC > 2		

**Table 6.12: Differentially Expressed Probesets Identified Using Different Filtering Parameters**

Further analysis was continued using the probesets marked in red. FC: fold change.

More genes were up-regulated than down-regulated in AML T cells. In addition, there was found to be a large overlap in the probesets that were differentially expressed in CD4 and CD8 cells. For CD4, this represented 35% of the probesets and for CD8, 63% (**Figure 6.9**). This is not an unfamiliar phenomenon. When the data from CLL compared with healthy T cells previously published was reanalysed using Bioconductor with a FDR<0.05, 43% of the probesets differentially expressed in CD4 cells were also differentially expressed in CD8 cells and 8% of the CD8 probesets were also present in the CD4 list.

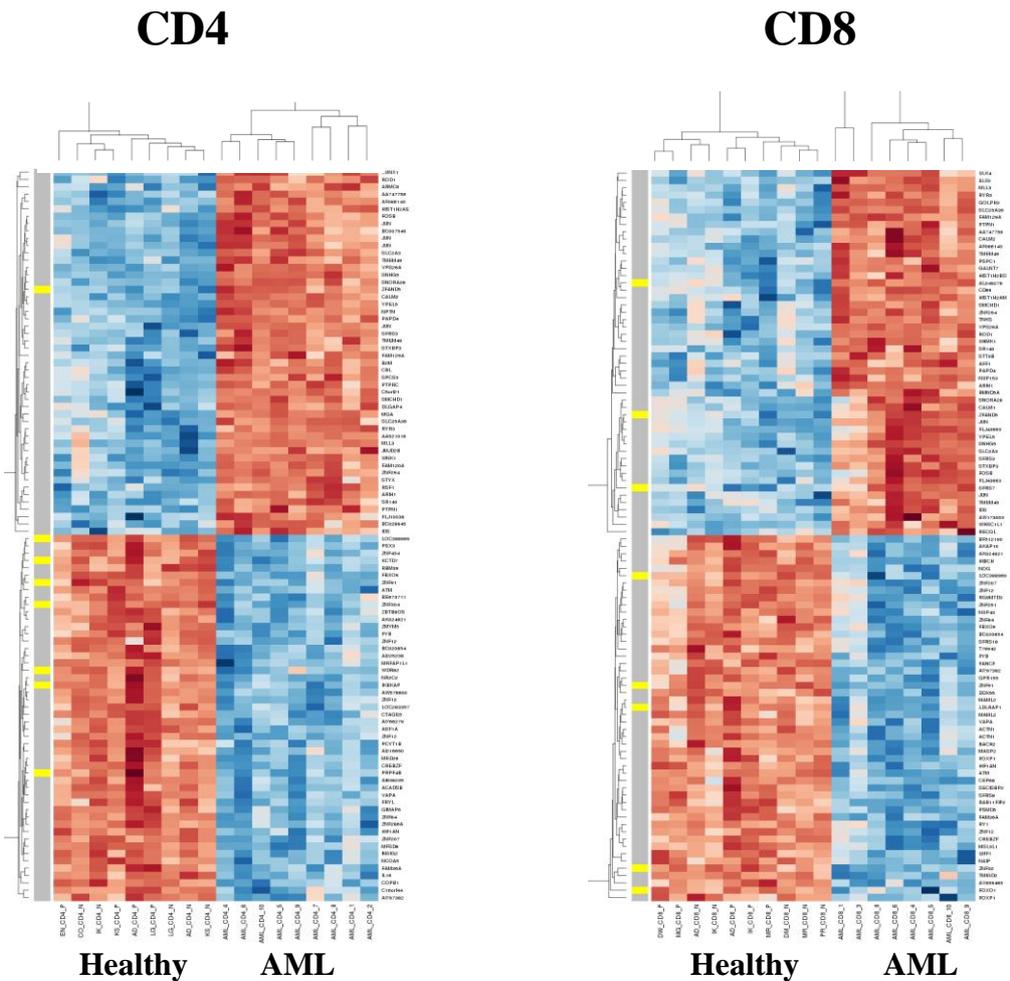


**Figure 6.9: Venn Diagram Illustrating the Distribution of Differentially Regulated Probesets**

Filtering criteria used were  $FDR < 0.01$ ,  $Fold\ Change > 2$ . Up-regulated genes are shown in red and down-regulated in blue.

### 6.4.1.3 Supervised Clustering

Supervised analysis was performed using the top 50 most significantly up and down-regulated genes. The heatmap generated is shown in **Figure 6.10** below.



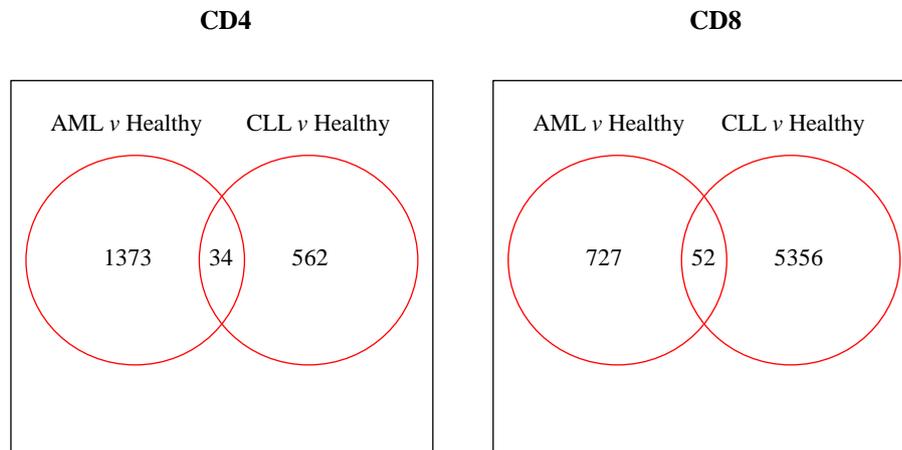
**Figure 6.10: Supervised Analysis: AML *versus* Healthy T cells**

Heatmap created using the top 50 most significantly up and down-regulated genes for CD4 and for CD8 in the comparison AML *v* Healthy. Up-regulated genes are in red and down-regulated in blue. The yellow bars on the otherwise grey vertical bar highlight genes that were also found to be differentially expressed in the CLL *v* Healthy experiment

#### 6.4.1.4 Comparison of Differentially Expressed Genes Between AML and CLL T Cells

The AML dataset was compared to that generated in the CLL *versus* healthy T cell comparison performed using Bioconductor. For CD4 T cells in CLL, of 1039 probesets found to be differentially expressed, only 34 (2.4% of AML CD4 probesets) were common to the AML and CLL gene-lists. For CD8 CLL T cells, 5851 probesets were identified as differentially expressed of which 52 were also

present in the AML gene-list (6.7% of AML probesets) (**Figure 6.11**). The full gene-lists can be found in **Appendix C**.



**Figure 6.11: Common Genes in AML and CLL T Cell Datasets**

Pathway analysis of these lists using Ingenuity software did not indicate involvement of any one particular pathway. Although it was interesting to note that four genes from the CD8 list, *KRAS*, *PLEC1*, *TTN* and *HGF*, are all involved in actin organisation although only *PLEC1* moved in the same direction in both the CLL and AML T cell datasets. Therefore, this analysis suggests that the gene expression changes observed in AML T cells are different to those seen in CLL.

#### 6.4.1.5 Impact of Clinical and Biological Parameters on AML T Cell Gene Expression Patterns

It was questioned whether the gene expression profile of the T cells in a patient with AML varied depending on clinical or biological parameters. Therefore, the AML T cell gene expression profiles were also examined on their own to investigate if certain characteristics resulted in differential gene expression patterns. Prognostic category (favourable or intermediate), response to first line

therapy (CR or fail), relapse (yes or no), absolute T cell count (above or below median value) and absolute CD3+56+ cell count (above or below median value) were investigated. This supervised analysis using an FDR <0.05 and fold change >2 resulted in the identification of a total of only four genes shown in **Table 6.13**.

Probeset	logFC	FDR	Analysis	Symbol	Acc	
					Number	Description
242782_x_at	1.559505	0.03374	Favourable v Intermediate Relapse v	TMEM198	BF307733	transmembrane protein 198
218359_at	2.562301	0.0005	No Relapse Relapse v	NRSN2	NM_024958	neurensin 2
243784_s_at	-3.84362	0.008415	No Relapse Relapse v		AW085489	NA
1559109_a_at	-3.16812	0.043727	No Relapse		BE147896	NA

**Table 6.13: Differentially Expressed Genes Found on Supervised Analysis of the AML T Cell Samples Only**

FC: fold change, FDR: false discovery rate.

Firstly, this is a very small list of genes therefore this data would suggest that it is unlikely that any T cell factor is impacting on patient clinical outcome. Nor would it seem that either the absolute T cell count or the quantity of our population of interest, CD3+56+ cells, makes any difference to the gene expression profile seen. The significance of the four genes found is difficult to interpret. Only one gene was found in the favourable *versus* intermediate prognostic group analysis. *TMEM198* has only a single reference in PubMed that is of no significance. More interesting are the three genes found to be differentially expressed in the relapse *versus* no relapse comparison. *NRSN2* may play a role in maintenance and transport of vesicles and has previously been studied in neural tissue (Nakanishi, *et al* 2006) which may have relevance for T cell cytotoxic capability. Furthermore investigation of AW085489 using the UCSC browser (<http://genome.ucsc.edu/>) suggests the probe binds to the 5'UTR of *FUBP3*, a potent transcriptional activator. A family member *FUPB1* is known to bind to the Far Upstream Element (FUSE) region of the *MYC* proto-oncogene.

#### 6.4.1.6 Impact of T Cell Purity on Differential Gene Expression Pattern

As the purity of T cells varied across samples, a linear model was used to assess the impact of T cell purity on the gene expression changes. If there were a large number of genes whose expression covaried with purity then it might be expected that the differences between AML and healthy T cells might be driven by this factor instead. A linear model was fitted to the expression data (log-expression values) for each probe. A line of best fit was overlaid onto a scatter plot displaying the purity of sample 'I' on the x-axis and the expression on the y-axis, defined as:

$$y_I = a + bx_I + e_I$$

where  $a$  = the intercept of the line with the y-axis (average expression value if there is no purity effect),  $b$  = the slope of the line,  $e$  = random error. Genes that had a purity dependence were defined as having a slope ( $b$ ) which was non-zero. If many such genes were identified in this fashion, the implication would be that a proportion of the differentially expressed genes found in the AML *versus* healthy comparison were there due to purity effect. Using an FDR < 0.05, no significant genes were found for either CD4 or CD8 suggesting that purity does not have an impact on the gene-lists generated.

#### 6.4.1.7 Pathway Analysis

The ten most up and down-regulated genes in AML compared with healthy T cells are listed in **Tables 6.14 and 6.15**.

<b>FOSB</b>	FBJ murine osteosarcoma viral oncogene homolog B
<b>JUN</b>	JUN oncogene
<b>HIST1H2AE</b>	histone cluster , H2ae
<b>THRAP3</b>	thyroid hormon receptor associated protein 3
<b>C13ORF15</b>	chromosome 13 open reading frame15
<b>HIST1H2BC</b>	histone cluster 1, H2bc
<b>SOX4</b>	SRY (sex determining region) box 4
<b>FOS</b>	V-fos FBJ murine osteosarcoma viral oncogene
<b>BAT2D1</b>	BAT2 domain containing 1
<b>YPEL5</b>	yippee-like 5 (Drosophila)
<b>LTF</b>	lactotransferrin
<b>HBB</b>	haemoglobin beta
<b>HBA2</b>	haemoglobin A2
<b>KLRC2</b>	killer cell lectin-type receptor subfamily C, member 2
<b>ID3</b>	inhibitor of DNA binding 3
<b>PCDH9</b>	protocadherin 9
<b>LRRN3</b>	leucine rich repeat neuronal 9
<b>PECR</b>	peroxisomal trans-2-enoyl-CoA reductase
<b>ANKS1A</b>	ankyrin repeat and sterile alpha motif domain contain 1A
<b>FYB</b>	FYN binding protein

**Table 6.14: CD4 AML v Healthy - Top 10 Up-regulated (red) and Down-regulated (blue) Genes**

Genes indicated in bold have biological relevance in T cells.

*JUN* is an oncogene that forms part of the AP1 transcription factor family. It dimerizes with *FOS* to form AP1, a critical component of the T cell activation pathway. *FOSB* is also part of the Fos gene family and also forms dimers with members of the Jun family to form AP1. *KLRC2* (or *NKG2C*) is an inhibitory receptor most frequently expressed by NK cells. *ID3* is preferentially expressed in T<sub>H</sub>1 cells (Lund, *et al* 2005) and *FYB* is involved in the TCR signalling pathway as part of the FYN and SLP-76 signalling cascade (da Silva, *et al* 1997).

<b>FOSB</b>	FBJ murine osteosarcoma viral oncogene homolog B
<b>JUN</b>	JUN oncogene
HIST1H2AE	histone cluster 1, H2ae
HIST1H2AM	histone cluster 1, H2am
<b>FOS</b>	V-fos FBJ murine osteosarcoma viral oncogene
HIST1H2BG	histone cluster 1, H2bg
C10ORF46	chromosome 10 open reading frame 46
HNRPA3	heterogeneous nuclear ribonucleoprotein A3
NXT1	NTF2-like export factor 1
HIST1H2BH	histone cluster 1, H2bh
LTF	lactotransferrin
HBA1	haemoglobin alpha 1
HBB	haemoglobin beta
HBA2	haemoglobin alpha 2
LRRN3	leucine rich repeat neuronal 3
DSC1	desmocollin 1
ORM1	Orosomuroid 1
<b>RUNX2</b>	runt-related transcription factor 2
DECR2	2,4-dienoyl CoA reductase 2, peroxisomal
<b>RTN1</b>	reticulon 1

**Table 6.15: CD8 AML  $\nu$  Healthy - Top 10 Up-regulated (red) and Down-regulated (blue) Genes**

Genes indicated in bold have biological relevance in T cells.

*FOSB*, *JUN* and *FOS* have been discussed above. *RUNX2* is involved in T cell development accelerating development to a CD8 immature single positive stage (Vaillant, *et al* 2002) and *RTN1* modulates the anti-apoptotic activity of Bcl-xl and Bcl-2 (Tagami, *et al* 2000).

Examining the gene-lists revealed a large number of transmembrane receptors and other genes of potential interest in T cells. These are listed in **Tables 6.16 and 6.17**.

---

**TRANSMEMBRANE RECEPTORS /**

KLRC2 (NKG2-C)	killer cell lectin-like receptor subfamily C, member 2
KLRD1 (CD94)	killer cell lectin-like receptor subfamily D, member 1
MR1	major histocompatibility complex, class I-related
CLEC2D	C-type lectin domain family 2, member D
IGHM	immunoglobulin heavy constant mu
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)
FCGR1B	Fc fragment of IgG, high affinity Ib, receptor (CD64)
LILRB3	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3
B2M	beta-2-microglobulin
CCR1	chemokine (C-C motif) receptor 1
IFNGR1	interferon gamma receptor 1
TGFBR2	transforming growth factor, beta receptor II (70/80kDa)
LTBR	lymphotoxin beta receptor (TNFR superfamily, member 3)
CLEC7A (dectin 1)	C-type lectin domain family 7, member A
MSR1	macrophage scavenger receptor 1

**CELL SURFACE MOLECULES**

CD47	CD47 molecule
CD48	CD48 molecule
CD9	CD9 molecule
CD36	CD36 molecule (thrombospondin receptor)
CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)
CD68	CD68 molecule
CD69	CD69 molecule

**INTEGRINS**

ITGA6	integrin, alpha 6
ITFG2	integrin alpha FG-GAP repeat containing 2
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)

**SECRETED MOLECULES**

TNFSF13	tumor necrosis factor (ligand) superfamily, member 13
IL18	interleukin 18 (interferon-gamma-inducing factor)

**CELL CYCLE COMPONENTS**

ATM	ataxia telangiectasia mutated
CCNC	cyclin C
CCND2	cyclin D2
CCNI	cyclin I
CCNL1	cyclin L1
CDC14A	CDC14 cell division cycle 14 homolog A ( <i>S. cerevisiae</i> )
CDKN1A	cyclin dependent kinase inhibitor 1A (p21)

**CALCIUM SIGNALLING**

RCAN2	regulator of calcineurin 2
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive
NFATC2IP	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein

---

CALM1	calmodulin 1
CALM2	calmodulin 2
<b>T CELL ACTIVATION</b>	
TAGAP	T-cell activation RhoGTPase activating protein
PIK3CB	phosphoinositide-3-kinase, catalytic, beta polypeptide
PIK3CG	phosphoinositide-3-kinase, catalytic, gamma polypeptide
<b>VESICLE TRANSPORT</b>	
VAPA	VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa
LAMP2	lysosomal-associated membrane protein 2
<b>TRANSCRIPTION FACTORS</b>	
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
JUN	jun oncogene
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
<b>MICRO RNAs</b>	
MIRN21	microRNA 21

---

**Table 6.16: CD4 AML v Healthy – Genes of Potential Relevance**

Down-regulated genes in AML T cells compared with healthy T cells are indicated in blue. Up-regulated genes are indicated in red.

---

<b>TRANSMEMBRANE RECEPTORS</b>	
CR2	complement component (3d/Epstein Barr virus) receptor 2
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)
IL6R	interleukin 6 receptor
CCR7	chemokine (C-C motif) receptor 7
IGF1R	insulin-like growth factor 1 receptor
MR1	major histocompatibility complex, class I-related
IFNGR1	interferon gamma receptor 1
B2M	beta-2-microglobulin
<b>CELL SURFACE MOLECULES</b>	
CD48	CD48 molecule
CD69	CD69 molecule
<b>INTEGRINS</b>	
ITGA6	integrin, alpha 6
ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
<b>CELL CYCLE COMPONENTS</b>	
ATM	ataxia telangiectasia mutated
CCNL1	cyclin L1
CDK6	cyclin-dependent kinase 6
CDC14A	CDC14 cell division cycle 14, homolog A
CDC2	cell division cycle 2, G1 to S and G2 to M
CDCA7	cell division cycle associated7
<b>CALCIUM SIGNALLING</b>	
NFATC2IP	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein
CALM1	calmodulin 1
CALM2	calmodulin 2
<b>T CELL ACTIVATION</b>	
PRKCA	protein kinase C, alpha

---

PIK3CG	phosphoinositide-3-kinase, catalytic gamma polypeptide
PIK3R1	phosphoinositide-3-kinase regulatory subunit 1
<b>VESICLE TRANSPORT</b>	
VAPA	VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa
LYST	lysosomal trafficking regulator
VTI1A	vesicle transport through interaction with t-SNAREs homolog 1A (yeast)
<b>TRANSCRIPTION FACTORS</b>	
JUNB	jun B proto-oncogene
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
JUN	jun oncogene
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
<b>ACTIN CYTOSKELETON</b>	
ACTN1	actinin, alpha 1
ABLIM1	actin binding LIM protein 1
FILIP1	filamin A interacting protein 1
TRAK2	trafficking protein, kinesin binding 2

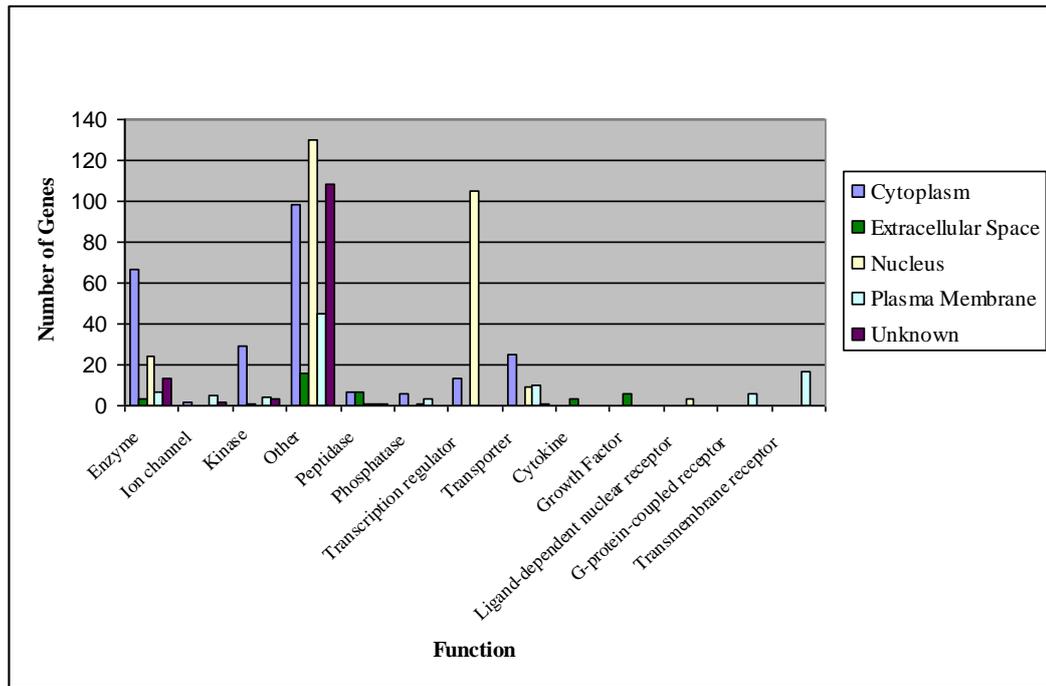
---

**Table 6.17: CD8 AML  $\nu$  Healthy - Genes of Potential Relevance**

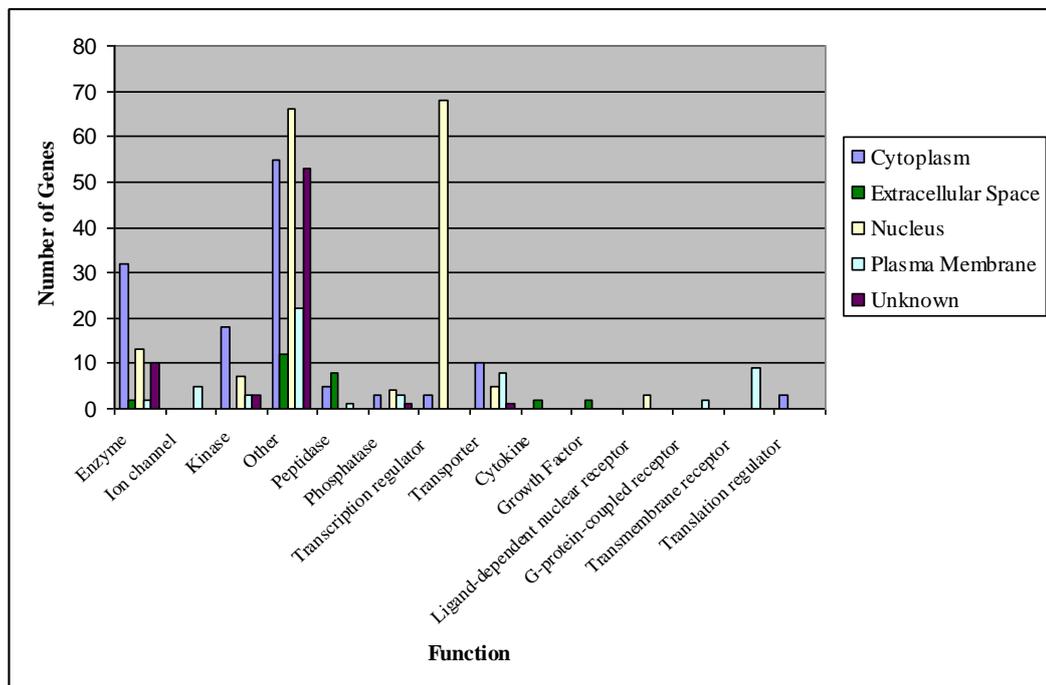
Down-regulated genes in AML T cells compared with healthy T cells are indicated in blue. Up-regulated genes are indicated in red.

Of particular interest were the genes noted to be involved in the actin cytoskeleton that were picked up in the CD8 gene-list. Subsequent analysis of the CLL dataset had identified abnormalities in the actin cytoskeletal pathways in both CD4 and CD8 cells that had led to further functional investigation of these defects by visualising actin polarisation in immunological synapses (Ramsay, *et al* 2008a).

To obtain biological meaning from these gene-lists, data was first analysed using Ingenuity software. Globally, for both CD4 and CD8, many genes fell into the function ‘other’ and location ‘unknown’. Transcription regulators, enzymes, kinases and transporters were most well represented within both lists with the most frequent locations being the nucleus and cytoplasm (**Figures 6.12 and 6.13**).



**Figure 6.12: CD4 AML v Healthy - Location and Function of Differentially Expressed Genes**



**Figure 6.13: CD8 AML v Healthy - Location and Function of Differentially Expressed Genes**

Biological processes and potential pathway involvement of the differentially expressed genes was subsequently examined. Again, much similarity was noted between CD4 and CD8. Gene expression, cell proliferation and haematological system development and function featured in both lists as notably, did cancer. IL-2 and TCR signalling were also common relevant pathways (**Tables 6.18 and 6.19**).

<b>Top Functions</b>	<b>Top Pathways</b>
Gene Expression	IL-2 Signalling
Cancer	EGF Signalling
Cell Morphology	B cell receptor Signalling
Cell Death	GM-CSF Signalling
Cellular Development	PDGF Signalling
Haematological System Development and Function	JAK/STAT Signalling

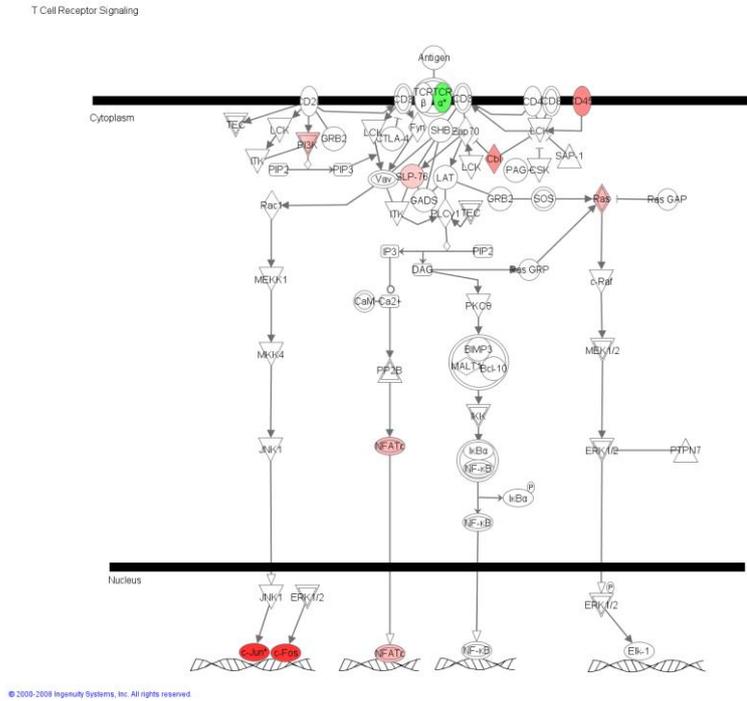
**Table 6.18: CD4 AML v Healthy - Top Functions and Pathways Identified by Ingenuity**

<b>Top Functions</b>	<b>Top Pathways</b>
Cancer	PDGF Signalling
Cell Morphology	IGF-1 Signalling
Connective Tissue Disorders	EGF Signalling
Gene Expression	14-3-3-mediated Signalling
Cellular Development	TCR Signalling
Haematological System Development and Function	IL-2 Signalling

**Table 6.19: CD8 AML v Healthy - Top Functions and Pathways Identified by Ingenuity**

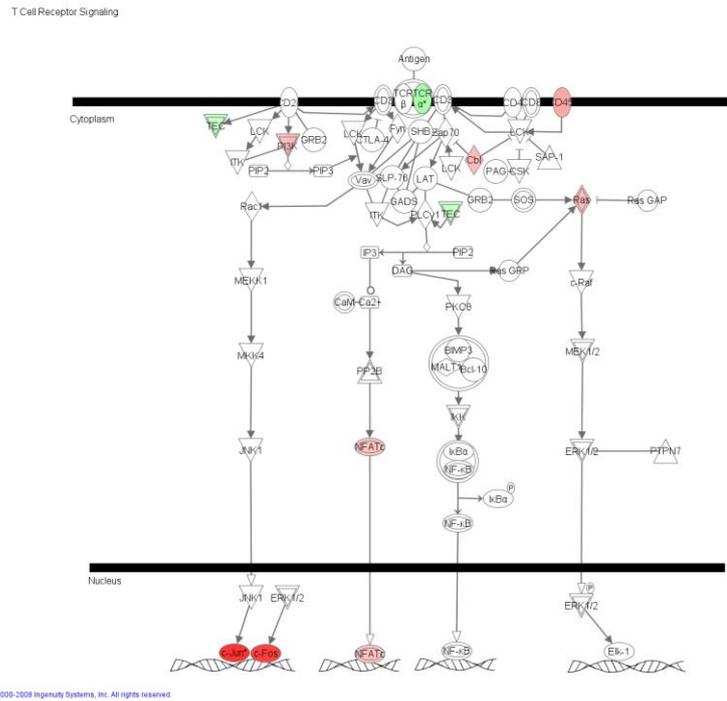
Genes involved in the TCR signalling pathways are illustrated (**Figures 6.14 and 6.15**). Of note, contrary to initial expectation, only in CD4 T cells were significant

numbers of genes involved in the actin cytoskeleton pathway (as defined by Ingenuity software) (**Figure 6.16**).



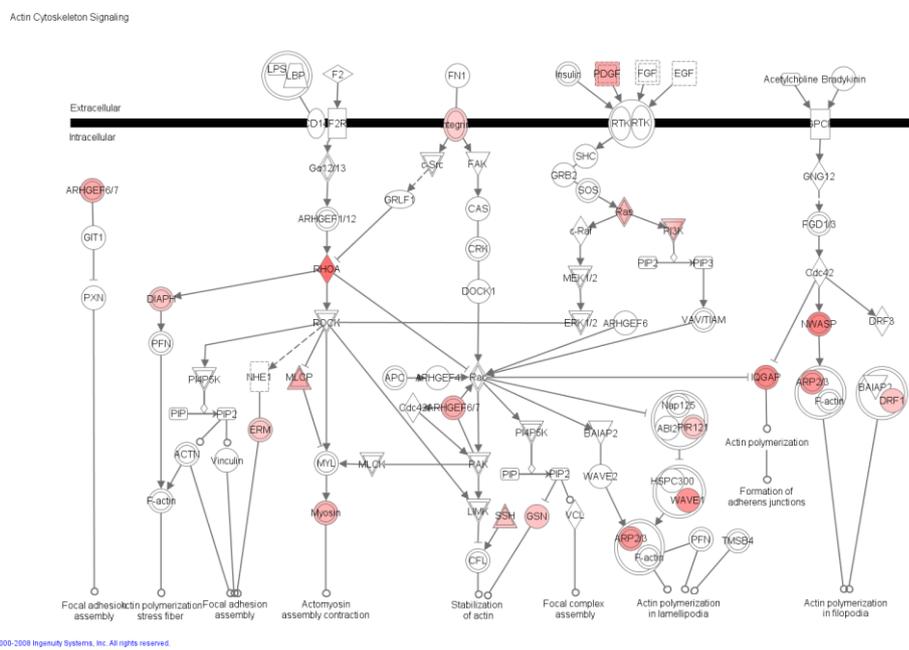
**Figure 6.14: TCR Signalling Pathway for CD4 AML v Healthy analysis.**

Genes from within the gene-list are distinguished in colour with red indicating up-regulation and green, down-regulation.



**Figure 6.15: TCR Signalling Pathway for CD8 AML v Healthy Analysis**

Genes from within the gene-list are distinguished in colour with red indicating up-regulation and green, down-regulation.



**Figure 6.16: Actin Cytoskeleton Signalling Pathway for CD4 AML v Healthy Analysis**

Genes from within the gene-list are distinguished in colour with red indicating up-regulation.

To try to identify which genes within the lists were relevant to T cells, a search was performed on PubMed of each gene name in association with 'T cell'. From the list of 1407 genes for CD4, 179 were found to have associations with T cells. From the list of 779 genes for CD8, 120 were found to have associations with T cells. These genes were then categorised into signalling pathways (**Table 6.20**). For both CD4 and CD8, the pathways that genes were most frequently associated with were TCR signalling and activation.

CD4		CD8	
Signalling Pathway	Genes	Signalling Pathway	Genes
TCR signalling	25	T cell activation	27
WNT signalling	16	TCR signalling	14
Actin cytoskeleton	12	Proliferation	9
T cell activation	10	AP1 signalling	8
Adhesion/migration	9	Cytokine signalling	8
Receptors	8	Apoptosis	6
TH1 v TH2	7	WNT signalling	6
AP1 pathway	6	Motility	5
Co-stimulation	6	Ras signalling	4
IL-2 signalling	6	Ca <sup>2+</sup> signalling	3
Apoptosis	6	Vesicle transport	3
Cytokine signalling	6	Lymphocyte development	3
Vesicle transport	5	Actin cytoskeleton	3
TNF signalling	5	SDF1 $\alpha$ induced chemotaxis	3
TGF $\beta$ signalling	4	Cell survival/homeostasis	3
MHC	4	TGF $\beta$ signalling	2
Lectins	3	Adhesion	2
CD4 down-regulation	3	MHC	2
Extracellular matrix	3	Complement	2
Ca <sup>2+</sup> signalling	3	DNA repair	2
Memory v Effector	3	Immune synapse	2
Cytotoxicity	2	Cytotoxicity	2
Cell fate	2	TCR	2
NOTCH signalling	2	Co-stimulation	1
Homeostasis T cell numbers	2	Reduced transcription	1
MicroRNA	2	NOTCH signalling	1
DNA repair	2	Transport	1
IL-4 signalling	1	T cell homing	1
Cell survival	1	TNF signalling	1
Recombination of TCR	1	Tumour-specific T cells	1
Proliferation	1	NK-like killing	1
Transcription	1	PDGF signalling	1
		IFN $\alpha$ signalling	1
		IFN $\gamma$ signalling	1
		BMP signalling	1

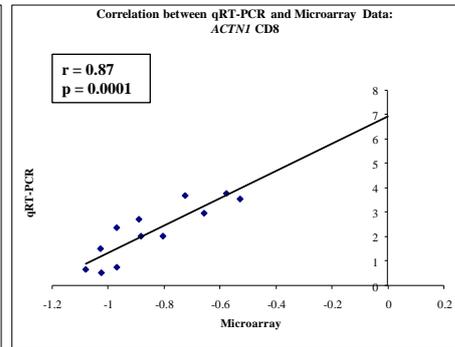
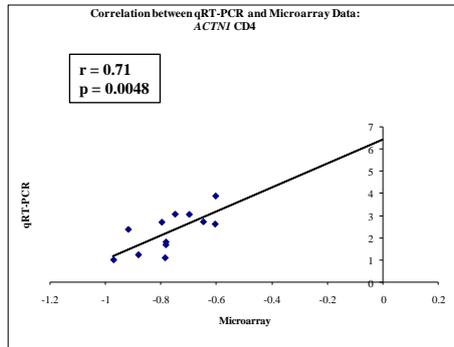
**Table 6.20: PubMed Analysis of Gene Function**

This data suggests that T cells from patients with AML are more likely to be activated in comparison with healthy T cells. However, a comparison was made with the gene-lists from Wang et al. (Wang, *et al* 2008) that had looked at the pattern of gene expression on normal T cell activation. Only 6.8% of the CD4 AML  $\nu$  Healthy gene-list and 10.7% of the CD8 AML  $\nu$  Healthy list were present in the list of genes differentially expressed on T cell activation. This indicates that the gene expression changes seen in T cells from patients with AML are not simply due to the normal signalling that occurs on T cell activation.

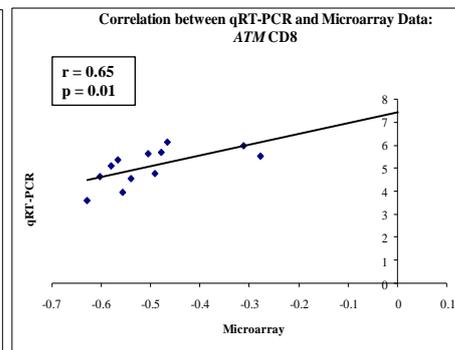
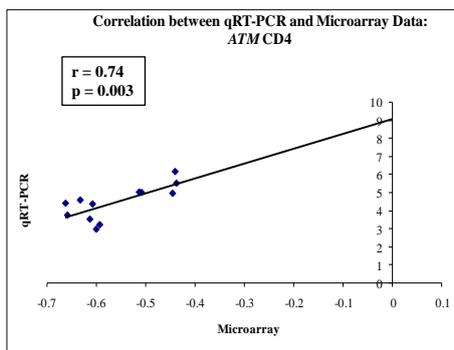
#### **6.4.2 qRT-PCR Validation**

Only one out of the 10 correlations tested failed to show a statistically significant correlation between the microarray result and the qRT-PCR result. This was CD48 in CD8 cells which was shown to have a -1.2 log(2) fold change in the microarray experiment (**Figure 6.17**).

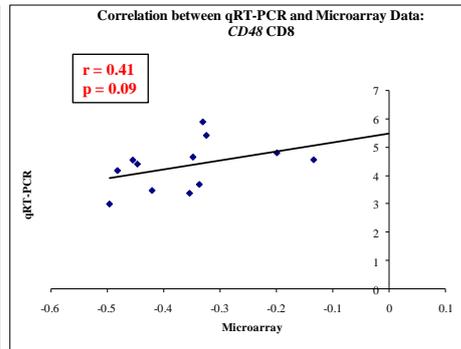
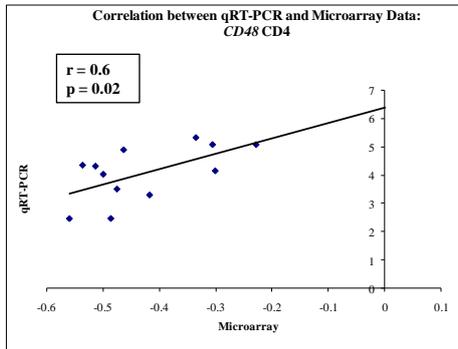
**ACTN1**



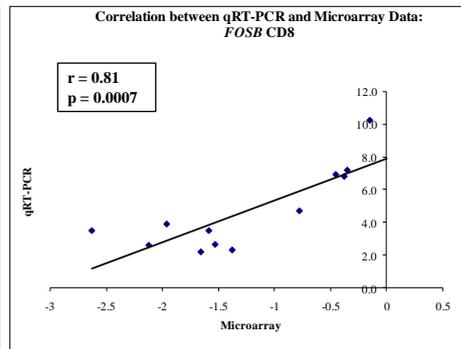
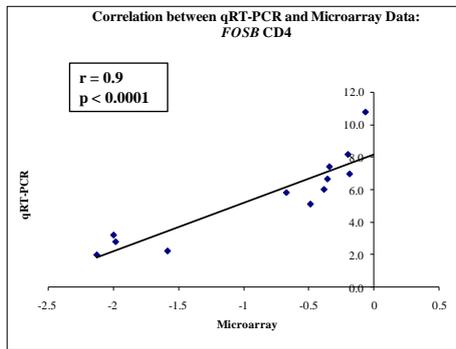
**ATM**

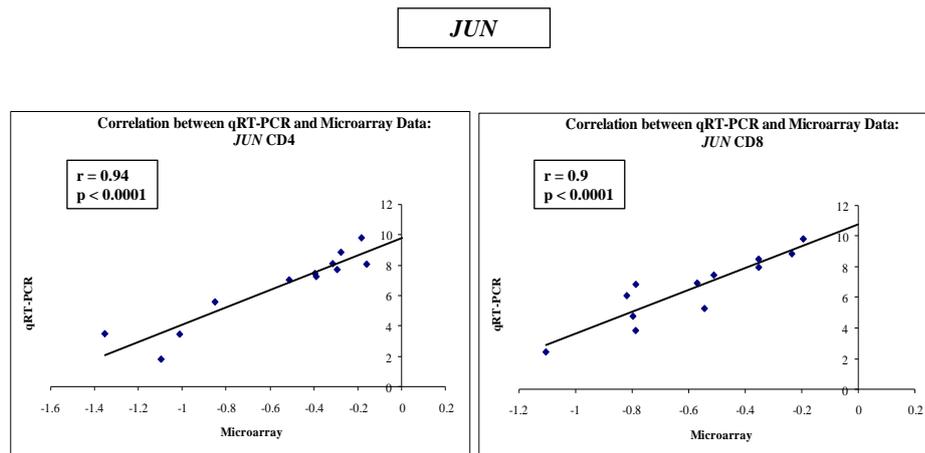


**CD48**



**FOSB**



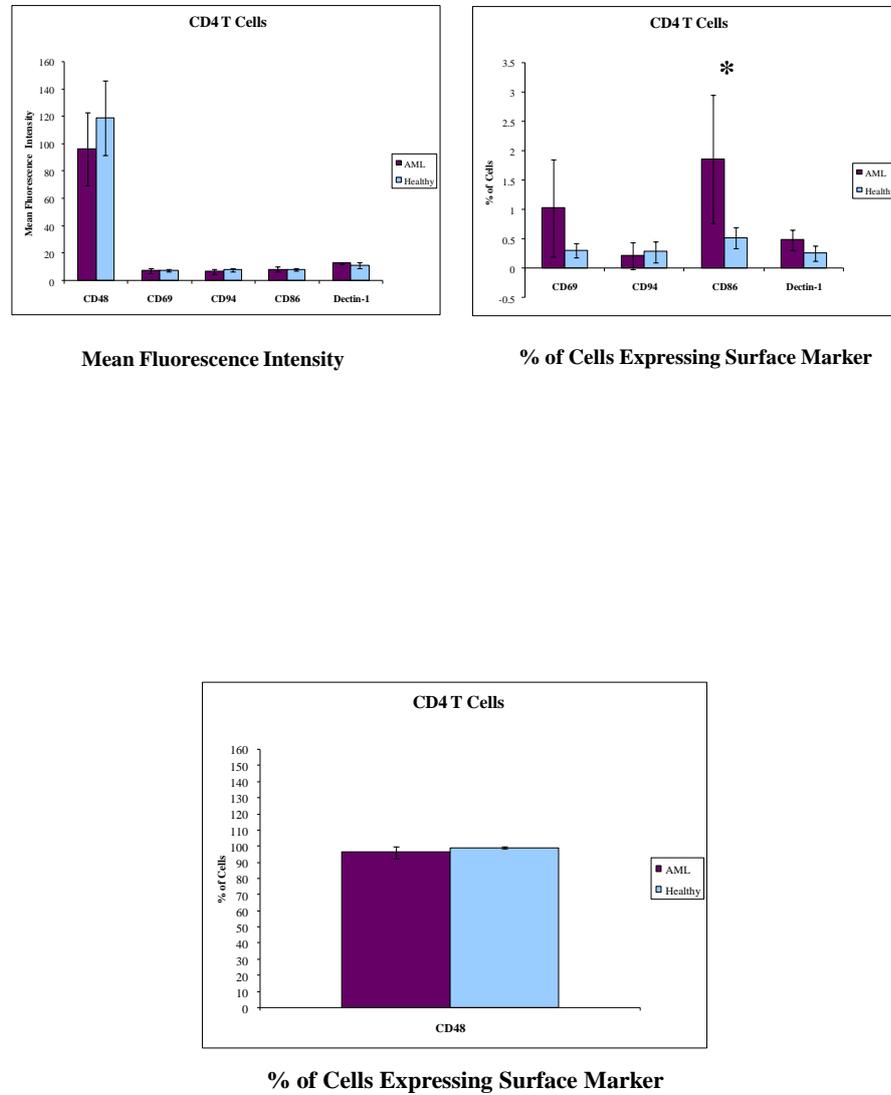


**Figure 6.17: qRT-PCR Validation of Microarray Data**

Correlations between microarray and qRT-PCR data are shown for CD4 and CD8 T cells for the genes *ACTN1*, *ATM*, *CD48*, *FOSB* and *JUN*.

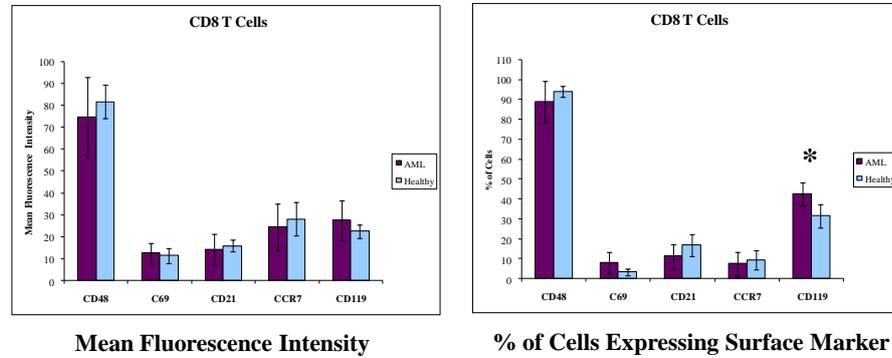
### 6.4.3 Protein Validation

Five cell surface markers found to have significant differential expression between AML and healthy T cells by gene expression were examined by flow cytometry in CD4 cells and five in CD8. Both mean fluorescence intensity (MFI) (reflecting the level of expression on each cell) and the percentage of cells expressing the marker (reflecting the level of expression within the population of T cells) were assessed. All markers were found to move in the expected direction (up or down in AML cells compared with healthy) except for CD69 MFI on CD4 cells. However, the only statistically significant difference in expression was seen for the percentage of CD4 cells expressing CD86 and the percentage of CD8 cells expressing CD119 (**Figure 6.18 and 6.19**).



**Figure 6.18: CD4 T Cells - Protein Validation of Microarray Data**

Flow cytometric assessment of the expression of the surface markers CD48, CD69, CD94, CD86 and Dectin-1 found to be differentially expressed in CD4 cells in AML patients compared with healthy donors. Both MFI and the mean percentage of cells expressing the marker were assessed. \* indicates p value < 0.05.



**Figure 6.19: CD8 T Cells - Protein Validation of Microarray Data**

Flow cytometric assessment of the expression of the surface markers CD48, CD69, CD21, CCR7 and CD119 found to be differentially expressed in CD8 cells in AML patients compared with healthy donors. Both MFI and the mean percentage of cells expressing the marker were assessed. \* indicates  $p$  value  $< 0.05$ .

## 6.4 Discussion

These experiments have demonstrated that there are global differences in gene expression between AML and healthy T cells for both CD4 and CD8. The focus of these gene expression changes appears to be the nucleus with an up-regulation of molecules involved in gene transcription. A primary pathway that appears to be affected is TCR signalling and T cell activation. The costimulatory molecule *CD86* is unregulated in CD4 T cells in AML as is the accessory molecule *GRAP*. Molecules involved in calcium signalling include *CALM1*, *CALM2*, *RYR3*, *STXBP3* and *ITPR1*. Two molecules related to NFAT were found to be up-regulated; *NFATC2IP* and *NFAT5*. Three crucial downstream signalling pathways had genes represented; MAPkinase pathway – *MAP3K8*, *MAP4K4*, *MAP3K3*; PI3K and protein kinase C pathway; *PI3CG* and *PRKCA* and the AP1 pathway; *JUN*, *JUNB*, *KLF6*, *TNPO1*, *FOSB* and *FOS*. Up-regulation of the early cell surface activation marker, *CD69* is seen. One terminal event of T cell activation is proliferation and the cell cycle genes *CCND2*, *CDC2* and *CDK6* are up-regulated in AML T cells. However the other product of T cell activation, the production of IL-2 is not apparent. Nonetheless, these changes do not correlate with those described by Wang and colleagues in an *in vitro* T cell activation gene expression assessment. Some changes are fundamentally different; *FOSB* and *JUN* were the most up-regulated genes found in both cell types yet they appeared down-regulated in the Wang paper. Thus the gene expression changes seen in AML T cells cannot be ascribed to T cell activation alone. Anergy is closely related to activation: NFAT activation in the absence of concurrent activation of transcription factors such as AP1 or NFκB results in anergy. However, a search for 14 anergy-related genes (Dominguez-Villar, *et al* 2007) within the AML v Healthy gene-lists revealed the differential expression of only two, *JMJD2B* and *RAB10*.

An important observation from the genes relevant in T cells is the frequency of genes associated with the actin cytoskeleton and cellular polarisation on activation. For CD4, *WASL*, *WAVE*, *ABII*, *ARP2*, *IQGAP1*, *EVL*, *SNX9* and *CAPZA1* are all either associated with actin or involved in actin remodelling whereas *CTBP2* is a cytoskeletal regulator, *CTNNA1* is a component of the

cytoskeleton and *MYH9* is associated with LFA-1 at the uropod of migrating T cells and links directly to the cytoskeleton. All of these are up-regulated apart from *EVL*. For CD8 cells, the list is shorter but *ACTN1* and *CAPZA1* are actin binding proteins, *FILIP1* interacts with filamin1 and is therefore involved in intracellular motility processes and *PLEC1* is a cytoskeletal organiser. All of these are down-regulated apart from *CAPZA1*. There are also genes involved in the related function of T cell motility; for CD4, *OPA1*, *RAP1B* are up-regulated and the adaptor protein *RASSF5* is down-regulated; for CD8, *DOCK2* and *OPA1* are up-regulated. Of interest, the lymphocyte homing receptor, *CCR7*, is down-regulated in CD8 cells. Several genes involved in cell adhesion are up-regulated in CD4 cells; *RHOA*, *RHOB*, *ADAM28*, *LGALS8* and *LPXN*. MicroRNA21 was noted to be up-regulated in CD4 AML T cells. However, it has not so far been implicated in regulation of immune cell function (Baltimore, *et al* 2008) although levels have been shown to be up-regulated in healthy CD8 cells (Wu, *et al* 2007). These observations would suggest that there may be defects in actin cytoskeletal remodelling and T cell motility in AML T cells.

The issue regarding contaminating tumour cells impacting on the gene expression profile was addressed. Firstly, every attempt was made to obtain as pure a T cell population as possible. This required several modifications of the standard cell separation protocol. The cell populations eventually used for microarrays had a mean purity of 85.9% for CD4 and 92.3% for CD8. Secondly, a biostatistical linear model was used in analysis. This suggested that the variation in T cell purity had no impact on the gene expression changes seen. In addition, the CLL study had also noted in supervised analyses no significant difference in the gene expression profiles based on cell purity less than or greater than 85%. The biological function cancer did feature prominently in pathway analysis of both CD4 and CD8 gene-lists. Nonetheless, these genes are normal genes involved in cell cycle control, proliferation and apoptosis so although they are associated with cancer they are not specific to it.

The wide overlap in the gene-lists for CD4 and CD8 cells was notable but not unexpected. A similar phenomenon was noted in the CLL data and published data

has shown CD8 T cells to have an expression signature of 23 genes whereas for CD4 cells it is only four genes indicating that the vast majority of gene expression is similar (Palmer, *et al* 2006).

It was also of note that the gene expression changes documented were not the same as those seen in CLL T cells both in terms of the numbers of common genes and also pathways. Neither the RAS-dependent JNK MAPK pathway nor cytotoxicity pathways featured prominently in the AML *versus* healthy comparison. This is perhaps not surprising. CLL is an indolent condition in contrast to the aggressive nature of AML. By the time patients with CLL come into contact with medical professionals often long periods of time will have elapsed since their condition began allowing many months of interaction with the immune system. In contrast, patients with AML will present within days or weeks of their malignancy developing resulting in much less time for tumour–T cell interactions to take place.

The microarray data correlated well with qRT-PCR data in the five genes examined across both cell populations with the exception of CD48 in CD8. This is unlikely to be due to the phenomenon of cross-hybridizing transcripts as a correlation was seen in CD4 cells. More likely is that the lack of correlation seen is due to the low fold change of CD48 in CD8 cells (-1.2 logFC). Small variations in mRNA levels are much more accurately detected by qRT-PCR than microarray expression scores resulting in discrepant correlation coefficients (Dallas, *et al* 2005).

An attempt was made to validate the gene expression changes seen by protein expression using flow cytometric assessment of differentially expressed surface markers. Unfortunately, only two out of the 10 markers examined, CD86 on CD4 cells and CD119 on CD8 cells, demonstrated the expected statistically significant difference in expression. All other markers, except for CD69 MFI on CD4 cells, showed a trend in the right direction. Flow cytometry would generally be considered to be more sensitive than western blotting for assessing protein.

However, using antibodies directed towards cell surface markers may not accurately reflect the total protein content of the cell due to cellular trafficking.

There are several issues that may impact on the validity of the data generated. Firstly, the AML GeneChips were processed at a time point much later than the normal chips. This could result in a potential batch effect; that is, the differences observed could be due to differences in processing rather than biology. However, although for practical purposes only a small number of genes could be validated by qRT-PCR, nonetheless, for this small selection, the changes observed by microarray were found to be true. Secondly, the AML and healthy samples were not age and sex matched. This was largely due to the fact that most AML patients are elderly (the median age for these samples was 62) and finding age-matched healthy donors was difficult from the donor pool available. With regards to sex, the choice of AML samples was limited to the patients from whom sufficient T cells could be obtained for the analysis, allowing no room for manoeuvre and the healthy GeneChips had already been processed by the time AML patients were selected.

In summary, these experiments have demonstrated a global difference in the gene expression profile between AML and healthy T cells with changes particularly noted in TCR signalling and activation suggesting both CD4 and CD8 T cells in AML are undergoing some form of activation via the TCR. However, this does not result in the expected changes in gene expression seen in published *in vitro* experiments suggesting some form of aberrant signalling. Numerous genes were found to be involved in actin cytoskeletal signalling and cellular motility indicating these functions may be impaired in AML T cells. Finally, the gene expression changes noted, both in terms of the number of common genes and pathways involved were different to those observed in CLL leading to the conclusion that there is a different molecular basis to the T cells defects observed in these very different diseases.

The changes noted in the expression of genes involved in the actin cytoskeletal pathway in both CD4 and CD8 T cells in AML led to a functional examination of this pathway in AML T cells as will be described in the next chapter.

## Chapter 7

### Immunological Synapse Formation in AML Patients

#### 7.1 Introduction

The functional implications of the gene expression changes previously noted in T cells in AML now required investigation. As genes within the actin cytoskeletal pathway had been found to be differentially regulated in AML compared with healthy T cells this was the pathway chosen for further study.

The actin cytoskeleton is critical for T cell activation. Firstly, actin filaments are fundamental for the large-scale molecular rearrangements necessary for the formation of the immunological synapse and secondly, the actin scaffold is required for the recruitment and stabilisation of signalling complexes involved in downstream signalling pathways (Dustin and Cooper 2000). On TCR engagement with appropriate MHC-peptide complexes, polarisation of the T cell actin cortical cytoskeleton occurs with a reorientation towards the T cell – APC interface where F-actin subsequently accumulates.

Previous work from our group demonstrated that the actin cytoskeletal pathway was differentially regulated in T cells from CLL patients compared with healthy T cells. We subsequently showed that immunological synapse formation was defective in CD4 and CD8 T cells in CLL due to impaired actin polymerisation (Ramsay, *et al* 2008a). We also established that recruitment of key actin regulatory proteins such as Cdc42, WASp, Filamin-A and Dynamin-2 to the immune synapse was inhibited. This impairment in immunological synapse formation was seen when CLL T cells were conjugated with autologous CLL B cells and also healthy allogeneic B cells. These defects could also be induced in healthy allogeneic T cells by culturing them with CLL cells. We went on to demonstrate the same defect in T cells from the E $\mu$ -TCL1 transgenic mouse model of CLL as the mice developed leukaemia (Gorgun 2009).

This led to the hypothesis that a similar defect in immune synapse formation may be present in T cells in both AML and CLL even though the differentially regulated genes noted in the actin cytoskeletal signalling pathway were not the same in AML T cells compared with CLL T cells.

## 7.2 Aims

The aim of this part of the study was to examine the functional consequences of the gene expression changes noted in AML T cells. Two questions were posed. Firstly, could AML blasts act as APCs to form immunological synapses when conjugated with healthy T cells? Secondly, could AML T cells form effective immune synapses with autologous blasts?

## 7.3 Materials and Methods

The cell conjugation assay used is described in detail in section 2.13. In brief, APCs were stained with Cell Tracker Blue CMAC (blue) then either pulsed or not pulsed with a cocktail of superantigen. T cells were then conjugated with B cells or AML blasts at a ratio of 1:2 for 15 minutes. This is optimal time for the formation of mature immune synapses established within the literature and from experience within our laboratory. After plating onto coverslips, cell conjugates were fixed and permeabilised prior to staining with rhodamine phalloidin (red) which stains F-actin. This results in T cells stained with a red outline and clear delineation of actin polarisation at the T cell-APC interface indicative of an immunological synapse. Conjugates were also stained with an antibody directed towards phosphotyrosine signalling molecules (green) to examine the downstream effect of immune synapse formation. Confocal imaging of the fixed and stained cell conjugates could then performed at a convenient time.

Healthy B cells were obtained by positive selection from buffy coat preparations supplied by the National Blood Service. Healthy CD4 and CD8 T cells were obtained by negative selection from the same buffy coats. Since these samples are anonymised no demographic data was available on these individuals. For both T and B cell separation, Miltenyi MACS® beads were used. AML T cells were

isolated by negative selection using StemCell Technologies antibody cocktails and equipment with the method optimised in chapter 4 (described in section 2.6.3). T cells were isolated from fresh leucopheresis specimens. Cells were then frozen post-separation and subsequently thawed and revived on the day of the experiment. Details of the patients are listed in **Table 7.1**.

	Age (years)	Sex	FAB Type	Prognostic Group
AML1	40.9	M	M5	Intermediate
AML2	54.3	M	secondary	
AML3	45.7	F	M2	Intermediate
AML4	27.8	F	M5	Intermediate
AML5	63	M	M1	Intermediate
AML6	66	M	M4	Favourable

**Table 7.1: Characteristics of AML Patients**

The experiments were set up to answer the two questions in the manner listed in **Tables 7.2-7.3**.

Coverslip	APC	sAg	T cell	
1	Healthy B allo	+	Healthy CD4	Control
2	Healthy B allo	-	Healthy CD4	Control
3	Healthy B allo	+	Healthy CD8	Control
4	Healthy B allo	-	Healthy CD8	Control
5	Blasts allo	+	Healthy CD4	Test
6	Blasts allo	-	Healthy CD4	Test
7	Blasts allo	+	Healthy CD8	Test
8	Blasts allo	-	Healthy CD8	Test

**Table 7.2: Experimental Set-up: Do Blasts Form Immune Synapses with Healthy T Cells?**

APC: antigen-presenting cell, allo: allogeneic, sAg: superantigen.

Coverslip	APC	sAg	T cell	
1	Healthy B auto	+	Healthy CD4	Control
2	Healthy B auto	-	Healthy CD4	Control
3	Healthy B auto	+	Healthy CD8	Control
4	Healthy B auto	-	Healthy CD8	Control
5	Auto Blasts	+	AML CD4	Test
6	Auto Blasts	-	AML CD4	Test
7	Auto Blasts	+	AML CD8	Test
8	Auto Blasts	-	AML CD8	Test

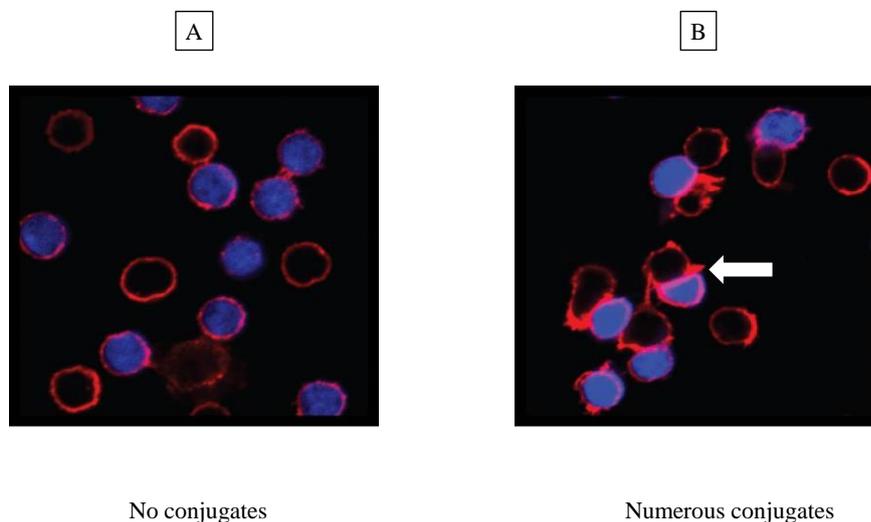
**Table 7.3: Experimental Set-up: Do AML T cells Form Immune Synapses with Autologous Blasts?**

APC: antigen-presenting cell, allo: allogeneic, sAg: superantigen.

In all cases, at least three AML cases were tested. A healthy control was included in each experiment. Healthy control data had been generated on many occasions previously by the group during the work on CLL. As this data was consistent with

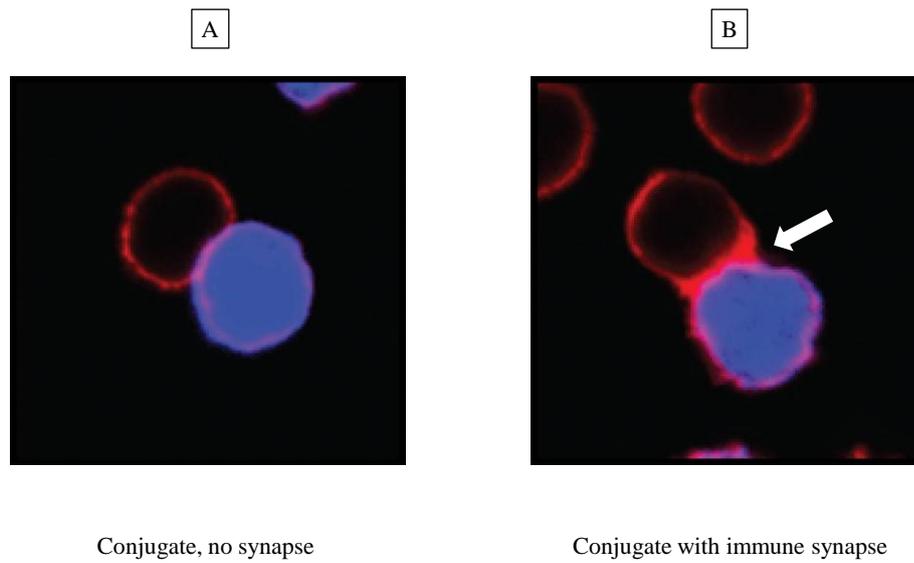
the control data generated here, all control data was combined to allow statistical analyses to be performed.

Images of a minimum of 50 cell conjugates were acquired and subsequently scored independently by three investigators. Each coverslip was assessed for the percentage of total T cells forming conjugates with APCs (**Figure 7.1**), the percentage of conjugates that resulted in immunological synapse formation (**Figure 7.2**) and the percentage of conjugates in which phosphotyrosine signalling was apparent at the immune synapse (**Figure 7.3**).



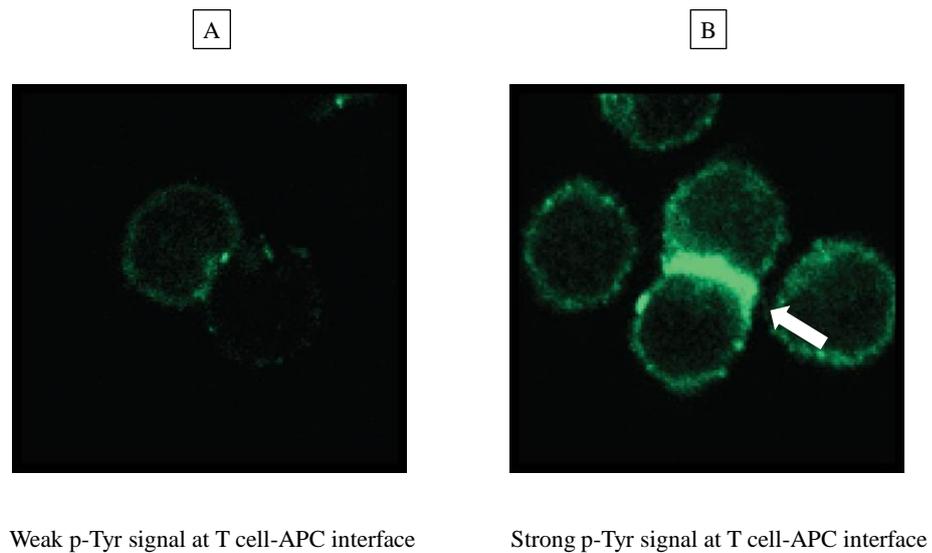
**Figure 7.1: Immunofluorescence Images of Cell Conjugates.**

Blue (CMAC): APCs; red (rhodamine phalloidin): F-actin. (A) shows T cells and APCs failing to form conjugates. (B) shows numerous conjugates, one of which is indicated by the white arrow.



**Figure 7.2: Immunofluorescence Images of Immune Synapses**

Blue (CMAC): APCs; red (rhodamine phalloidin): F-actin. (A) shows a conjugate between an APC and a T cell but no actin polarisation to form an immune synapse. (B) shows a strong F-actin band at the T cell-APC interface (white arrow) indicative of an immunological synapse.



**Figure 7.3: Immunofluorescence Images of Phosphotyrosine (p-Tyr) Signalling**

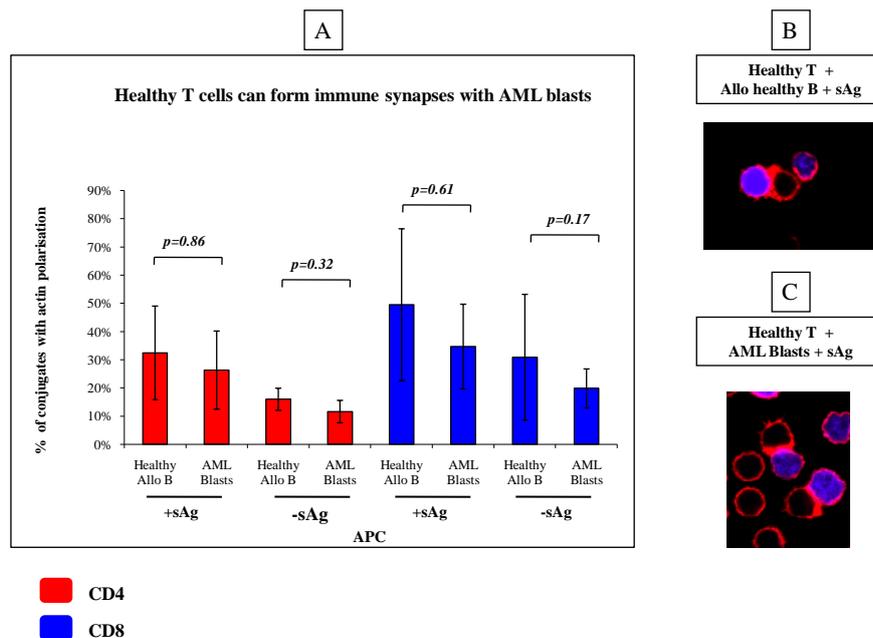
Green (Alexa 488): phosphotyrosine molecules. (A) shows only weak phosphotyrosine signalling at the T cell-APC interface. (B) shows recruitment of large quantities of phosphotyrosine molecules to the immune synapse indicated by the white arrow.

All statistical analysis was performed using Excel. T-tests were 2-tailed with samples assumed to have unequal variance.

## 7.4 Results

### 7.4.1 Do Blasts Form Immune Synapses with Healthy T cells?

Firstly, the ability of AML blasts to act as APCs in a cell conjugation assay with healthy allogeneic CD4 and CD8 T cells was examined. Contrary to expectation, this showed that AML blasts were as good at forming immunological synapses with healthy T cells as healthy allogeneic B cells (**Figure 7.4**). There was no apparent correlation between the FAB type of the blasts and the ability to form immune synapses with T cells.

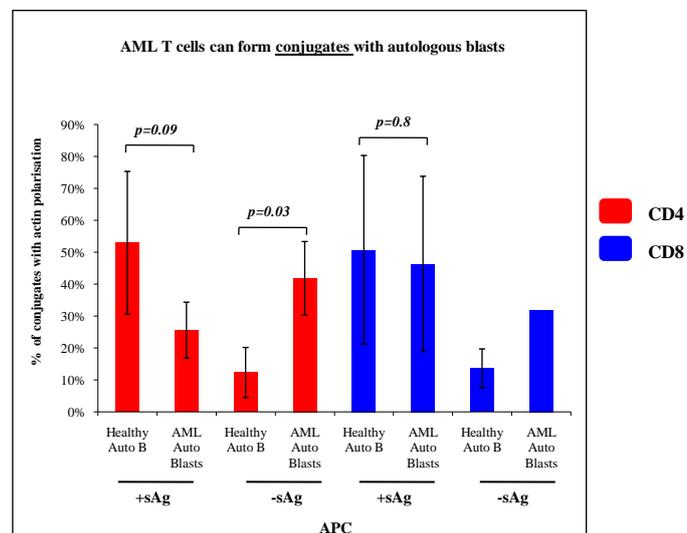


**Figure 7.4: Healthy T Cells can Form Immune Synapses with AML Blasts**

Bar chart (A) shows the percentage of cell conjugates that resulted in actin polarisation within the T cell to form an immune synapse when healthy T cells were conjugated with the APCs listed. Data presented represents the mean with error bars corresponding to standard deviation in five AML patients and at least two healthy controls. In the immunofluorescence images (B) and (C), T cells are red only (rhodamine phalloidin) and APCs/blasts are blue (CMAC). Strong F-actin immune synapses are visible with both healthy allogeneic B cells (B) and AML blasts (C). sAg: superantigen.

### 7.4.2 Do AML T Cells Interact with Autologous Blasts?

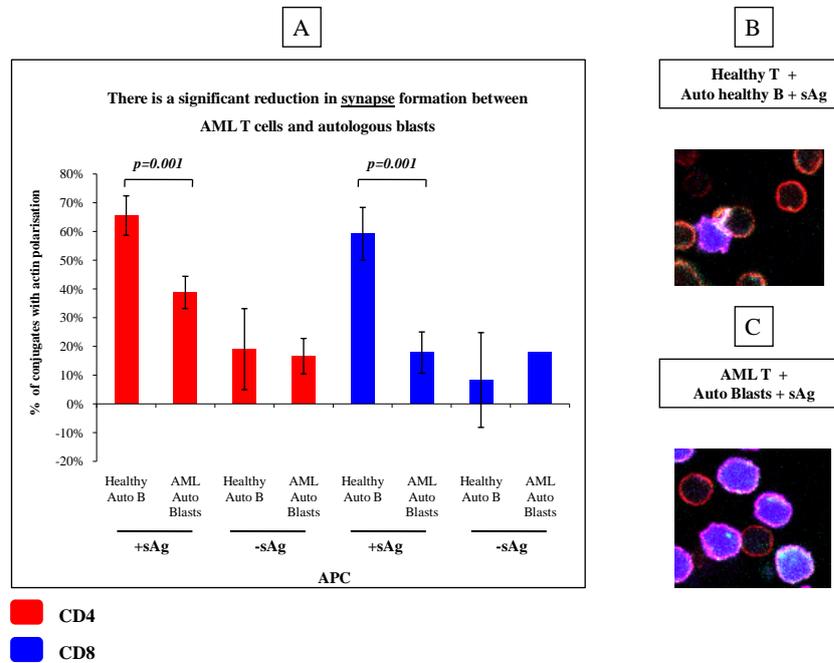
Secondly, the interaction between the T cells in AML patients with their autologous tumour cells was examined. Contrary to the situation seen in CLL, AML T cells were capable of forming cellular conjugates with autologous blasts. In fact, in the case where blasts were not pulsed with superantigen, AML CD4 cells were significantly better than healthy CD4 cells at forming cell conjugates ( $p=0.03$ ) (Figure 7.5).



**Figure 7.5: AML T Cells can Form Cell Conjugates with Autologous AML Blasts**

Data presented represents the mean with error bars corresponding to standard deviation in at least three AML patients (except CD8 minus superantigen where  $n=1$  due to lack of available T cells) and four healthy controls.

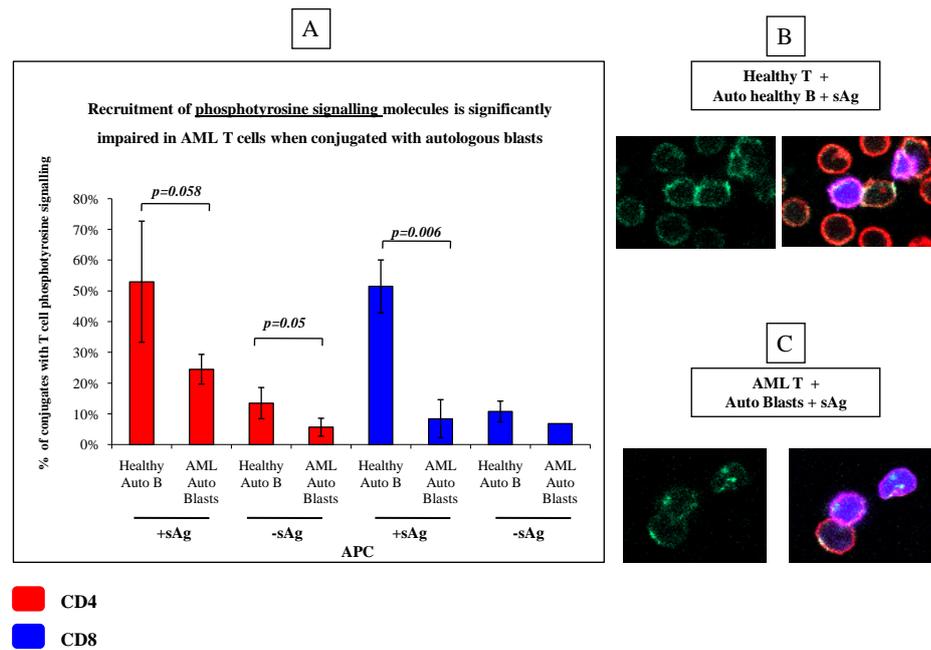
Despite this capacity to interact with the blasts, there was a significant reduction in the ability of AML T cells to then form immunological synapses at the sites of cell contact (**Figure 7.6**).



**Figure 7.6: Immune Synapse Formation between AML T Cells and Autologous AML Blasts**

Bar chart (A) shows the percentage of cell conjugates that resulted in actin polarisation within the T cell to form an immune synapse when healthy or AML T cells were conjugated with the APCs listed. All healthy autologous B cell APCs were conjugated with healthy T cells. Data presented represents the mean with error bars corresponding to standard deviation in at least three AML patients (except CD8 minus superantigen where  $n=1$  due to lack of available T cells) and four healthy controls. In the immunofluorescence images (B) and (C), T cells are red only (rhodamine phalloidin), APCs/blasts are blue (CMAC) and phosphotyrosine signalling molecules green (Alexa 488). These images illustrate reduced immune synapse formation between AML T cells and autologous AML blasts (C) compared with healthy T cells and autologous B cells (B). sAg: superantigen.

Furthermore, this reduced ability to polarise actin to form immunological synapses was associated with reduced recruitment of phosphotyrosine signalling molecules to the synapse when T cells from AML patients were conjugated with autologous blasts. This reached statistical significance for CD4 cells without superantigen ( $p=0.05$ ) and CD8 cells with superantigen ( $p=0.006$ ) (**Figure 7.7**).

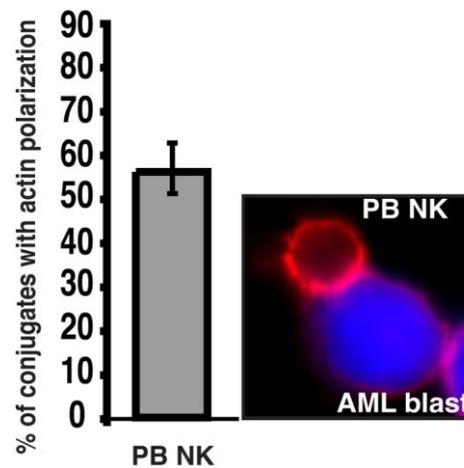


**Figure 7.7: Phosphotyrosine Signalling at the Immune Synapse between AML T Cells and Autologous AML Blasts**

Bar chart (A) shows the percentage of cell conjugates that resulted in accumulation of phosphotyrosine signalling molecules at the T cell-APC interface when healthy or AML T cells were conjugated with the APCs listed. All healthy autologous B cell APCs were conjugated with healthy T cells. Data presented represents the mean with error bars corresponding to standard deviation in at least three AML patients (except CD8 minus superantigen where  $n=1$  due to lack of available T cells) and four healthy controls. In the immunofluorescence images (B) and (C), T cells are red only (rhodamine phalloidin), APCs/blasts are blue (CMAC) and phosphotyrosine signalling molecules green (Alexa 488). Images on the left show phosphotyrosine signalling alone. Those on the right are the merged image of all three colours for the same area of the slide. These images illustrate reduced recruitment of phosphotyrosine signalling molecules to the T cell-APC interface when AML T cells were conjugated with autologous AML blasts (C) compared with when healthy T cells were conjugated with healthy autologous B cells (B). sAg: superantigen.

## 7.5 Discussion

Even though these experiments were initiated to look at T cell function in AML, the first question had to be to examine whether the tumour cells in AML could act as APCs with healthy T cells in an equivalent fashion to CLL B cells. Were the tumour cells completely invisible to T cells or were they capable of inducing immunological synapse formation? Despite the fact that myeloblasts have low expression of MHC Class I and co-stimulatory molecules (Brouwer, *et al* 2000, Vollmer, *et al* 2003, Whiteway, *et al* 2003) the data presented here suggests that they are as good as healthy allogeneic B cells at forming immunological synapses with healthy allogeneic CD4 and CD8 T cells. This came as a surprise but was supported by data generated within our group that looked at the ability of AML blasts to form immune synapses with healthy peripheral blood NK cells (**Figure 7.8**).



**Figure 7.8: AML Blasts can Form Immune Synapses with Healthy NK Cells**

PB: peripheral blood, NK: natural killer cell. Blue (CMAC): APC (in this case a myeloblast); red (rhodamine phalloidin): F-actin.

Previous data has shown that myeloblasts inhibit healthy T cell proliferative and cytokine responses (Buggins, *et al* 1999). Therefore whatever inhibitory signal is being sent by myeloblasts does not impact on the ability of a normal T cell to polarise actin to form the necessary unit for subsequent T cell activation, the immune synapse.

Having established that no defect in immune synapse formation is seen with the blasts and healthy T cells, attention then turned to the T cells in AML. In contrast to the situation seen in CLL, when AML T cells are conjugated with autologous AML tumour cells they are as capable as healthy T cells at forming a cell conjugate. However, like T cells in CLL, their ability to polarise actin to form an immunological synapse is significantly reduced. Furthermore, this results in reduced recruitment of upstream phosphotyrosine signalling molecules to the TCR at the centre of the immunological synapse.

These observations indicate a functional defect of T cells in patients presenting with AML and lead to speculation that this is induced by myeloblasts. This would suggest that within a patient, T cells can recognise and form a conjugate with tumour cells but their ability to form immune synapses is impaired. This results in a lack of recruitment of upstream phosphotyrosine signalling molecules which would be expected to have a profound impact on downstream TCR signalling and subsequent T cell activation. Although this T cell defect is not as marked as that observed in CLL, where T cells have a significantly reduced ability to form conjugates with autologous B CLL cells, this data would suggest that there is a common means by which T cell function is impaired in these very different diseases.

## Chapter 8

### Discussion

For much of the 20<sup>th</sup> century, cancer research has focused on the tumour cell. Much effort has been expended to identify the genomic events that accumulate within a normal cell resulting in the uncontrolled growth, invasion and metastasis that characterise cancer. What is now apparent is that these genetic changes are complex and heterogeneous with no single underlying mechanism responsible for every cancer. This diversity would suggest that the original hope for an all-encompassing ‘cure for cancer’ that targets the tumour cell is unrealistic. Current treatment strategies for cancer remain based on drugs that target all dividing cells with the result that off-target side-effects occur and make it unlikely that the tumour stem cell is destroyed. As a result, modern rational drug design has aimed to develop chemicals that target the tumour cell specifically. However, the early enthusiasm for ‘magic bullets’ sparked by the remarkable success of Imatinib in CML that acts as a specific inhibitor of the bcr-abl tyrosine kinase has yet to be followed by other similarly successful agents (O'Brien, *et al* 2003). This is largely due to the fact that tumour-specific targets are difficult to identify and when they are, the agents developed may not have the expected clinical efficacy as was seen with the FLT3 inhibitors in AML (Illmer and Ehninger 2007). Using antibodies to target tumour cells has proved useful. These may simply label the cancer cell for destruction by the immune system or alternatively toxins or radioisotopes can be tagged to antibodies allowing more directed cytotoxicity. The targeting of the B cell surface molecule CD20 with the monoclonal antibody Rituximab has arguably been the most significant event in the modern history of B cell lymphoma treatment. However, as a single agent its effects are modest. It is only when combined with chemotherapy that significant improvements in event-free and overall survival are observed (Coiffier, *et al* 2002). New approaches to treating cancer are required if the goal of cure is to be achieved.

There is now unambiguous data that the immune system has a crucial role to play in the development of cancer. According to Schreiber's 'Three E' hypothesis of cancer immunoediting, in the initial elimination phase, immune cells recognise and destroy transformed cells. In some cases, this process may fail and an equilibrium phase follows in which the transformed cell remains but its proliferative capacity is held in check by the immune system. As a result of the genomic instability of the tumour cell, mutant daughter cells may arise. The immune system subsequently selects for mutant tumour cells less visible to the immune system by destroying those daughter cells it can see. Therefore, at some point, the tumour 'escapes' the restraining immune response and begins to proliferate uncontrollably (Dunn, *et al* 2002). If the tumour cells could once again be perceived by the immune system, a powerful anti-tumour response may occur. The potential of the immune system to eliminate minimal residual disease and maintain long-term remissions is already apparent in the graft-*versus*-leukaemia effect seen in allogeneic stem cell transplantation. However, this treatment has limited applicability and is associated with high toxicity.

In 1894 the American surgeon William Coley, often regarded as the father of immunotherapy, noted that tumour regression could be induced by stimulating the immune system with bacterial toxins (Coley 1894). Since that time interest in immunotherapy has waxed and waned. Initial promise has frequently been followed by disappointing and sometimes catastrophic results such as were seen in the clinical trial of the CD28 superagonist antibody TGN1412 at Northwick Park Hospital in 2006 (Suntharalingam, *et al* 2006). However, as the field of immunology has progressed so hopes for finding a means of controlling and re-directing the immune system have grown. Most recently, much interest has been directed towards vaccine therapy in cancer. However, results reported thus far are disappointing. The use of adoptive T cell therapy using genetically engineered autologous T cells has shown some exciting anecdotal results in metastatic melanoma but data thus far are limited (Morgan, *et al* 2006). These brilliantly engineered vaccines and T cells that have been years in the designing theoretically should work – vaccines, by re-educating the patient's own immune system to recognise the tumour cell as foreign and autologous T cells by direct cytotoxicity.

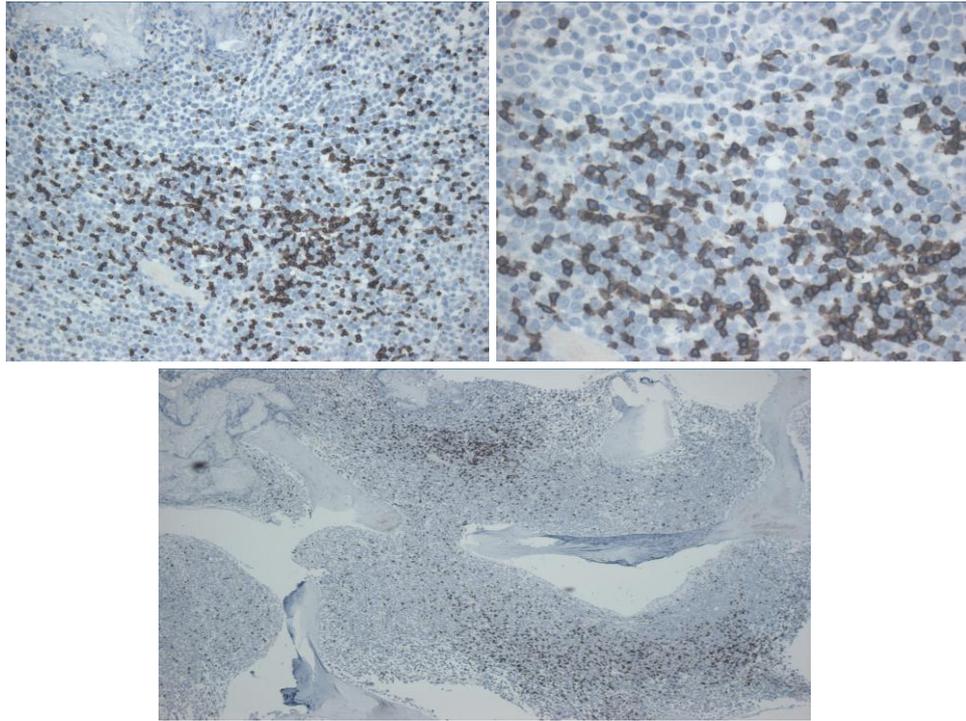
Nonetheless, the potent anti-cancer GVL effect seen in the allogeneic haematopoietic transplant setting has not so far been reproduced when autologous T cells are being re-educated towards self – recognition that lacks the benefit of minor histocompatibility antigen mismatch. Therefore, a better understanding of the impact of the tumour cell on the immune system is critical if these techniques are to eventually prove successful.

These issues have begun to be explored by our group in CLL. Data has been published demonstrating that the T cells in CLL have a differential gene expression pattern when compared with normal healthy T cells. Genes impacting on  $T_H1$  differentiation are differentially expressed in CD4 cells and on cytoskeletal formation, vesicle trafficking and cytotoxicity in CD8 cells. These gene expression changes could be induced in healthy allogeneic T cells by direct contact with CLL B cells (Gorgun 2005). Similar gene expression changes were found in the T cells in the E $\mu$ -TCL1 transgenic mouse model of CLL (Gorgun 2009). It was also observed that genes involved in the actin cytoskeletal pathway were differentially expressed in both CD4 and CD8 T cells in CLL. This led to an investigation of the functional implications of these gene expression changes. As the actin cytoskeleton is critical for the formation of the immunological synapse between a T cell and an APC, actin polymerisation was examined in cell conjugation experiments. It was found that immunological synapse formation is impaired in both CD4 and CD8 cells in CLL and that this actin polymerisation defect could be induced in healthy allogeneic T cells by culturing them with CLL cells (Ramsay, *et al* 2008a).

The question then arose as to whether these T cell defects were unique to CLL or were common to other malignancies. Although AML represents a very different disease to CLL it does offer the same opportunity for widespread interaction between tumour cells and T cells within the peripheral blood and bone marrow that is seen in CLL. Also, due to the widespread interest within the Medical Oncology laboratory in AML, numerous patient samples were available within the Tissue Bank with associated clinical data. In addition, in view of the poor prognosis associated with relapse in conjunction with evidence for a GVL effect,

AML represents a disease in which outcome may be improved by immune manipulation to eradicate minimal residual disease and prevent relapse. To date, the only studies to examine T cells in AML have either been on patients in remission or post stem cell transplantation or functional studies investigating the impact of AML blasts on healthy or remission T cells.

In view of the limited data available on T cells in patients presenting with AML (Ersvaer, *et al* 2007, Panoskaltsis, *et al* 2003, Vidriales, *et al* 1993) a flow cytometric analysis of T cell subsets in newly diagnosed AML patients was the starting point for this thesis. This revealed that absolute T cell numbers, rather than being decreased as is generally perceived or normal as reported in the studies listed, were increased with the CD8 subset showing the greater amplification. This phenomenon did not represent a general proliferative response of the bone marrow. Why should this be? T cells are not part of the malignant clone and therefore their increased number in the peripheral blood of AML patients may either represent a specific proliferation of T cells in response to local environmental growth signals secondary to the myeloblast proliferation. Alternatively, it may represent a redistribution of T cells as they are ‘crowded out’ of the bone marrow compartment by the mass of proliferating blasts. However, this is less likely to be the case as CD3 staining of bone marrow trephines illustrates the presence of numerous T cells (**Figure 8.1**). If this does denote a proliferative T cell response, it would suggest that rather than the picture of a burnt-out anergic immune system associated with cancer, that this is an active reaction to the malignancy that is attempting, though failing, to contain its spread. This is also supported by the oligoclonality of the T cell populations in AML suggesting a polarisation towards particular antigens.



**Figure 8.1: T Cells in a Bone Marrow Trepine from a Patient Presenting with AML**

CD3 positive T cells are stained brown.

It was observed that a population of CD56-expressing T cells was significantly increased in the peripheral blood of patients with AML. These cells were not true NKT cells but did express certain NK receptors. Instead, their phenotype was that of effector cytotoxic T lymphocytes with high expression of activation markers but lower expression of cytotoxic granules compared to the same cell population in healthy individuals which may indicate an impaired cytotoxic potential in AML. As this cell population has already been identified as having anti-tumour cytotoxicity (Ohkawa, *et al* 2001, Pittet, *et al* 2000) it was hypothesized that these cells could represent an immunosurveillance mechanism. They were found to return to normal levels when patients entered CR however a change in their absolute number was not a prelude to relapse in the single individual examined. This does not negate the hypothesis. A functional assessment of the ability of isolated CD3+56+ cells to kill autologous blasts is an obvious next step.

True NKT cells present a new and exciting avenue in cancer immunotherapy. Reduced absolute numbers or numbers secreting IFN $\gamma$  have been reported to be reduced in the peripheral blood of patients with a variety of malignancies (Fujii, *et al* 2003a, Molling, *et al* 2005, Tahir, *et al* 2001, Yoneda, *et al* 2005). In this thesis, absolute numbers of NKT cells were not found to be significantly different in the peripheral blood of patients with AML compared with healthy individuals. However, the relative number of NKT cells in the double-negative ‘anti-cancer’ T<sub>H</sub>1 subset was found to be significantly higher in AML patients compared with healthy controls again suggesting an immunosurveillance role for this population. This observation also points to an idea of an immune system that is attempting unsuccessfully to contain the aggressive spread of malignant cells.

Further analysis of T cells in presentation AML specimens required the ability to isolate T cell fractions in relatively high purity. This proved extraordinarily difficult to achieve but led to an understanding why much previous work on AML T cells had either used remission T cells or observed the effect of AML blasts on healthy T cells. One reason why this proved so problematic is the heterogeneous nature of AML blasts. No single surface marker is sufficiently widely expressed to give good immunomagnetic blast depletion. With much work, we developed a method of negatively selecting CD4 and CD8 T cells from presentation AML specimens using a cocktail of antibodies. This technique was subsequently used to isolate T cells for the functional studies performed. However, this method is extremely costly and whether it is economical to obtain large numbers of T cells using this technique in future studies remains doubtful.

The initial goal of this study had been to perform gene expression profiling on negatively selected CD4 and CD8 T cells from presentation AML specimens. As the method of negative selection took so much time to optimise, attention turned to the possibility of using positive selection to isolate the T cells. This method was associated with concerns about the potential for activating the cell of interest by cross-linking surface antigens and resulting in altered gene transcription. At the time this study was initiated there was no published data directly comparing the impact of positive *versus* negative immunomagnetic cell selection on cellular gene

expression profile. Therefore this experiment was undertaken in the course of this project. Data presented here on a total of 19 healthy volunteers demonstrated the minimal impact of the method of cell selection on the gene expression profile of the cell in agreement with a subsequent study published in 2007 that looked at the same question using cells from three individuals and custom arrays containing 10,515 genes (Lyons, *et al* 2007). This led to the use of positive selection for isolating T cells from presentation AML specimens for microarray analysis. A very small percentage of probesets were found to be differentially expressed in positively selected CD8 T cells and some of these genes were associated with T cells or T cell activation. However, this finding was made using only one of the three software packages used for analysis. In view of this small impact of positive selection on CD8 T cell gene expression in conjunction with the published functional data available (Bernard, *et al* 2002, Berndt, *et al* 1998, Chuck, *et al* 1993, Milia, *et al* 1997, Stanciu, *et al* 1996, Wang, *et al* 1994) it was felt appropriate to use the negative selection technique for isolating T cells for the analyses of T cell function performed.

Gene expression profiling was performed on positively selected AML CD4 and CD8 T cells and compared to those from normal healthy controls. Importantly, the gene expression changes observed were validated by qRT-PCR with further validation at a protein level performed by flow cytometry. Major differences were found between the AML and healthy T cells which impacted on the way in which the data could be analysed. T cell signalling and activation pathways appeared to be primarily affected in both CD4 and CD8 cells. Although when the differentially regulated gene-lists were compared to those generated from T cells activated *ex vivo* (Wang, *et al* 2008) little overlap was seen suggesting that the gene expression changes seen in AML T cells are not purely due to the normal signalling seen on T cell activation. Anergy was explored and discarded as an alternative explanation for the gene expression changes by comparison of gene-lists from a source in the literature (Dominguez-Villar, *et al* 2007). Thus both CD4 and CD8 T cells in AML appear to be undergoing some form of aberrant activation process. On comparison of the gene-lists with those generated from the CLL dataset, little commonality was observed. This leads to the conclusion that

although T cells in both of these malignancies show different gene expression patterns to healthy T cells, they differ in what those changes are. In view of the very different natures of CLL and AML this is not an unpredictable conclusion but did support the viewpoint that different cancers do not adopt a similar final pathway to down-regulate host T cell mediated immune responses.

Although the genes themselves were different, it was noteworthy that in both CLL and AML numerous genes were differentially regulated that were involved with the actin cytoskeleton and cellular polarisation. Since this observation led to the finding of impaired immunological synapse formation in CLL T cells, it was therefore felt to be relevant to use the same cell conjugation assay to examine the functional ability of AML CD4 and CD8 T cells to polarise actin appropriately to form immune synapses with APCs. These experiments demonstrated that, contrary to expectation, AML blasts could act as APCs to induce T cell immunological synapse formation in cell conjugation assays with healthy allogeneic T cells. However, when AML T cells were conjugated with autologous blasts, although they were able to form conjugates, their ability to form immune synapses was impaired. This was associated with reduced recruitment of phosphotyrosine signalling molecules to the immune synapse. This is in keeping with the observed gene expression changes noted. The differentially regulated gene profiles of T cells in AML were suggestive that the cells were undergoing signalling via the TCR but the pattern of gene expression changes observed was aberrant. Thus T cells may be interacting with myeloblasts and forming cell conjugates but subsequent downstream signalling events including actin reorganisation to form the immunological synapse is impaired. The implications of this could be that although T cells are recognising malignant cells their means of destroying them are being thwarted. This would explain the clinical situation when patients relapse with AML post allogeneic SCT despite GVHD where alloreactive T cells are clearly failing to control the disease. The question remains as to the identity of the signal from the myeloblasts that induces these changes. Previous work from King's College London has identified an unknown inhibitory protein secreted by blasts that does not require cell contact to induce its functional effects on T cells (Buggins, *et al* 2001). In contrast, in CLL, direct contact is

required between the tumour cell and the T cell to induce both the gene expression and immune synapse formation defects observed (Gorgun, *et al* 2005, Ramsay, *et al* 2008a). Therefore, although actin cytoskeletal reorganisation may be a fundamental pathway affected in both AML and CLL T cells, the means by which this defect is induced may differ in these diseases. This first requires further exploration in AML. Can an immune synapse formation defect be induced in healthy T cells by first culturing them with AML blasts? If so, is the same thing seen when a transwell culture system is used to prevent contact between the blasts and T cells? The experiments performed by the group from King's College should be repeated and if confirmed, subsequent proteomic studies performed to identify the nature of the inhibitory molecule secreted by blasts. These studies were beyond the scope of this thesis.

This work was initiated to examine if defects observed in T cells in one malignancy, CLL, were unique to that disease or common to others. AML, although also a haematological malignancy and therefore by its nature allowing many opportunities for contact between tumour cells and T cells, is nonetheless a very different disease. CLL is in general a chronic disorder that many individuals will die with rather than from. The clonal lymphoproliferation may be present for months or even years before it becomes clinically apparent. This allows much time for interaction between tumour and T cells. In contrast, AML is an aggressive, progressive disorder in which patients will present within weeks if not days of disease development. Therefore, it does not come as much of a surprise that the gene expression changes observed in the T cells from these two clinically heterogeneous diseases are not the same. From a functional standpoint however, immunological synapse formation does appear to be similarly affected in AML T cells as in CLL although not to the same extent. These observations require further consolidation. It is of note that work from our group has demonstrated that similar immune synapse defects seen in CLL T cells are also apparent in T cells from affected lymph nodes of patients with follicular lymphoma, an equivalently chronic disorder to CLL (Ramsay 2008b). The consequence of these findings is to elucidate some mechanism of reversing the observed defects. In CLL and follicular lymphoma, work is already underway to attempt to reverse the immune

synapse defects observed with the immunomodulatory drug lenalidomide showing some effect (Ramsay 2008b, Ramsay, *et al* 2008a). It is of interest that this drug has already demonstrated efficacy in AML (Fehniger, *et al* 2009). Following on from this, the question will need to be asked as to whether reversing the defect in T cell actin repolarisation results in immune recognition of tumour cells and subsequent improved cytotoxicity before clinical efficacy can be addressed. Nonetheless, these encouraging results that may lead to a time when cancers are treated not only by targeting the tumour but also the T cell.

In summary, this thesis has provided technical information on methods of separating T cells from presentation AML specimens and the impact of the method of immunomagnetic cell selection on the gene expression profile of T cells. In addition, a picture has been obtained of AML at presentation as not only an aggressive proliferating mass of myeloblasts but also an associated florid immune response. The resulting T cells can form conjugates with tumour cells but subsequent immune synapse formation and recruitment of downstream signalling molecules is inhibited. This is associated with a gene expression profile that indicates aberrant TCR signalling and T cell activation patterns. Therefore, T cells in patients with AML are different from healthy T cells and also from T cells in patients with CLL. However, the common strand of impaired actin cytoskeletal formation in immunological synapse formation may represent a means of improving T cell function in these malignancies. These ongoing developments mean that in the future we may well at last develop effective treatments for cancer thus finally contradicting that ancient writer of the Egyptian papyri from 3600 years ago (Breasted 1922).

## Future Work

Firstly, the data on immune synapse formation between T cells presenting with AML and autologous blasts requires extension. Preliminary data suggest that the defect in synapse formation resides primarily in the T cells and not in the leukaemic blasts. Evaluation of more patients, to cover the variety of AML FAB types as well as molecular subtypes, require evaluation. The next question would be to address if AML blasts can induce the observed synapse defect in healthy allogeneic T cells by culturing them either in direct contact or in a transwell culture system to establish if a diffusible factor is responsible for the changes observed. In view of the data from CLL, ongoing experiments are addressing if the immunomodulatory drug lenolidomide can repair the demonstrated T cell immune defect. A subsequent demonstration of improved T cell cytotoxicity against AML blasts would then be required.

With regards to the population of CD3+CD56+ cells identified to be increased in the peripheral blood of patients presenting with AML, an assessment of their function is an obvious first line of enquiry as their absolute number may not be as critical as their functional efficacy. Cytotoxicity assays using AML blasts as the target cells and ELISPOT assessment of IFN $\gamma$  production in response to culture with AML blasts would be valuable.

Finally, no further progress seems to have been made in the identification of the protein secreted by AML blasts that reduces T cell activation since the work published by Buggins and colleagues in 2001. In the light of the data presented here, it would be worthwhile repeating their experiments and confirming their data with a subsequent proteomic investigation to identify the nature of the secreted protein. This work is beyond the scope of the present thesis.

## Appendix A: Company Addresses

<b>Abgene UK</b>	ABgene House, Blenheim Road, Epsom. KT19 9AP
<b>Affymetrix UK Ltd.</b>	Voyager, Mercury Park, Wycombe Lane, Wooburn Green, High Wycombe. HP10 0HH
<b>Agilent Technologies UK Ltd.</b>	710 Wharfedale Road, Winnersh Triangle, Wokingham, Berkshire. RG41 5TP
<b>Amersham (GE Healthcare)</b>	Pollards Wood, Nightingales Lane, Chalfont St. Giles, Bucks. HP8 4SP
<b>Ancell Corporation</b>	P.O. Box 87, 243 3 <sup>rd</sup> Street N, Bayport MN 55003 USA
<b>Applied Biosystems</b>	Lingley House, 120 Birchwood Boulevard, Warrington. WA3 7QH
<b>Axis-Shield PoC</b>	P.O. Box 6863 Rodelokka, N-0504, Oslo, Norway
<b>BD Biosciences</b>	The Danby Building, Edmund Halley Road, Oxford Science Park, Oxford. OX4 4DQ
<b>Beckman Coulter</b>	Oakley Court, Kingsmead Business Park, London Road, High Wycombe, Buckinghamshire. HP11 1JU
<b>Carl Zeiss Ltd</b>	15-20 Woodfield Rd., Welwyn Garden City, Hertfordshire. AL7 1JQ
<b>CRUK Media Production Lab</b>	Clare Hall Laboratories, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3LD
<b>Dako UK Ltd</b>	Cambridge House, St. Thomas Place, Ely, Cambridgeshire CB7 4EX

<b>eBioscience Inc.</b>	10255 Science Center Drive, San Diego, CA 92121 USA
<b>Fisher Scientific</b>	2000 Park Lane Drive, Pittsburgh, PA 15275 USA
<b>Hund Wetzlar</b>	Wilhelm-Will-Str. 7, Wetzlar, 35580 Germany
<b>Immunotech</b>	130, Avenue de Lattre de Tassigny, BP177, 13276 Marseille Cedex 9, France
<b>Ingenuity Systems, Inc.</b>	1700 Seaport Blvd., Third Floor, Redwood City, CA 94063 USA
<b>Invitrogen Ltd</b>	3 Fountain Drive, Inchinnan Business Park, Paisley. PA4 9RF
<b>InVivoScribe Technologies</b>	Le Forum - Bât B, 515 Avenue de la Tramontane, ZI Athelia IV, 13600 La Ciotat, France
<b>Millipore (UK) Ltd</b>	2-4 Fleming Road, Kirkton Campus, Livingston. EH54 7BN
<b>Miltenyi Biotec Ltd</b>	Almac House, Church Lane, Bisley, Surrey. GU24 9DR
<b>MJ Research</b>	590 Lincoln Street, Waltham, 02451 USA
<b>Nalgene</b>	Nalge (Europe) Limited, Unit 1a, Thorn Business Park, Hereford. HR2 6JT
<b>NanoDrop Products</b>	3411 Silverside Rd, Bancroft Building, Wilmington, DE 19810 USA
<b>National Blood Service</b>	Colindale Avenue, Colindale, London. NW9 5BG

---

<b>NIH Tetramer Facility</b>	Emory University Vaccine Center, 954 Gatewood Road, Atlanta, GA 30329 USA
<b>PAA Laboratories Ltd.</b>	Termare Close, Houndstone Business Park, Yeovil, Somerset. BA22 8YG
<b>Partek Incorporated</b>	12747 Olive Blvd., Suite 205, St. Louis, Missouri 63141 USA
<b>Promega UK Ltd</b>	Delta House, Southampton Science Park, Southampton, Hampshire. SO16 7NS
<b>Qiagen Ltd</b>	QIAGEN HOUSE, Fleming Way, Crawley, West Sussex. RH10 9NQ
<b>R&amp;D Systems Europe Ltd</b>	19 Barton Lane, Abingdon Science Park, Abingdon. OX14 3NB
<b>Sanquin</b>	Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands
<b>Sigma Aldrich</b>	The Old Brickyard, New Road, Gillingham, Dorset. SP8 4XT
<b>StemCell Technologies</b>	Miniparc POLYTEC, Batiment Tramontane, RDC, 60 rue des Berges, 38000 Grenoble, France
<b>Syngene Europe</b>	Beacon House, Nuffield Road, Cambridge. CB4 1TF
<b>TAAB Laboratories Equipment Ltd</b>	3 Minerva House, Calleva Park, Aldermaston, Berkshire. RG7 8NA
<b>Thermo Fisher Scientific Inc.</b>	81 Wyman Street, Waltham, MA 02454 USA
<b>Tree Star Inc</b>	340 A Street Bd. 1#203 Ashland, Oregon 97520 USA

## Appendix B: Antibodies

Antibody	Clone	Isotype	Company	Catalog Number
CD2-PE	RPA-2.10	Mouse IgG1,k	BD	555327
CD3-PECy7	SK7	Mouse IgG1k	BD Pharmingen	557851
CD3-PE	UCHT1	Mouse IgG1k	BD Pharmingen	555333
CD3-FITC	UCHT1	Mouse IgG1,k	BD Pharmingen	555332
CD3-APC	UCHT1	Mouse IgG1,k	BD Pharmingen	555335
CD4-PE	RPA-T4	Mouse IgG1k	BD Pharmingen	555347
CD4-PERCP	SK3	Mouse IgG1	BD	345770
CD4-FITC	13B8.2	Mouse IgG1	Immunotech	IM0448
CD8-FITC	SK1	Mouse IgG1	BD	345772
CD8-PE	SK1	Mouse IgG1,k	BD	345773
CD8-PERCP	SK1	Mouse IgG1	BD	345774
CD11c-PE	HI10a- EMPTY	Mouse IgG1	BD Pharmingen	555375
CD13-APC	WM15	Mouse IgG1,k	BD Pharmingen	557454
CD14-FITC	RMO52	Mouse IgG2a	Immunotech	IM0645U
CD16-PE	B73.1	Mouse IgG1,k	BD	332779
CD16-FITC	3G8	Mouse IgG1,k	BD Pharmingen	555406
CD16-FITC	NKP15	Mouse IgG1	BD	335035
CD19-APC	HIB19	Mouse IgG1,k	BD Pharmingen	555415
CD20-FITC	LT20	Mouse IgG1	Miltenyi	130-091-108
CD21-PE	B-ly4	Mouse IgG1,k	BD Pharmingen	555422
CD25-PE	4E3	Mouse IgG2b	Miltenyi	130-091-024
CD25-APC	M-A251	Mouse IgG1,k	BD Pharmingen	555434
CD27-PE	M-T271	Mouse IgG1,k	BD Pharmingen	555441
CD28-PE	CD28.2	Mouse IgG1	Immunotech	IM2071U
CD33-PE	AC104.3E3	Mouse IgG1	Miltenyi	130-091-732
CD33-FITC	HIM3-4	Mouse IgG1,k	BD Pharmingen	555626
CD34-PERCP	8G12	Mouse IgG1	BD	345803
CD36-APC	CB38	Mouse IgM,k	BD Pharmingen	550956
CD38-PECy7	HB7	Mouse IgG1	BD	335825
CD45RA-FITC	HI100	Mouse IgG2b,k	BD Pharmingen	555488
CD45RO-PE	UCHL1	Mouse IgG2a,k	BD Pharmingen	555493
CD48-FITC	TÜ145	Mouse IgM,k	BD Pharmingen	555759
CD56-PE	B159	Mouse IgG1,k	BD Pharmingen	555516
CD56-APC	B159	Mouse IgG1,k	BD Pharmingen	555518

CD57-FITC	NK-1	Mouse IgM,k	BD Pharmingen	555619
CD69-FITC	FN50	Mouse IgG1,k	BD Pharmingen	555530
CD69-PE	FN50	Mouse IgG1,k	BD Pharmingen	555531
CD86-FITC	GL1	Rat IgG2a,k	BD Pharmingen	553691
CD94-FITC	HP-3D9	Mouse IgG1,k	BD Pharmingen	555888
CD117-PE	YB5.B8	Mouse IgG1	BD Pharmingen	555714
CD119-PE	GIR-208	Mouse IgG1	eBioscience	12-1199
CD122-PE	Mik- $\beta$ 3	Mouse IgG1,k	BD Pharmingen	554525
CD123-PE	9F5	Mouse IgG1	BD Pharmingen	555644
CD244-FITC	2B4	Mouse IgG2a,k	BD Pharmingen	550815
CCR7-PE	3D12	Rat IgG2a,k	eBioscience	12-1979
Dectin 1/CLEC7A -PE	259931	Mouse IgG2B	R&D Systems	FAB1859P
Granzyme B-PE	CLB-GB11	Mouse IgG1	Pelicluster (Sanquin)	M2289
HLA-DR-PE	Immu-357	Mouse IgG1	Immunotech	IM1639
IL-4-PE	8D4-8	Mouse IgG1	BD Pharmingen	554516
IL-2-PE	MQ1-17H12	Rat IgG2a	BD Pharmingen	554566
TNF $\alpha$ -FITC	MAb11	Mouse IgG1	BD Pharmingen	554512
IFN $\gamma$ -FITC	B27	Mouse IgG1	BD Pharmingen	554700
NKG2D-APC	1D11	Mouse IgG1,k	BD Pharmingen	558071
Perforin-FITC	Delta G9	Mouse IgG2b	Ancell	358-040
TCR $\alpha\beta$ -FITC	T10B9.1A-31	Mouse IgM,k	BD Pharmingen	555547
TCR $\gamma\delta$ -FITC	B1	Mouse IgG1,k	BD Pharmingen	559878
V $\alpha$ 24-PE	C15	Mouse IgG1	Immunotech	2290
V $\beta$ 11-FITC	C21	Mouse IgG2a	Immunotech	1586
Mouse IgG1,k isotype control-FITC	MOPC-21		BD Pharmingen	555909
Mouse IgG1,k isotype control-APC	MOPC-21		BD Pharmingen	555751
Mouse IgG1,k isotype control-PERCP	MOPC-21		BD Pharmingen	559425
Mouse IgG1,k isotype control-PECy7	MOPC-21		BD Pharmingen	557646
Mouse IgG2a,k isotype control-PE	G155-178		BD Pharmingen	559319
Mouse IgG2b,k isotype control-APC	27-35		BD Pharmingen	555745
Mouse IgG2b,k isotype control-FITC	27-35		BD Pharmingen	555742

---

Mouse IgG2b,k isotype control-PE	27-35	BD Pharmingen	555058
Mouse IgM,k isotype control-FITC	G155-228	BD Pharmingen	555583
Rat IgG2a,k Isotype control-PE	R35-95	BD Pharmingen	554689

---

## Appendix C: Common Differentially Expressed Genes in AML and CLL Datasets

### CD4

Probeset	Symbol	Description	CLL	AML
211947_s_at	<b>BAT2D1</b>	BAT2 domain containing 1	Blue	Red
204285_s_at	<b>PMAIP1</b>	phorbol-12-myristate-13-acetate-induced protein 1	Red	Red
207540_s_at	<b>SYK</b>	spleen tyrosine kinase	Red	Red
201161_s_at	<b>CSDA</b>	cold shock domain protein A	Red	Red
204286_s_at	<b>PMAIP1</b>	phorbol-12-myristate-13-acetate-induced protein 1	Red	Red
208268_at	<b>ADAM28</b>	ADAM metalloproteinase domain 28	Red	Red
217282_at	<b>MAN1A2</b>	mannosidase, alpha, class 1A, member 2	Blue	Red
204713_s_at	<b>F5</b>	coagulation factor V (proaccelerin, labile factor)	Blue	Red
201560_at	<b>CLIC4</b>	chloride intracellular channel 4	Red	Red
210479_s_at	<b>RORA</b>	RAR-related orphan receptor A	Blue	Red
201160_s_at	<b>CSDA</b>	cold shock domain protein A	Red	Red
210384_at	<b>PRMT2</b>	protein arginine methyltransferase 2	Blue	Red
213650_at	<b>GOLGA8A</b>	golgi autoantigen, golgin subfamily a, 8A	Red	Red
208690_s_at	<b>PDLIM1</b>	PDZ and LIM domain 1 (elfin)	Red	Red
210426_x_at	<b>RORA</b>	RAR-related orphan receptor A	Blue	Red
212007_at	<b>UBXD2</b>	UBX domain containing 2	Blue	Red
214723_x_at	<b>ANKRD36B</b>	ankyrin repeat domain 36B	Blue	Red
210407_at	<b>PPM1A</b>	protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform	Blue	Red
217620_s_at	<b>PIK3CB</b>	phosphoinositide-3-kinase, catalytic, beta polypeptide	Blue	Red
220940_at	<b>ANKRD36B</b>	ankyrin repeat domain 36B	Blue	Red
211944_at	<b>BAT2D1</b>	BAT2 domain containing 1	Blue	Red
217607_x_at	<b>EIF4G2</b>	eukaryotic translation initiation factor 4 gamma, 2	Blue	Red
216682_s_at	<b>FAM48A</b>	family with sequence similarity 48, member A	Red	Red
211824_x_at	<b>NLRP1</b>	NLR family, pyrin domain containing 1	Blue	Red
216850_at	<b>SNRPN</b>	small nuclear ribonucleoprotein polypeptide N	Blue	Red
214298_x_at	<b>SEPT6</b>	septin 6	Blue	Red
222279_at	<b>RP3-</b>	hypothetical protein FLJ35429	Red	Blue

377H14.5			
213666_at	<b>SEPT6</b>	septin 6	
215921_at	<b>LOC388237</b>	hypothetical LOC388237	
212921_at	<b>SMYD2</b>	SET and MYND domain containing 2	
214823_at	<b>ZNF204</b>	zinc finger protein 204 (pseudogene)	
215859_at	<b>NCLN</b>	nicalin homolog (zebrafish)	
218793_s_at	<b>SCML1</b>	sex comb on midleg-like 1 (Drosophila)	
220936_s_at	<b>H2AFJ</b>	H2A histone family, member J	

## CD8

Probesets	Symbol	Description	CLL	AML
217649_at	<b>ZFAND5</b>	zinc finger, AN1-type domain 5		
214516_at	<b>HIST1H4B</b>	histone cluster 1, H4b		
219654_at	<b>PTPLA</b>	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A		
215224_at	<b>RPL23</b>	ribosomal protein L23		
216474_x_at	<b>TPSAB1</b>	tryptase alpha/beta 1		
202708_s_at	<b>HIST2H2BE</b>	histone cluster 2, H2be		
210407_at	<b>PPM1A</b>	protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform		
206847_s_at	<b>HOXA7</b>	homeobox A7		
218847_at	<b>IGF2BP2</b>	insulin-like growth factor 2 mRNA binding protein 2		
201348_at	<b>GPX3</b>	glutathione peroxidase 3 (plasma)		
203557_s_at	<b>PCBD1</b>	pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha		
213649_at	<b>SFRS7</b>	splicing factor, arginine/serine-rich 7, 35kDa		
219588_s_at	<b>NCAPG2</b>	non-SMC condensin II complex, subunit G2		
AFFX-				
HUMRGE/M10098_M_at		NA		
211944_at	<b>BAT2D1</b>	BAT2 domain containing 1		
210742_at	<b>CDC14A</b>	CDC14 cell division cycle 14 homolog A (S. cerevisiae)		

213517_at	<b>PCBP2</b>	poly(rC) binding protein 2		
216022_at		NA		
201354_s_at	<b>FUT1</b>	fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase, H blood group)		
208610_s_at	<b>SRRM2</b>	serine/arginine repetitive matrix 2		
210755_at	<b>HGF</b>	hepatocyte growth factor (hepapoietin A; scatter factor)		
214352_s_at	<b>KRAS</b>	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog		
208129_x_at	<b>RUNX1</b>	runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)		
202124_s_at	<b>TRAK2</b>	trafficking protein, kinesin binding 2		
211572_s_at	<b>SLC23A2</b>	solute carrier family 23 (nucleobase transporters), member 2		
216203_at	<b>SPTLC2</b>	serine palmitoyltransferase, long chain base subunit 2		
210479_s_at	<b>RORA</b>	RAR-related orphan receptor A		
210528_at	<b>MR1</b>	major histocompatibility complex, class I-related		
208195_at	<b>TTN</b>	titin		
211824_x_at	<b>NLRP1</b>	NLR family, pyrin domain containing 1		
218638_s_at	<b>SPON2</b>	spondin 2, extracellular matrix protein		
206828_at	<b>TXK</b>	TXK tyrosine kinase		
216971_s_at	<b>PLEC1</b>	plectin 1, intermediate filament binding protein 500kDa		
215921_at	<b>LOC388237</b>	hypothetical LOC388237		
217950_at	<b>NOSIP</b>	nitric oxide synthase interacting protein		
221790_s_at	<b>LDLRAP1</b>	low density lipoprotein receptor adaptor protein 1		
219139_s_at	<b>CROCCL2</b>	ciliary rootlet coiled-coil, rootletin-like 2		
221878_at	<b>LOC388969</b>	hypothetical LOC388969		
204319_s_at	<b>RGS10</b>	regulator of G-protein signaling 10		
202723_s_at	<b>FOXO1</b>	forkhead box O1		

57082_at	<b>LDLRAP1</b>	low density lipoprotein receptor adaptor protein 1	Red	Blue
221397_at	<b>TAS2R10</b>	taste receptor, type 2, member 10	Blue	Blue
218793_s_at	<b>SCML1</b>	sex comb on midleg-like 1 (Drosophila)	Blue	Blue
216330_s_at	<b>POU6F1</b>	POU class 6 homeobox 1	Blue	Blue
215555_at	<b>C1orf63</b>	chromosome 1 open reading frame 63	Blue	Blue
204105_s_at	<b>NRCAM</b>	neuronal cell adhesion molecule	Blue	Blue
215692_s_at	<b>MPPED2</b>	metallophosphoesterase domain containing 2	Blue	Blue
219664_s_at	<b>DECR2</b>	2,4-dienoyl CoA reductase 2, peroxisomal	Red	Blue
211696_x_at	<b>HBB</b>	hemoglobin, beta	Red	Blue
214414_x_at	<b>HBA1</b>	hemoglobin, alpha 1	Red	Blue
211745_x_at	<b>HBA2</b>	hemoglobin, alpha 2	Red	Blue
209116_x_at	<b>HBB</b>	hemoglobin, beta	Red	Blue

Red: up-regulated in patient T cells compared with healthy T cells, blue: down-regulated in patient T cells compared with healthy controls.

## References

- Abbas, A.K., Lichtman, A.H. (2000) *Cellular and Molecular Immunology*. Elsevier Science (USA).
- Acuto, O. & Cantrell, D. (2000) T cell activation and the cytoskeleton. *Annu Rev Immunol*, **18**, 165-184.
- Agrawal, B., Krantz, M.J., Reddish, M.A. & Longenecker, B.M. (1998) Cancer-associated MUC1 mucin inhibits human T-cell proliferation, which is reversible by IL-2. *Nat Med*, **4**, 43-49.
- Akasaki, Y., Liu, G., Chung, N.H., Ehtesham, M., Black, K.L. & Yu, J.S. (2004) Induction of a CD4+ T regulatory type 1 response by cyclooxygenase-2-overexpressing glioma. *J Immunol*, **173**, 4352-4359.
- Alexander, J.P., Kudoh, S., Melsop, K.A., Hamilton, T.A., Edinger, M.G., Tubbs, R.R., Sica, D., Tuason, L., Klein, E., Bukowski, R.M. & et al. (1993) T-cells infiltrating renal cell carcinoma display a poor proliferative response even though they can produce interleukin 2 and express interleukin 2 receptors. *Cancer Res*, **53**, 1380-1387.
- Almand, B., Clark, J.I., Nikitina, E., van Beynen, J., English, N.R., Knight, S.C., Carbone, D.P. & Gabrilovich, D.I. (2001) Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol*, **166**, 678-689.
- Almand, B., Resser, J.R., Lindman, B., Nadaf, S., Clark, J.I., Kwon, E.D., Carbone, D.P. & Gabrilovich, D.I. (2000) Clinical significance of defective dendritic cell differentiation in cancer. *Clin Cancer Res*, **6**, 1755-1766.
- Amadori, S., Suci, S., Willemze, R., Mandelli, F., Selleslag, D., Stauder, R., Ho, A., Denzlinger, C., Leone, G., Fabris, P., Muus, P., Vignetti, M., Hagemeijer, A., Beeldens, F., Anak, O. & De Witte, T. (2004) Sequential administration of gemtuzumab, azacitidine and conventional chemotherapy as first line therapy in elderly patients with acute myeloid leukemia: a phase II study (AML-15) of the EORTC and GIMEMA leukemia groups. *Haematologica*, **89**, 950-956.
- Andersen, M.H., Svane, I.M., Kvistborg, P., Nielsen, O.J., Balslev, E., Reker, S., Becker, J.C. & Straten, P.T. (2005) Immunogenicity of Bcl-2 in patients with cancer. *Blood*, **105**, 728-734.
- Apperley, J., Carreras, E., Gluckman, E., Gratwohl, A., Masszi, T. (2008) *The EBMT Handbook - Haematopoietic Stem Cell Transplantation*. ESH-EBMT.
- Arai, S., Sheehan, K., Moore, S., Laport, G., Johnston, L., Lowsky, R., Miklos, D., Goldstein, K., Weng, W., Shizuru, J., Horning, S., Negrin, R. (2007) Autologous Cytokine-Induced Killer Cells as Post-Transplant Cellular Immunotherapy. *Blood (ASH Annual Meeting Abstracts)*, **110**, Abstract #580
- Asirvatham, A.L., Galligan, S.G., Schillace, R.V., Davey, M.P., Vasta, V., Beavo, J.A. & Carr, D.W. (2004) A-kinase anchoring proteins interact with phosphodiesterases in T lymphocyte cell lines. *J Immunol*, **173**, 4806-4814.

- Bae, J., Martinson, J.A. & Klingemann, H.G. (2004) Heteroclitic CD33 peptide with enhanced anti-acute myeloid leukemic immunogenicity. *Clin Cancer Res*, **10**, 7043-7052.
- Baer, M.R., George, S.L., Caligiuri, M.A., Sanford, B.L., Bothun, S.M., Mrozek, K., Kolitz, J.E., Powell, B.L., Moore, J.O., Stone, R.M., Anastasi, J., Bloomfield, C.D. & Larson, R.A. (2008) Low-dose interleukin-2 immunotherapy does not improve outcome of patients age 60 years and older with acute myeloid leukemia in first complete remission: Cancer and Leukemia Group B Study 9720. *J Clin Oncol*, **26**, 4934-4939.
- Bain, B.J. (2002) *Blood Cells. A Practical Guide*. Blackwell Publishing.
- Bain, B.J., Clark, D. M., Lampert, I.A., Wilkins, B.S. (2001) *Bone Marrow Pathology*. Blackwell Science.
- Baltimore, D., Boldin, M.P., O'Connell, R.M., Rao, D.S. & Taganov, K.D. (2008) MicroRNAs: new regulators of immune cell development and function. *Nat Immunol*, **9**, 839-845.
- Bamias, A., Tsiatas, M.L., Kafantari, E., Liakou, C., Rodolakis, A., Voulgaris, Z., Vlahos, G., Papageorgiou, T., Tsitsilonis, O., Bamia, C., Papatheodoridis, G., Politi, E., Archimandritis, A., Antsaklis, A. & Dimopoulos, M.A. (2007) Significant differences of lymphocytes isolated from ascites of patients with ovarian cancer compared to blood and tumor lymphocytes. Association of CD3+CD56+ cells with platinum resistance. *Gynecol Oncol*, **106**, 75-81.
- Banat, G.A., Ihlow, K., Usluoglu, N., Hoppmann, S., Hoeck, M. & Pralle, H. (2003) Core-binding factor-beta positive acute myeloid leukaemia cells induce T-cell responses. *Br J Haematol*, **123**, 819-829.
- Barrett, S.G., Hansen, K.S. & Bainton, D.F. (1981) Differentiation of cell surface receptors on normal human bone marrow myeloid precursors. *Br J Haematol*, **48**, 491-500.
- Beckman, E.M., Porcelli, S.A., Morita, C.T., Behar, S.M., Furlong, S.T. & Brenner, M.B. (1994) Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells. *Nature*, **372**, 691-694.
- Behl, D., Porrata, L.F., Markovic, S.N., Letendre, L., Pruthi, R.K., Hook, C.C., Tefferi, A., Elliot, M.A., Kaufmann, S.H., Mesa, R.A. & Litzow, M.R. (2006) Absolute lymphocyte count recovery after induction chemotherapy predicts superior survival in acute myelogenous leukemia. *Leukemia*, **20**, 29-34.
- Bendelac, A., Lantz, O., Quimby, M.E., Yewdell, J.W., Bennink, J.R. & Brutkiewicz, R.R. (1995) CD1 recognition by mouse NK1+ T lymphocytes. *Science*, **268**, 863-865.
- Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A., Gralnick, H.R. & Sultan, C. (1976) Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*, **33**, 451-458.
- Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A., Gralnick, H.R. & Sultan, C. (1985) Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med*, **103**, 620-625.
- Benyunes, M.C., Massumoto, C., York, A., Higuchi, C.M., Buckner, C.D., Thompson, J.A., Petersen, F.B. & Fefer, A. (1993) Interleukin-2 with or without lymphokine-activated killer cells as consolidative immunotherapy

- after autologous bone marrow transplantation for acute myelogenous leukemia. *Bone Marrow Transplant*, **12**, 159-163.
- Bergmann, L., Miething, C., Maurer, U., Brieger, J., Karakas, T., Weidmann, E. & Hoelzer, D. (1997) High levels of Wilms' tumor gene (wt1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome. *Blood*, **90**, 1217-1225.
- Bernard, F., Jaleco, S., Dardalhon, V., Steinberg, M., Yssel, H., Noraz, N., Taylor, N. & Kinet, S. (2002) Ex vivo isolation protocols differentially affect the phenotype of human CD4<sup>+</sup> T cells. *J Immunol Methods*, **271**, 99-106.
- Berndt, C., Mopps, B., Angermuller, S., Gierschik, P. & Krammer, P.H. (1998) CXCR4 and CD4 mediate a rapid CD95-independent cell death in CD4<sup>+</sup> T cells. *Proc Natl Acad Sci U S A*, **95**, 12556-12561.
- Beyer, M., Kochanek, M., Darabi, K., Popov, A., Jensen, M., Endl, E., Knolle, P.A., Thomas, R.K., von Bergwelt-Baildon, M., Debey, S., Hallek, M. & Schultze, J.L. (2005) Reduced frequencies and suppressive function of CD4<sup>+</sup>CD25<sup>hi</sup> regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. *Blood*, **106**, 2018-2025.
- Beyer, M., Kochanek, M., Giese, T., Endl, E., Weihrauch, M.R., Knolle, P.A., Classen, S. & Schultze, J.L. (2006) In vivo peripheral expansion of naive CD4<sup>+</sup>CD25<sup>high</sup> FoxP3<sup>+</sup> regulatory T cells in patients with multiple myeloma. *Blood*, **107**, 3940-3949.
- Billingham, R.E. (1966) The biology of graft-versus-host reactions. *Harvey Lect*, **62**, 21-78.
- Billingham, R.E., Brent, L. & Medawar, P.B. (1953) Actively acquired tolerance of foreign cells. *Nature*, **172**, 603-606.
- Billingham, R.E., Brent, L., Medawar, P.B.M. (1956) Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. *Philos Trans R Soc Lond B*, **239**, 357-414.
- Birkeland, S.A., Storm, H.H., Lamm, L.U., Barlow, L., Blohme, I., Forsberg, B., Eklund, B., Fjeldborg, O., Friedberg, M., Frodin, L. & et al. (1995) Cancer risk after renal transplantation in the Nordic countries, 1964-1986. *Int J Cancer*, **60**, 183-189.
- Blaise, D., Attal, M., Pico, J.L., Reiffers, J., Stoppa, A.M., Bellanger, C., Molina, L., Nedellec, G., Vernant, J.P., Legros, M., Gabus, R., Huguet, F., Brandely, M., Hercend, T., Olive, D. & Maraninchi, D. (1997) The use of a sequential high dose recombinant interleukin 2 regimen after autologous bone marrow transplantation does not improve the disease free survival of patients with acute leukemia transplanted in first complete remission. *Leuk Lymphoma*, **25**, 469-478.
- Bogen, B. (1996) Peripheral T cell tolerance as a tumor escape mechanism: deletion of CD4<sup>+</sup> T cells specific for a monoclonal immunoglobulin idiotype secreted by a plasmacytoma. *Eur J Immunol*, **26**, 2671-2679.
- Bonini, C., Ferrari, G., Verzeletti, S., Servida, P., Zappone, E., Ruggieri, L., Ponzoni, M., Rossini, S., Mavilio, F., Traversari, C. & Bordignon, C. (1997) HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science*, **276**, 1719-1724.
- Bonnet, D. & Dick, J.E. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*, **3**, 730-737.

- Bonyhadi, M., Frohlich, M., Rasmussen, A., Ferrand, C., Grosmaire, L., Robinet, E., Leis, J., Maziarz, R.T., Tiberghien, P. & Berenson, R.J. (2005) In vitro engagement of CD3 and CD28 corrects T cell defects in chronic lymphocytic leukemia. *J Immunol*, **174**, 2366-2375.
- Boyer, M.W., Vallera, D.A., Taylor, P.A., Gray, G.S., Katsanis, E., Gorden, K., Orchard, P.J. & Blazar, B.R. (1997) The role of B7 costimulation by murine acute myeloid leukemia in the generation and function of a CD8+ T-cell line with potent in vivo graft-versus-leukemia properties. *Blood*, **89**, 3477-3485.
- Breasted, J.H. (1922) The Edwin Smith Papyrus. *New York Historical Society*.
- Brossart, P., Schneider, A., Dill, P., Schammann, T., Grunebach, F., Wirths, S., Kanz, L., Buhring, H.J. & Brugger, W. (2001) The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. *Cancer Res*, **61**, 6846-6850.
- Brossay, L., Chioda, M., Burdin, N., Koezuka, Y., Casorati, G., Dellabona, P. & Kronenberg, M. (1998) CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med*, **188**, 1521-1528.
- Brouwer, R.E., Zwinderman, K.H., Kluin-Nelemans, H.C., van Luxemburg-Heijs, S.A., Willemze, R. & Falkenburg, J.H. (2000) Expression and induction of costimulatory and adhesion molecules on acute myeloid leukemic cells: implications for adoptive immunotherapy. *Exp Hematol*, **28**, 161-168.
- Bruggemann, M., White, H., Gaulard, P., Garcia-Sanz, R., Gameiro, P., Oeschger, S., Jasani, B., Ott, M., Delsol, G., Orfao, A., Tiemann, M., Herbst, H., Langerak, A.W., Spaargaren, M., Moreau, E., Groenen, P.J., Sambade, C., Feroni, L., Carter, G.I., Hummel, M., Bastard, C., Davi, F., Delfau-Larue, M.H., Kneba, M., van Dongen, J.J., Beldjord, K. & Molina, T.J. (2007) Powerful strategy for polymerase chain reaction-based clonality assessment in T-cell malignancies Report of the BIOMED-2 Concerted Action BHM4 CT98-3936. *Leukemia*, **21**, 215-221.
- Brune, M., Castaigne, S., Catalano, J., Gehlsen, K., Ho, A.D., Hofmann, W.K., Hogge, D.E., Nilsson, B., Or, R., Romero, A.I., Rowe, J.M., Simonsson, B., Spearing, R., Stadtmauer, E.A., Szer, J., Wallhult, E. & Hellstrand, K. (2006) Improved leukemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukemia: results of a randomized phase 3 trial. *Blood*, **108**, 88-96.
- Bruserud, O. (1998b) Cellular immune responses in acute leukaemia patients with severe chemotherapy-induced leucopenia; characterization of the cytokine repertoire of clonogenic T cells. *Cancer Immunol Immunother*, **46**, 221-228.
- Bruserud, O., Ulvestad, E., Berentsen, S., Bergheim, J. & Nesthus, I. (1998a) T-lymphocyte functions in acute leukaemia patients with severe chemotherapy-induced cytopenia: characterization of clonogenic T-cell proliferation. *Scand J Immunol*, **47**, 54-62.
- Buckley, R.H. (2000) Primary immunodeficiency diseases due to defects in lymphocytes. *N Engl J Med*, **343**, 1313-1324.
- Budd, R.C., Miescher, G.C., Howe, R.C., Lees, R.K., Bron, C. & MacDonald, H.R. (1987) Developmentally regulated expression of T cell receptor beta

- chain variable domains in immature thymocytes. *J Exp Med*, **166**, 577-582.
- Buggins, A.G., Hirst, W.J., Pagliuca, A. & Mufti, G.J. (1998) Variable expression of CD3-zeta and associated protein tyrosine kinases in lymphocytes from patients with myeloid malignancies. *Br J Haematol*, **100**, 784-792.
- Buggins, A.G., Lea, N., Gaken, J., Darling, D., Farzaneh, F., Mufti, G.J. & Hirst, W.J. (1999) Effect of costimulation and the microenvironment on antigen presentation by leukemic cells. *Blood*, **94**, 3479-3490.
- Buggins, A.G., Milojkovic, D., Arno, M.J., Lea, N.C., Mufti, G.J., Thomas, N.S. & Hirst, W.J. (2001) Microenvironment produced by acute myeloid leukemia cells prevents T cell activation and proliferation by inhibition of NF-kappaB, c-Myc, and pRb pathways. *J Immunol*, **167**, 6021-6030.
- Burnet, F.M. (1957) Cancer - a biological approach. *Brit Med J*, **1**, 841-847.
- Burnett, A.K. & Knapper, S. (2007) Targeting Treatment in AML. *Hematology Am Soc Hematol Educ Program*, 429-434.
- Burnett, A.K., Milligan, D., Prentice, A.G., Goldstone, A.H., McMullin, M.F., Hills, R.K. & Wheatley, K. (2007) A comparison of low-dose cytarabine and hydroxyurea with or without all-trans retinoic acid for acute myeloid leukemia and high-risk myelodysplastic syndrome in patients not considered fit for intensive treatment. *Cancer*, **109**, 1114-1124.
- Burnett, A.K., Wheatley, K., Goldstone, A.H., Stevens, R.F., Hann, I.M., Rees, J.H. & Harrison, G. (2002) The value of allogeneic bone marrow transplant in patients with acute myeloid leukaemia at differing risk of relapse: results of the UK MRC AML 10 trial. *Br J Haematol*, **118**, 385-400.
- Cassileth, P.A., Harrington, D.P., Appelbaum, F.R., Lazarus, H.M., Rowe, J.M., Paietta, E., Willman, C., Hurd, D.D., Bennett, J.M., Blume, K.G., Head, D.R. & Wiernik, P.H. (1998) Chemotherapy compared with autologous or allogeneic bone marrow transplantation in the management of acute myeloid leukemia in first remission. *N Engl J Med*, **339**, 1649-1656.
- Ceredig, R., Lynch, F. & Newman, P. (1987) Phenotypic properties, interleukin 2 production, and developmental origin of a "mature" subpopulation of Lyt-2- L3T4- mouse thymocytes. *Proc Natl Acad Sci U S A*, **84**, 8578-8582.
- Chan, L., Hardwick, N., Darling, D., Galea-Lauri, J., Gaken, J., Devereux, S., Kemeny, M., Mufti, G. & Farzaneh, F. (2005) IL-2/B7.1 (CD80) fusogene transduction of AML blasts by a self-inactivating lentiviral vector stimulates T cell responses in vitro: a strategy to generate whole cell vaccines for AML. *Mol Ther*, **11**, 120-131.
- Chan, L., Hardwick, N.R., Guinn, B.A., Darling, D., Gaken, J., Galea-Lauri, J., Ho, A.Y., Mufti, G.J. & Farzaneh, F. (2006) An immune edited tumour versus a tumour edited immune system: Prospects for immune therapy of acute myeloid leukaemia. *Cancer Immunol Immunother*, **55**, 1017-1024.
- Charrad, R.S., Gadhoun, Z., Qi, J., Glachant, A., Allouche, M., Jasmin, C., Chomienne, C. & Smadja-Joffe, F. (2002) Effects of anti-CD44 monoclonal antibodies on differentiation and apoptosis of human myeloid leukemia cell lines. *Blood*, **99**, 290-299.
- Charrad, R.S., Li, Y., Delpech, B., Balitrand, N., Clay, D., Jasmin, C., Chomienne, C. & Smadja-Joffe, F. (1999) Ligation of the CD44 adhesion molecule reverses blockage of differentiation in human acute myeloid leukemia. *Nat Med*, **5**, 669-676.

- Chen, F., Ogawa, K., Nagarajan, R.P., Zhang, M., Kuang, C. & Chen, Y. (2003) Regulation of TG-interacting factor by transforming growth factor-beta. *Biochem J*, **371**, 257-263.
- Chen, L., Ashe, S., Brady, W.A., Hellstrom, I., Hellstrom, K.E., Ledbetter, J.A., McGowan, P. & Linsley, P.S. (1992) Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell*, **71**, 1093-1102.
- Cheng, F., Wang, H.W., Cuenca, A., Huang, M., Ghansah, T., Brayer, J., Kerr, W.G., Takeda, K., Akira, S., Schoenberger, S.P., Yu, H., Jove, R. & Sotomayor, E.M. (2003) A critical role for Stat3 signaling in immune tolerance. *Immunity*, **19**, 425-436.
- Chikamatsu, K., Sakakura, K., Whiteside, T.L. & Furuya, N. (2007) Relationships between regulatory T cells and CD8+ effector populations in patients with squamous cell carcinoma of the head and neck. *Head Neck*, **29**, 120-127.
- Chuck, R.S., Cantor, C.R. & Tse, D.B. (1993) Effect of CD4 engagement on CD4-T cell receptor complexes. *Cell Immunol*, **152**, 211-219.
- Chung, C.D., Patel, V.P., Moran, M., Lewis, L.A. & Miceli, M.C. (2000) Galectin-1 induces partial TCR zeta-chain phosphorylation and antagonizes processive TCR signal transduction. *J Immunol*, **165**, 3722-3729.
- Cignetti, A., Bryant, E., Allione, B., Vitale, A., Foa, R. & Cheever, M.A. (1999) CD34(+) acute myeloid and lymphoid leukemic blasts can be induced to differentiate into dendritic cells. *Blood*, **94**, 2048-2055.
- Clemente, C.G., Mihm, M.C., Jr., Bufalino, R., Zurrida, S., Collini, P. & Cascinelli, N. (1996) Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer*, **77**, 1303-1310.
- Clift, R.A., Buckner, C.D., Thomas, E.D., Kopecky, K.J., Appelbaum, F.R., Tallman, M., Storb, R., Sanders, J., Sullivan, K., Banaji, M. & et al. (1987) The treatment of acute non-lymphoblastic leukemia by allogeneic marrow transplantation. *Bone Marrow Transplant*, **2**, 243-258.
- Cohavy, O. & Targan, S.R. (2007) CD56 marks an effector T cell subset in the human intestine. *J Immunol*, **178**, 5524-5532.
- Coiffier, B., Lepage, E., Briere, J., Herbrecht, R., Tilly, H., Bouabdallah, R., Morel, P., Van Den Neste, E., Salles, G., Gaulard, P., Reyes, F., Lederlin, P. & Gisselbrecht, C. (2002) CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *N Engl J Med*, **346**, 235-242.
- Coley, W.B. (1894) Treatment of inoperable malignant tumour with the toxins of erysipelas and *Bacillus prodigiosus*. *Trans Am Surg Assoc*, **12**, 183-196.
- Cooper, M.A., Fehniger, T.A., Turner, S.C., Chen, K.S., Ghaheri, B.A., Ghayur, T., Carson, W.E. & Caligiuri, M.A. (2001) Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood*, **97**, 3146-3151.
- Cortes, J.E., Kantarjian, H.M., O'Brien, S., Giles, F., Keating, M.J., Freireich, E.J. & Estey, E.H. (1999) A pilot study of interleukin-2 for adult patients with acute myelogenous leukemia in first complete remission. *Cancer*, **85**, 1506-1513.
- Costello, R.T., Sivori, S., Mallet, F., Sainty, D., Arnoulet, C., Reviron, D., Gastaut, J.A., Moretta, A. & Olive, D. (2002a) A novel mechanism of

- antitumor response involving the expansion of CD3+/CD56+ large granular lymphocytes triggered by a tumor-expressed activating ligand. *Leukemia*, **16**, 855-860.
- Costello, R.T., Sivori, S., Marcenaro, E., Lafage-Pochitaloff, M., Mozziconacci, M.J., Reviron, D., Gastaut, J.A., Pende, D., Olive, D. & Moretta, A. (2002b) Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. *Blood*, **99**, 3661-3667.
- Csako, G., Binder, R.A., Kales, A.N. & Neefe, J.R. (1980) Cloning of human lymphocytes reactive with autologous leukemia cells. *Cancer Res*, **40**, 3218-3221.
- Curiel, T.J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Evdemon-Hogan, M., Conejo-Garcia, J.R., Zhang, L., Burow, M., Zhu, Y., Wei, S., Kryczek, I., Daniel, B., Gordon, A., Myers, L., Lackner, A., Disis, M.L., Knutson, K.L., Chen, L. & Zou, W. (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*, **10**, 942-949.
- da Silva, A.J., Li, Z., de Vera, C., Canto, E., Findell, P. & Rudd, C.E. (1997) Cloning of a novel T-cell protein FYB that binds FYN and SH2-domain-containing leukocyte protein 76 and modulates interleukin 2 production. *Proc Natl Acad Sci U S A*, **94**, 7493-7498.
- Dallas, P.B., Gottardo, N.G., Firth, M.J., Beesley, A.H., Hoffmann, K., Terry, P.A., Freitas, J.R., Boag, J.M., Cummings, A.J. & Kees, U.R. (2005) Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR -- how well do they correlate? *BMC Genomics*, **6**, 59.
- Dannull, J., Su, Z., Rizzieri, D., Yang, B.K., Coleman, D., Yancey, D., Zhang, A., Dahm, P., Chao, N., Gilboa, E. & Vieweg, J. (2005) Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest*, **115**, 3623-3633.
- Datta, S.K. (1996) Role of natural effector cells in the prevention of radiation-induced leukemogenesis. *Biomed Pharmacother*, **50**, 125-131.
- Dave, S.S., Wright, G., Tan, B., Rosenwald, A., Gascoyne, R.D., Chan, W.C., Fisher, R.I., Braziel, R.M., Rimsza, L.M., Grogan, T.M., Miller, T.P., LeBlanc, M., Greiner, T.C., Weisenburger, D.D., Lynch, J.C., Vose, J., Armitage, J.O., Smeland, E.B., Kvaloy, S., Holte, H., Delabie, J., Connors, J.M., Lansdorp, P.M., Ouyang, Q., Lister, T.A., Davies, A.J., Norton, A.J., Muller-Hermelink, H.K., Ott, G., Campo, E., Montserrat, E., Wilson, W.H., Jaffe, E.S., Simon, R., Yang, L., Powell, J., Zhao, H., Goldschmidt, N., Chiorazzi, M. & Staudt, L.M. (2004) Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. *N Engl J Med*, **351**, 2159-2169.
- Davey, M.P., Meyer, M.M., Munkirs, D.D., Babcock, D., Braun, M.P., Hayden, J.B. & Bakke, A.C. (1991) T-cell receptor variable beta genes show differential expression in CD4 and CD8 T cells. *Hum Immunol*, **32**, 194-202.
- Davis, M.M. & Bjorkman, P.J. (1988) T-cell antigen receptor genes and T-cell recognition. *Nature*, **334**, 395-402.
- De Rosa, S.C., Herzenberg, L.A. & Roederer, M. (2001) 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. *Nat Med*, **7**, 245-248.

- Dellabona, P., Padovan, E., Casorati, G., Brockhaus, M. & Lanzavecchia, A. (1994) An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. *J Exp Med*, **180**, 1171-1176.
- Delluc, S., Tourneur, L., Michallet, A.S., Boix, C., Varet, B., Fradelizi, D., Guillet, J.G. & Buzyn, A. (2005) Autologous peptides eluted from acute myeloid leukemia cells can be used to generate specific antileukemic CD4 helper and CD8 cytotoxic T lymphocyte responses in vitro. *Haematologica*, **90**, 1050-1062.
- Derre, L., Corvaisier, M., Charreau, B., Moreau, A., Godefroy, E., Moreau-Aubry, A., Jotereau, F. & Gervois, N. (2006) Expression and release of HLA-E by melanoma cells and melanocytes: potential impact on the response of cytotoxic effector cells. *J Immunol*, **177**, 3100-3107.
- Dhodapkar, M.V., Geller, M.D., Chang, D.H., Shimizu, K., Fujii, S., Dhodapkar, K.M. & Krasovsky, J. (2003a) A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. *J Exp Med*, **197**, 1667-1676.
- Dhodapkar, M.V., Krasovsky, J., Osman, K. & Geller, M.D. (2003b) Vigorous premalignancy-specific effector T cell response in the bone marrow of patients with monoclonal gammopathy. *J Exp Med*, **198**, 1753-1757.
- Dieckmann, D., Bruett, C.H., Ploettner, H., Lutz, M.B. & Schuler, G. (2002) Human CD4(+)CD25(+) regulatory, contact-dependent T cells induce interleukin 10-producing, contact-independent type 1-like regulatory T cells [corrected]. *J Exp Med*, **196**, 247-253.
- Doherty, D.G., Norris, S., Madrigal-Estebas, L., McEntee, G., Traynor, O., Hegarty, J.E. & O'Farrelly, C. (1999) The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. *J Immunol*, **163**, 2314-2321.
- Dominguez-Villar, M., Munoz-Suano, A., Anaya-Baz, B., Aguilar, S., Novalbos, J.P., Giron, J.A., Rodriguez-Iglesias, M. & Garcia-Cozar, F. (2007) Hepatitis C virus core protein up-regulates anergy-related genes and a new set of genes, which affects T cell homeostasis. *J Leukoc Biol*, **82**, 1301-1310.
- Dong, H., Strome, S.E., Salomao, D.R., Tamura, H., Hirano, F., Flies, D.B., Roche, P.C., Lu, J., Zhu, G., Tamada, K., Lennon, V.A., Celis, E. & Chen, L. (2002) Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*, **8**, 793-800.
- Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J. & Schreiber, R.D. (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol*, **3**, 991-998.
- Dustin, M.L. & Cooper, J.A. (2000) The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. *Nat Immunol*, **1**, 23-29.
- Edelman, G.M. & Crossin, K.L. (1991) Cell adhesion molecules: implications for a molecular histology. *Annu Rev Biochem*, **60**, 155-190.
- Ehrlich, P. (1900) The Croonian Lecture: "On immunity with special reference to cell life". *Proc R Soc*, **66**, 424-448.
- Ersvaer, E., Hampson, P., Wendelbo, O., Lord, J.M., Gjertsen, B.T. & Bruserud, O. (2007) Circulating T cells in patients with untreated acute myelogenous

- leukemia are heterogeneous and can be activated through the CD3/TCR complex. *Hematology*, **12**, 199-207.
- Evel-Kabler, K., Song, X.T., Aldrich, M., Huang, X.F. & Chen, S.Y. (2006) SOCS1 restricts dendritic cells' ability to break self tolerance and induce antitumor immunity by regulating IL-12 production and signaling. *J Clin Invest*, **116**, 90-100.
- Falini, B., Mecucci, C., Tiacci, E., Alcalay, M., Rosati, R., Pasqualucci, L., La Starza, R., Diverio, D., Colombo, E., Santucci, A., Bigerna, B., Pacini, R., Pucciarini, A., Liso, A., Vignetti, M., Fazi, P., Meani, N., Pettrossi, V., Saglio, G., Mandelli, F., Lo-Coco, F., Pelicci, P.G. & Martelli, M.F. (2005) Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med*, **352**, 254-266.
- Fehniger, T.A., Byrd, J.C., Marcucci, G., Abboud, C.N., Kefauver, C., Payton, J.E., Vij, R. & Blum, W. (2009) Single-agent lenalidomide induces complete remission of acute myeloid leukemia in patients with isolated trisomy 13. *Blood*, **113**, 1002-1005.
- Ferradini, L., Mackensen, A., Genevee, C., Bosq, J., Duvillard, P., Avril, M.F. & Hercend, T. (1993) Analysis of T cell receptor variability in tumor-infiltrating lymphocytes from a human regressive melanoma. Evidence for in situ T cell clonal expansion. *J Clin Invest*, **91**, 1183-1190.
- Feuer, G., Stewart, S.A., Baird, S.M., Lee, F., Feuer, R. & Chen, I.S. (1995) Potential role of natural killer cells in controlling tumorigenesis by human T-cell leukemia viruses. *J Virol*, **69**, 1328-1333.
- Fowlkes, B.J., Kruisbeek, A.M., Ton-That, H., Weston, M.A., Coligan, J.E., Schwartz, R.H. & Pardoll, D.M. (1987) A novel population of T-cell receptor alpha beta-bearing thymocytes which predominantly expresses a single V beta gene family. *Nature*, **329**, 251-254.
- Fozza, C., Bellizzi, S., Bonfigli, S., Campus, P.M., Dore, F. & Longinotti, M. (2004) Cytogenetic and hematological spontaneous remission in a case of acute myelogenous leukemia. *Eur J Haematol*, **73**, 219-222.
- Frassanito, M.A., Cusmai, A. & Dammacco, F. (2001) Deregulated cytokine network and defective Th1 immune response in multiple myeloma. *Clin Exp Immunol*, **125**, 190-197.
- Fujii, S., Fujimoto, K., Shimizu, K., Ezaki, T., Kawano, F., Takatsuki, K., Kawakita, M. & Matsuno, K. (1999) Presentation of tumor antigens by phagocytic dendritic cell clusters generated from human CD34+ hematopoietic progenitor cells: induction of autologous cytotoxic T lymphocytes against leukemic cells in acute myelogenous leukemia patients. *Cancer Res*, **59**, 2150-2158.
- Fujii, S., Shimizu, K., Klimek, V., Geller, M.D., Nimer, S.D. & Dhodapkar, M.V. (2003a) Severe and selective deficiency of interferon-gamma-producing invariant natural killer T cells in patients with myelodysplastic syndromes. *Br J Haematol*, **122**, 617-622.
- Fujii, S., Shimizu, K., Smith, C., Bonifaz, L. & Steinman, R.M. (2003b) Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med*, **198**, 267-279.
- Gabrilovich, D.I., Chen, H.L., Girgis, K.R., Cunningham, H.T., Meny, G.M., Nadaf, S., Kavanaugh, D. & Carbone, D.P. (1996) Production of vascular

- endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med*, **2**, 1096-1103.
- Gale, R.P., Buchner, T., Zhang, M.J., Heinecke, A., Champlin, R.E., Dicke, K.A., Gluckman, E., Good, R.A., Gratwohl, A., Herzig, R.H., Keating, A., Klein, J.P., Marmont, A.M., Prentice, H.G., Rowlings, P.A., Sobocinski, K.A., Speck, B., Weiner, R.S. & Horowitz, M.M. (1996) HLA-identical sibling bone marrow transplants vs chemotherapy for acute myelogenous leukemia in first remission. *Leukemia*, **10**, 1687-1691.
- Galea-Lauri, J. (2002) Immunological weapons against acute myeloid leukaemia. *Immunology*, **107**, 20-27.
- Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pages, C., Tosolini, M., Camus, M., Berger, A., Wind, P., Zinzindohoue, F., Bruneval, P., Cugnenc, P.H., Trajanoski, Z., Fridman, W.H. & Pages, F. (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*, **313**, 1960-1964.
- Garrido, S.M., Appelbaum, F.R., Willman, C.L. & Banker, D.E. (2001) Acute myeloid leukemia cells are protected from spontaneous and drug-induced apoptosis by direct contact with a human bone marrow stromal cell line (HS-5). *Exp Hematol*, **29**, 448-457.
- Gasser, O., Missiou, A., Eken, C. & Hess, C. (2005) Human CD8+ T cells store CXCR1 in a distinct intracellular compartment and up-regulate it rapidly to the cell surface upon activation. *Blood*, **106**, 3718-3724.
- Gerlini, G., Tun-Kyi, A., Dudli, C., Burg, G., Pimpinelli, N. & Nestle, F.O. (2004) Metastatic melanoma secreted IL-10 down-regulates CD1 molecules on dendritic cells in metastatic tumor lesions. *Am J Pathol*, **165**, 1853-1863.
- Germain, R.N. (1994) MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell*, **76**, 287-299.
- Germain, R.N. (2002) T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol*, **2**, 309-322.
- Godfrey, D.I., Hammond, K.J., Poulton, L.D., Smyth, M.J. & Baxter, A.G. (2000) NKT cells: facts, functions and fallacies. *Immunol Today*, **21**, 573-583.
- Gorgun, G., Holderried, T.A., Zahrieh, D., Neuberg, D. & Gribben, J.G. (2005) Chronic lymphocytic leukemia cells induce changes in gene expression of CD4 and CD8 T cells. *J Clin Invest*, **115**, 1797-1805.
- Gorgun, G., Holderried, T., Le Dieu, R., Zahrieh, D., Gribben, J. (2005) CLL cells in TCL1 mice induce similar defects in CD4 and CD8 T cells to those observed in patients with CLL. *Blood*, **106**, 19a, abstract #50.
- Gorgun, G., Ramsay, A.G., Holderried, T.A.W., Zahrieh, D., Le Dieu, R., Liu, F., Quackenbush, J., Croce, C.M., Gribben, J.G. (2009) E $\mu$ -TCL1 mice represent a model for immunotherapeutic reversal of chronic lymphocytic leukemia-induced T cell dysfunction. *PNAS* (in press).
- Grant, G.A. & Miller, J.F. (1965) Effect of neonatal thymectomy on the induction of sarcomata in C57 BL mice. *Nature*, **205**, 1124-1125.
- Gratwohl, A., Baldomero, H., Passweg, J. & Urbano-Ispizua, A. (2002) Increasing use of reduced intensity conditioning transplants: report of the 2001 EBMT activity survey. *Bone Marrow Transplant*, **30**, 813-831.
- Greiner, J., Li, L., Ringhoffer, M., Barth, T.F., Giannopoulos, K., Guillaume, P., Ritter, G., Wiesneth, M., Dohner, H. & Schmitt, M. (2005) Identification and characterization of epitopes of the receptor for hyaluronic acid-

- mediated motility (RHAMM/CD168) recognized by CD8+ T cells of HLA-A2-positive patients with acute myeloid leukemia. *Blood*, **106**, 938-945.
- Greiner, J., Ringhoffer, M., Simikopinko, O., Szmargowska, A., Huebsch, S., Maurer, U., Bergmann, L. & Schmitt, M. (2000) Simultaneous expression of different immunogenic antigens in acute myeloid leukemia. *Exp Hematol*, **28**, 1413-1422.
- Greiner, J., Ringhoffer, M., Taniguchi, M., Hauser, T., Schmitt, A., Dohner, H. & Schmitt, M. (2003) Characterization of several leukemia-associated antigens inducing humoral immune responses in acute and chronic myeloid leukemia. *Int J Cancer*, **106**, 224-231.
- Greiner, J., Ringhoffer, M., Taniguchi, M., Schmitt, A., Kirchner, D., Krahn, G., Heilmann, V., Gschwend, J., Bergmann, L., Dohner, H. & Schmitt, M. (2002) Receptor for hyaluronan acid-mediated motility (RHAMM) is a new immunogenic leukemia-associated antigen in acute and chronic myeloid leukemia. *Exp Hematol*, **30**, 1029-1035.
- Grimwade, D., Walker, H., Oliver, F., Wheatley, K., Harrison, C., Harrison, G., Rees, J., Hann, I., Stevens, R., Burnett, A. & Goldstone, A. (1998) The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood*, **92**, 2322-2333.
- Grunewald, J., Janson, C.H. & Wigzell, H. (1991) Biased expression of individual T cell receptor V gene segments in CD4+ and CD8+ human peripheral blood T lymphocytes. *Eur J Immunol*, **21**, 819-822.
- Guinn, B.A., Bland, E.A., Lodi, U., Liggins, A.P., Tobal, K., Petters, S., Wells, J.W., Banham, A.H. & Mufti, G.J. (2005) Humoral detection of leukaemia-associated antigens in presentation acute myeloid leukaemia. *Biochem Biophys Res Commun*, **335**, 1293-1304.
- Gumperz, J.E., Miyake, S., Yamamura, T. & Brenner, M.B. (2002) Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med*, **195**, 625-636.
- Hagemann, T., Wilson, J., Burke, F., Kulbe, H., Li, N.F., Pluddemann, A., Charles, K., Gordon, S. & Balkwill, F.R. (2006) Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype. *J Immunol*, **176**, 5023-5032.
- Hamann, D., Baars, P.A., Rep, M.H., Hooibrink, B., Kerkhof-Garde, S.R., Klein, M.R. & van Lier, R.A. (1997) Phenotypic and functional separation of memory and effector human CD8+ T cells. *J Exp Med*, **186**, 1407-1418.
- Hamann, D., Roos, M.T. & van Lier, R.A. (1999) Faces and phases of human CD8 T-cell development. *Immunol Today*, **20**, 177-180.
- Harousseau, J.L., Cahn, J.Y., Pignon, B., Witz, F., Milpied, N., Delain, M., Lioure, B., Lamy, T., Desablens, B., Guilhot, F., Caillot, D., Abgrall, J.F., Francois, S., Briere, J., Guyotat, D., Casassus, P., Audhuy, B., Tellier, Z., Hurteloup, P. & Herve, P. (1997) Comparison of autologous bone marrow transplantation and intensive chemotherapy as postremission therapy in adult acute myeloid leukemia. The Groupe Ouest Est Leucemies Aigues Myeloblastiques (GOELAM). *Blood*, **90**, 2978-2986.
- Hartmann, E., Wollenberg, B., Rothenfusser, S., Wagner, M., Wellisch, D., Mack, B., Giese, T., Gires, O., Endres, S. & Hartmann, G. (2003) Identification

- and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer. *Cancer Res*, **63**, 6478-6487.
- Hayday, A.C. (2000) [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol*, **18**, 975-1026.
- Hermans, I.F., Silk, J.D., Gileadi, U., Salio, M., Mathew, B., Ritter, G., Schmidt, R., Harris, A.L., Old, L. & Cerundolo, V. (2003) NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol*, **171**, 5140-5147.
- Hersh, E.M., Whitecar, J.P., Jr., McCredie, K.B., Bodey, G.P., Sr. & Freireich, E.J. (1971) Chemotherapy, immunocompetence, immunosuppression and prognosis in acute leukemia. *N Engl J Med*, **285**, 1211-1216.
- Heslop, H.E., Stevenson, F.K. & Molldrem, J.J. (2003) Immunotherapy of hematologic malignancy. *Hematology Am Soc Hematol Educ Program*, 331-349.
- Hingorani, R., Choi, I.H., Akolkar, P., Gulwani-Akolkar, B., Pergolizzi, R., Silver, J. & Gregersen, P.K. (1993) Clonal predominance of T cell receptors within the CD8+ CD45RO+ subset in normal human subjects. *J Immunol*, **151**, 5762-5769.
- Hirst, W.J., Buggins, A., Darling, D., Gaken, J., Farzaneh, F. & Mufti, G.J. (1997) Enhanced immune costimulatory activity of primary acute myeloid leukaemia blasts after retrovirus-mediated gene transfer of B7.1. *Gene Ther*, **4**, 691-699.
- Hoffbrand, A.V., Pettit, J.E. and Moss, P.A.H. (2002) *Essential Haematology*. Blackwell Science.
- Hoffman, S. & Edelman, G.M. (1983) Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. *Proc Natl Acad Sci U S A*, **80**, 5762-5766.
- Horowitz, M.M., Gale, R.P., Sondel, P.M., Goldman, J.M., Kersey, J., Kolb, H.J., Rimm, A.A., Ringden, O., Rozman, C., Speck, B. & et al. (1990) Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*, **75**, 555-562.
- Houston, A., Bennett, M.W., O'Sullivan, G.C., Shanahan, F. & O'Connell, J. (2003) Fas ligand mediates immune privilege and not inflammation in human colon cancer, irrespective of TGF-beta expression. *Br J Cancer*, **89**, 1345-1351.
- Hutchings, N.J., Clarkson, N., Chalkley, R., Barclay, A.N. & Brown, M.H. (2003) Linking the T cell surface protein CD2 to the actin-capping protein CAPZ via CMS and CIN85. *J Biol Chem*, **278**, 22396-22403.
- Ifrah, N., James, J.M., Viguie, F., Marie, J.P. & Zittoun, R. (1985) Spontaneous remission in adult acute leukemia. *Cancer*, **56**, 1187-1190.
- Illmer, T. & Ehninger, G. (2007) FLT3 kinase inhibitors in the management of acute myeloid leukemia. *Clin Lymphoma Myeloma*, **8 Suppl 1**, S24-34.
- Imai, K., Matsuyama, S., Miyake, S., Suga, K. & Nakachi, K. (2000) Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet*, **356**, 1795-1799.
- Jacobs, R., Hintzen, G., Kemper, A., Beul, K., Kempf, S., Behrens, G., Sykora, K.W. & Schmidt, R.E. (2001) CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *Eur J Immunol*, **31**, 3121-3127.

- Jaffe, E.S., Harris, N.L., Stein, H., Vardiman, J.W. (2001) *World Health Organization Classification of Tumours - Tumours of Haematopoietic and Lymphoid Tissues*. IARC Press.
- Jahn, B., Bergmann, L., Weidmann, E., Brieger, J., Fenchel, K., Schwulera, U., Hoelzer, D. & Mitrou, P.S. (1995) Bone marrow-derived T-cell clones obtained from untreated acute myelocytic leukemia exhibit blast directed autologous cytotoxicity. *Leuk Res*, **19**, 73-82.
- Jin, L., Hope, K.J., Zhai, Q., Smadja-Joffe, F. & Dick, J.E. (2006) Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat Med*, **12**, 1167-1174.
- Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., Koseki, H. & Taniguchi, M. (1997) CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science*, **278**, 1626-1629.
- Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Sato, H., Kondo, E., Harada, M., Koseki, H., Nakayama, T., Tanaka, Y. & Taniguchi, M. (1998) Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc Natl Acad Sci U S A*, **95**, 5690-5693.
- Kawarabayashi, N., Seki, S., Hatsuse, K., Ohkawa, T., Koike, Y., Aihara, T., Habu, Y., Nakagawa, R., Ami, K., Hiraide, H. & Mochizuki, H. (2000) Decrease of CD56(+)T cells and natural killer cells in cirrhotic livers with hepatitis C may be involved in their susceptibility to hepatocellular carcinoma. *Hepatology*, **32**, 962-969.
- Kell, W.J., Burnett, A.K., Chopra, R., Yin, J.A., Clark, R.E., Rohatiner, A., Culligan, D., Hunter, A., Prentice, A.G. & Milligan, D.W. (2003) A feasibility study of simultaneous administration of gemtuzumab ozogamicin with intensive chemotherapy in induction and consolidation in younger patients with acute myeloid leukemia. *Blood*, **102**, 4277-4283.
- Kelly, L.M. & Gilliland, D.G. (2002) Genetics of myeloid leukemias. *Annu Rev Genomics Hum Genet*, **3**, 179-198.
- Khanna, R., Bell, S., Sherritt, M., Galbraith, A., Burrows, S.R., Rafter, L., Clarke, B., Slaughter, R., Falk, M.C., Douglass, J., Williams, T., Elliott, S.L. & Moss, D.J. (1999) Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. *Proc Natl Acad Sci U S A*, **96**, 10391-10396.
- Khong, H.T. & Restifo, N.P. (2002) Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol*, **3**, 999-1005.
- King-Underwood, L., Renshaw, J. & Pritchard-Jones, K. (1996) Mutations in the Wilms' tumor gene WT1 in leukemias. *Blood*, **87**, 2171-2179.
- Kinjo, Y., Tupin, E., Wu, D., Fujio, M., Garcia-Navarro, R., Benhnia, M.R., Zajonc, D.M., Ben-Menachem, G., Ainge, G.D., Painter, G.F., Khurana, A., Hoebe, K., Behar, S.M., Beutler, B., Wilson, I.A., Tsuji, M., Sellati, T.J., Wong, C.H. & Kronenberg, M. (2006) Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol*, **7**, 978-986.
- Kinjo, Y., Wu, D., Kim, G., Xing, G.W., Poles, M.A., Ho, D.D., Tsuji, M., Kawahara, K., Wong, C.H. & Kronenberg, M. (2005) Recognition of

- bacterial glycosphingolipids by natural killer T cells. *Nature*, **434**, 520-525.
- Kitawaki, T., Kadowaki, N., Kondo, T., Ishikawa, T., Ichinohe, T., Teramukai, S., Fukushima, M., Kasai, Y., Maekawa, T. & Uchiyama, T. (2008) Potential of dendritic-cell immunotherapy for relapse after allogeneic hematopoietic stem cell transplantation, shown by WT1 peptide- and keyhole-limpet-hemocyanin-pulsed, donor-derived dendritic-cell vaccine for acute myeloid leukemia. *Am J Hematol*, **83**, 315-317.
- Klammer, M., Waterfall, M., Samuel, K., Turner, M.L. & Roddie, P.H. (2005) Fusion hybrids of dendritic cells and autologous myeloid blasts as a potential cellular vaccine for acute myeloid leukaemia. *Br J Haematol*, **129**, 340-349.
- Klein, G. (1966) Tumor Antigens. *Annu Rev Microbiol*, **20**, 223-252.
- Kobayashi, E., Motoki, K., Uchida, T., Fukushima, H. & Koezuka, Y. (1995) KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res*, **7**, 529-534.
- Koebel, C.M., Vermi, W., Swann, J.B., Zerafa, N., Rodig, S.J., Old, L.J., Smyth, M.J. & Schreiber, R.D. (2007) Adaptive immunity maintains occult cancer in an equilibrium state. *Nature*, **450**, 903-907.
- Kohrt, H.E., Nouri, N., Nowels, K., Johnson, D., Holmes, S. & Lee, P.P. (2005) Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer. *PLoS Med*, **2**, e284.
- Koneru, M., Schaer, D., Monu, N., Ayala, A. & Frey, A.B. (2005) Defective proximal TCR signaling inhibits CD8+ tumor-infiltrating lymphocyte lytic function. *J Immunol*, **174**, 1830-1840.
- Kottaridis, P.D., Gale, R.E., Frew, M.E., Harrison, G., Langabeer, S.E., Belton, A.A., Walker, H., Wheatley, K., Bowen, D.T., Burnett, A.K., Goldstone, A.H. & Linch, D.C. (2001) The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood*, **98**, 1752-1759.
- Kurt, R.A., Urba, W.J., Smith, J.W. & Schoof, D.D. (1998) Peripheral T lymphocytes from women with breast cancer exhibit abnormal protein expression of several signaling molecules. *Int J Cancer*, **78**, 16-20.
- Kusmartsev, S. & Gabrilovich, D.I. (2003) Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J Leukoc Biol*, **74**, 186-196.
- Kusmartsev, S., Nagaraj, S. & Gabrilovich, D.I. (2005) Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. *J Immunol*, **175**, 4583-4592.
- Lange, B.J., Smith, F.O., Feusner, J., Barnard, D.R., Dinndorf, P., Feig, S., Heerema, N.A., Arndt, C., Arceci, R.J., Seibel, N., Weiman, M., Dusenbery, K., Shannon, K., Luna-Fineman, S., Gerbing, R.B. & Alonzo, T.A. (2008) Outcomes in CCG-2961, a children's oncology group phase 3 trial for untreated pediatric acute myeloid leukemia: a report from the children's oncology group. *Blood*, **111**, 1044-1053.
- Lanier, L.L., Le, A.M., Civin, C.I., Loken, M.R. & Phillips, J.H. (1986) The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on

- human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol*, **136**, 4480-4486.
- Lanier, L.L., Testi, R., Bindl, J. & Phillips, J.H. (1989) Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J Exp Med*, **169**, 2233-2238.
- Lansdorp, P.M., Aalberse, R.C., Bos, R., Schutter, W.G. & Van Bruggen, E.F. (1986) Cyclic tetramolecular complexes of monoclonal antibodies: a new type of cross-linking reagent. *Eur J Immunol*, **16**, 679-683.
- Lantz, O. & Bendelac, A. (1994) An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med*, **180**, 1097-1106.
- Larson, R.A., Sievers, E.L., Stadtmauer, E.A., Lowenberg, B., Estey, E.H., Dombret, H., Theobald, M., Voliotis, D., Bennett, J.M., Richie, M., Leopold, L.H., Berger, M.S., Sherman, M.L., Loken, M.R., van Dongen, J.J., Bernstein, I.D. & Appelbaum, F.R. (2005) Final report of the efficacy and safety of gemtuzumab ozogamicin (Mylotarg) in patients with CD33-positive acute myeloid leukemia in first recurrence. *Cancer*, **104**, 1442-1452.
- Lee, A.M., Clear, A.J., Calaminici, M., Davies, A.J., Jordan, S., MacDougall, F., Matthews, J., Norton, A.J., Gribben, J.G., Lister, T.A. & Goff, L.K. (2006) Number of CD4+ cells and location of forkhead box protein P3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome. *J Clin Oncol*, **24**, 5052-5059.
- Lee, J.J., Choi, B.H., Kang, H.K., Kim, S.K., Nam, J.H., Yang, D.H., Kim, Y.K., Kim, H.J. & Chung, I.J. (2008) Monocyte-derived dendritic cells from HLA-matched allogeneic donors showed a greater ability to induce leukemic cell-specific T cells in comparison to leukemic cell-derived dendritic cells or monocyte-derived dendritic cells from AML patients. *Leuk Res*, **32**, 1653-1660.
- Lee, P.T., Benlagha, K., Teyton, L. & Bendelac, A. (2002) Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med*, **195**, 637-641.
- Lehe, C., Ghebeh, H., Al-Sulaiman, A., Al Qudaihi, G., Al-Hussein, K., Almohareb, F., Chaudhri, N., Alsharif, F., Al-Zahrani, H., Tbakhi, A., Aljurf, M. & Dermime, S. (2008) The Wilms' tumor antigen is a novel target for human CD4+ regulatory T cells: implications for immunotherapy. *Cancer Res*, **68**, 6350-6359.
- Leroy, H., Roumier, C., Huyghe, P., Biggio, V., Fenaux, P. & Preudhomme, C. (2005) CEBPA point mutations in hematological malignancies. *Leukemia*, **19**, 329-334.
- Levy, R., Robertson, M., Leonard, J., Vose, J., Denney, D. (2008) Results of a phase 3 trial evaluating safety and efficacy of specific immunotherapy, recombinant idiotype (ID) conjugated to KLH (ID-KLH) with GM-CSF compared to non-specific immunotherapy, KLH with GM-CSF in patients with follicular non-Hodgkin's lymphoma *Annals of Oncology*, **19**.
- Li, Y., Li, H., Wang, M.N., Lu, D., Bassi, R., Wu, Y., Zhang, H., Balderes, P., Ludwig, D.L., Pytowski, B., Kussie, P., Piloto, O., Small, D., Bohlen, P., Witte, L., Zhu, Z. & Hicklin, D.J. (2004) Suppression of leukemia

- expressing wild-type or ITD-mutant FLT3 receptor by a fully human anti-FLT3 neutralizing antibody. *Blood*, **104**, 1137-1144.
- Lim, S.H., Worman, C.P., Jewell, A.P. & Goldstone, A.H. (1991) Cellular cytotoxic function and potential in acute myelogenous leukaemia. *Leuk Res*, **15**, 641-644.
- Linn, Y.C. & Hui, K.M. (2003) Cytokine-induced killer cells: NK-like T cells with cytolytic specificity against leukemia. *Leuk Lymphoma*, **44**, 1457-1462.
- Liso, A., Castiglione, F., Cappuccio, A., Stracci, F., Schlenk, R.F., Amadori, S., Thiede, C., Schnittger, S., Valk, P.J., Dohner, K., Martelli, M.F., Schaich, M., Krauter, J., Ganser, A., Martelli, M.P., Bolli, N., Lowenberg, B., Haferlach, T., Ehninger, G., Mandelli, F., Dohner, H., Michor, F. & Falini, B. (2008) A one-mutation mathematical model can explain the age incidence of acute myeloid leukemia with mutated nucleophosmin (NPM1). *Haematologica*, **93**, 1219-1226.
- Liu, F.T. & Rabinovich, G.A. (2005) Galectins as modulators of tumour progression. *Nat Rev Cancer*, **5**, 29-41.
- Liyanage, U.K., Moore, T.T., Joo, H.G., Tanaka, Y., Herrmann, V., Doherty, G., Drebin, J.A., Strasberg, S.M., Eberlein, T.J., Goedegebuure, P.S. & Linehan, D.C. (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol*, **169**, 2756-2761.
- Lowdell, M.W., Craston, R. & Prentice, H.G. (1999) Generation of autologous immunity to acute myeloid leukaemia and maintenance of complete remission following interferon-alpha treatment. *Cytokines Cell Mol Ther*, **5**, 119-121.
- Lowdell, M.W., Craston, R., Samuel, D., Wood, M.E., O'Neill, E., Saha, V. & Prentice, H.G. (2002) Evidence that continued remission in patients treated for acute leukaemia is dependent upon autologous natural killer cells. *Br J Haematol*, **117**, 821-827.
- Lu, P.H. & Negrin, R.S. (1994) A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. *J Immunol*, **153**, 1687-1696.
- Lund, R., Ahlfors, H., Kainonen, E., Lahesmaa, A.M., Dixon, C. & Lahesmaa, R. (2005) Identification of genes involved in the initiation of human Th1 or Th2 cell commitment. *Eur J Immunol*, **35**, 3307-3319.
- Lyles, J.M., Norrild, B. & Bock, E. (1984) Biosynthesis of the D2 cell adhesion molecule: pulse-chase studies in cultured fetal rat neuronal cells. *J Cell Biol*, **98**, 2077-2081.
- Lyons, P.A., Koukoulaki, M., Hatton, A., Doggett, K., Woffendin, H.B., Chaudhry, A.N. & Smith, K.G. (2007) Microarray analysis of human leucocyte subsets: the advantages of positive selection and rapid purification. *BMC Genomics*, **8**, 64.
- Macdonald, D., Jiang, Y.Z., Gordon, A.A., Mahendra, P., Oskam, R., Palmer, P.A., Franks, C.R. & Barrett, A.J. (1990) Recombinant interleukin 2 for acute myeloid leukaemia in first complete remission: a pilot study. *Leuk Res*, **14**, 967-973.

- Makino, Y., Kanno, R., Ito, T., Higashino, K. & Taniguchi, M. (1995) Predominant expression of invariant V alpha 14+ TCR alpha chain in NK1.1+ T cell populations. *Int Immunol*, **7**, 1157-1161.
- Malmberg, K.J., Levitsky, V., Norell, H., de Matos, C.T., Carlsten, M., Schedvins, K., Rabbani, H., Moretta, A., Soderstrom, K., Levitskaya, J. & Kiessling, R. (2002) IFN-gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. *J Clin Invest*, **110**, 1515-1523.
- Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*, **23**, 549-555.
- Matarrese, P., Tinari, A., Mormone, E., Bianco, G.A., Toscano, M.A., Ascione, B., Rabinovich, G.A. & Malorni, W. (2005) Galectin-1 sensitizes resting human T lymphocytes to Fas (CD95)-mediated cell death via mitochondrial hyperpolarization, budding, and fission. *J Biol Chem*, **280**, 6969-6985.
- Maywald, O., Buchheidt, D., Bergmann, J., Schoch, C., Ludwig, W.D., Reiter, A., Hastka, J., Lengfelder, E. & Hehlmann, R. (2004) Spontaneous remission in adult acute myeloid leukemia in association with systemic bacterial infection-case report and review of the literature. *Ann Hematol*, **83**, 189-194.
- McLean, A.R. & Michie, C.A. (1995) In vivo estimates of division and death rates of human T lymphocytes. *Proc Natl Acad Sci U S A*, **92**, 3707-3711.
- Metelitsa, L.S., Naidenko, O.V., Kant, A., Wu, H.W., Loza, M.J., Perussia, B., Kronenberg, M. & Seeger, R.C. (2001) Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. *J Immunol*, **167**, 3114-3122.
- Metelitsa, L.S., Weinberg, K.I., Emanuel, P.D. & Seeger, R.C. (2003) Expression of CD1d by myelomonocytic leukemias provides a target for cytotoxic NKT cells. *Leukemia*, **17**, 1068-1077.
- Miescher, S., Whiteside, T.L., Carrel, S. & von Flidner, V. (1986) Functional properties of tumor-infiltrating and blood lymphocytes in patients with solid tumors: effects of tumor cells and their supernatants on proliferative responses of lymphocytes. *J Immunol*, **136**, 1899-1907.
- Milia, E., Di Somma, M.M., Majolini, M.B., Ulivieri, C., Somma, F., Piccolella, E., Telford, J.L. & Baldari, C.T. (1997) Gene activating and proapoptotic potential are independent properties of different CD4 epitopes. *Mol Immunol*, **34**, 287-296.
- Milojkovic, D., Buggins, A.G., Devereux, S., Thomas, N.S. & Mufti, G.J. (2005) Tumor supernatant from myeloid malignancies inhibits T-cell apoptosis and cell cycle entry independently. *Leukemia*, **19**, 1699-1702.
- Milojkovic, D., Devereux, S., Westwood, N.B., Mufti, G.J., Thomas, N.S. & Buggins, A.G. (2004) Antiapoptotic microenvironment of acute myeloid leukemia. *J Immunol*, **173**, 6745-6752.
- Miltenyi, S., Muller, W., Weichel, W. & Radbruch, A. (1990) High gradient magnetic cell separation with MACS. *Cytometry*, **11**, 231-238.
- Mizoguchi, H., O'Shea, J.J., Longo, D.L., Loeffler, C.M., McVicar, D.W. & Ochoa, A.C. (1992) Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science*, **258**, 1795-1798.

- Mocellin, S., Wang, E. & Marincola, F.M. (2001) Cytokines and immune response in the tumor microenvironment. *J Immunother*, **24**, 392-407.
- Mohty, M., Jarrossay, D., Lafage-Pochitaloff, M., Zandotti, C., Briere, F., de Lamballeri, X.N., Isnardon, D., Sainty, D., Olive, D. & Gaugler, B. (2001) Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairment. *Blood*, **98**, 3750-3756.
- Molling, J.W., Kolgen, W., van der Vliet, H.J., Boomsma, M.F., Kruijzena, H., Smorenburg, C.H., Molenkamp, B.G., Langendijk, J.A., Leemans, C.R., von Blomberg, B.M., Scheper, R.J. & van den Eertwegh, A.J. (2005) Peripheral blood IFN-gamma-secreting V $\alpha$ 24+V $\beta$ 11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. *Int J Cancer*, **116**, 87-93.
- Montagna, D., Maccario, R., Locatelli, F., Montini, E., Pagani, S., Bonetti, F., Daudt, L., Turin, I., Lisini, D., Garavaglia, C., Dellabona, P. & Casorati, G. (2006) Emergence of antitumor cytolytic T cells is associated with maintenance of hematologic remission in children with acute myeloid leukemia. *Blood*, **108**, 3843-3850.
- Monteiro, J., Hingorani, R., Choi, I.H., Silver, J., Pergolizzi, R. & Gregersen, P.K. (1995) Oligoclonality in the human CD8+ T cell repertoire in normal subjects and monozygotic twins: implications for studies of infectious and autoimmune diseases. *Mol Med*, **1**, 614-624.
- Montoya, C.J., Velilla, P.A., Chougnet, C., Landay, A.L. & Rugeles, M.T. (2006) Increased IFN-gamma production by NK and CD3+/CD56+ cells in sexually HIV-1-exposed but uninfected individuals. *Clin Immunol*, **120**, 138-146.
- Moretta, A., Bottino, C., Vitale, M., Pende, D., Cantoni, C., Mingari, M.C., Biassoni, R. & Moretta, L. (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytotoxicity. *Annu Rev Immunol*, **19**, 197-223.
- Morgan, R.A., Dudley, M.E., Wunderlich, J.R., Hughes, M.S., Yang, J.C., Sherry, R.M., Royal, R.E., Topalian, S.L., Kammula, U.S., Restifo, N.P., Zheng, Z., Nahvi, A., de Vries, C.R., Rogers-Freezer, L.J., Mavroukakis, S.A. & Rosenberg, S.A. (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*, **314**, 126-129.
- Munn, D.H., Shafizadeh, E., Attwood, J.T., Bondarev, I., Pashine, A. & Mellor, A.L. (1999) Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J Exp Med*, **189**, 1363-1372.
- Munn, D.H., Zhou, M., Attwood, J.T., Bondarev, I., Conway, S.J., Marshall, B., Brown, C. & Mellor, A.L. (1998) Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science*, **281**, 1191-1193.
- Nakagawa, R., Motoki, K., Ueno, H., Iijima, R., Nakamura, H., Kobayashi, E., Shimosaka, A. & Koezuka, Y. (1998) Treatment of hepatic metastasis of the colon26 adenocarcinoma with an alpha-galactosylceramide, KRN7000. *Cancer Res*, **58**, 1202-1207.
- Nakagomi, H., Petersson, M., Magnusson, I., Juhlin, C., Matsuda, M., Mellstedt, H., Taupin, J.L., Vivier, E., Anderson, P. & Kiessling, R. (1993) Decreased expression of the signal-transducing zeta chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma. *Cancer Res*, **53**, 5610-5612.

- Nakanishi, K., Ida, M., Suzuki, H., Kitano, C., Yamamoto, A., Mori, N., Araki, M. & Taketani, S. (2006) Molecular characterization of a transport vesicle protein Neurensin-2, a homologue of Neurensin-1, expressed in neural cells. *Brain Res*, **1081**, 1-8.
- Nakata, Y., Shetzline, S., Sakashita, C., Kalota, A., Rallapalli, R., Rudnick, S.I., Zhang, Y., Emerson, S.G. & Gewirtz, A.M. (2007) c-Myb contributes to G2/M cell cycle transition in human hematopoietic cells by direct regulation of cyclin B1 expression. *Mol Cell Biol*, **27**, 2048-2058.
- Nash, K.A., Mohammed, G., Nandapalan, N., Kernahan, J., Scott, R., Craft, A.W. & Toms, G.L. (1993) T cell function in children with acute lymphoblastic leukaemia. *Br J Haematol*, **83**, 419-427.
- Nestle, F.O., Burg, G., Fah, J., Wrone-Smith, T. & Nickoloff, B.J. (1997) Human sunlight-induced basal-cell-carcinoma-associated dendritic cells are deficient in T cell co-stimulatory molecules and are impaired as antigen-presenting cells. *Am J Pathol*, **150**, 641-651.
- Norris, S., Doherty, D.G., Collins, C., McEntee, G., Traynor, O., Hegarty, J.E. & O'Farrelly, C. (1999) Natural T cells in the human liver: cytotoxic lymphocytes with dual T cell and natural killer cell phenotype and function are phenotypically heterogenous and include Valpha24-JalphaQ and gammadelta T cell receptor bearing cells. *Hum Immunol*, **60**, 20-31.
- O'Brien, S.G., Guilhot, F., Larson, R.A., Gathmann, I., Baccarani, M., Cervantes, F., Cornelissen, J.J., Fischer, T., Hochhaus, A., Hughes, T., Lechner, K., Nielsen, J.L., Rousselot, P., Reiffers, J., Saglio, G., Shepherd, J., Simonsson, B., Gratwohl, A., Goldman, J.M., Kantarjian, H., Taylor, K., Verhoef, G., Bolton, A.E., Capdeville, R. & Druker, B.J. (2003) Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med*, **348**, 994-1004.
- Ohkawa, T., Seki, S., Dobashi, H., Koike, Y., Habu, Y., Ami, K., Hiraide, H. & Sekine, I. (2001) Systematic characterization of human CD8+ T cells with natural killer cell markers in comparison with natural killer cells and normal CD8+ T cells. *Immunology*, **103**, 281-290.
- Oka, Y., Tsuboi, A., Taguchi, T., Osaki, T., Kyo, T., Nakajima, H., Elisseeva, O.A., Oji, Y., Kawakami, M., Ikegame, K., Hosen, N., Yoshihara, S., Wu, F., Fujiki, F., Murakami, M., Masuda, T., Nishida, S., Shirakata, T., Nakatsuka, S., Sasaki, A., Udaka, K., Dohy, H., Aozasa, K., Noguchi, S., Kawase, I. & Sugiyama, H. (2004) Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci U S A*, **101**, 13885-13890.
- Old, L.J., Boyse, E.A. (1964) Immunology of Experimental Tumours. *Annu Rev Med*, **15**, 167-186.
- Orleans-Lindsay, J.K., Barber, L.D., Prentice, H.G. & Lowdell, M.W. (2001) Acute myeloid leukaemia cells secrete a soluble factor that inhibits T and NK cell proliferation but not cytolytic function--implications for the adoptive immunotherapy of leukaemia. *Clin Exp Immunol*, **126**, 403-411.
- Ortaldo, J.R., Winkler-Pickett, R.T., Yagita, H. & Young, H.A. (1991) Comparative studies of CD3- and CD3+ CD56+ cells: examination of morphology, functions, T cell receptor rearrangement, and pore-forming protein expression. *Cell Immunol*, **136**, 486-495.

- Oshimi, K., Oshimi, Y., Akutsu, M., Takei, Y., Saito, H., Okada, M. & Mizoguchi, H. (1986) Cytotoxicity of interleukin 2-activated lymphocytes for leukemia and lymphoma cells. *Blood*, **68**, 938-948.
- Owen, C.J., Toze, C.L., Koochin, A., Forrest, D.L., Smith, C.A., Stevens, J.M., Jackson, S.C., Poon, M.C., Sinclair, G.D., Leber, B., Johnson, P.R., Macheta, A., Yin, J.A., Barnett, M.J., Lister, T.A. & Fitzgibbon, J. (2008) Five new pedigrees with inherited RUNX1 mutations causing familial platelet disorder with propensity to myeloid malignancy (FPD/AML). *Blood*, **112**, 4639-4645.
- Padua, R.A., Larghero, J., Robin, M., le Pogam, C., Schlageter, M.H., Muszlak, S., Fric, J., West, R., Rousselot, P., Phan, T.H., Mudde, L., Teisserenc, H., Carpentier, A.F., Kogan, S., Degos, L., Pla, M., Bishop, J.M., Stevenson, F., Charron, D. & Chomienne, C. (2003) PML-RARA-targeted DNA vaccine induces protective immunity in a mouse model of leukemia. *Nat Med*, **9**, 1413-1417.
- Palmer, C., Diehn, M., Alizadeh, A.A. & Brown, P.O. (2006) Cell-type specific gene expression profiles of leukocytes in human peripheral blood. *BMC Genomics*, **7**, 115.
- Panoskaltzis, N., Reid, C.D. & Knight, S.C. (2003) Quantification and cytokine production of circulating lymphoid and myeloid cells in acute myelogenous leukaemia. *Leukemia*, **17**, 716-730.
- Pawelec, G., Da Silva, P., Max, H., Kalbacher, H., Schmidt, H., Bruserud, O., Zugel, U., Baier, W., Rehbein, A. & Pohla, H. (1995) Relative roles of natural killer- and T cell-mediated anti-leukemia effects in chronic myelogenous leukemia patients treated with interferon-alpha. *Leuk Lymphoma*, **18**, 471-478.
- Pearson-White, S. & McDuffie, M. (2003) Defective T-cell activation is associated with augmented transforming growth factor Beta sensitivity in mice with mutations in the Sno gene. *Mol Cell Biol*, **23**, 5446-5459.
- Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., Lider, O., Alon, R., Zipori, D. & Lapidot, T. (1999) Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science*, **283**, 845-848.
- Peralbo, E., Alonso, C. & Solana, R. (2007) Invariant NKT and NKT-like lymphocytes: two different T cell subsets that are differentially affected by ageing. *Exp Gerontol*, **42**, 703-708.
- Peralta, C.G., Han, V.K., Horrocks, J., Croy, B.A. & van den Heuvel, M.J. (2008) CD56bright cells increase expression of  $\alpha 4$  integrin at ovulation in fertile cycles. *J Leukoc Biol*, **84**, 1065-1074.
- Perillo, N.L., Pace, K.E., Seilhamer, J.J. & Baum, L.G. (1995) Apoptosis of T cells mediated by galectin-1. *Nature*, **378**, 736-739.
- Phan, G.Q., Yang, J.C., Sherry, R.M., Hwu, P., Topalian, S.L., Schwartzentruber, D.J., Restifo, N.P., Haworth, L.R., Seipp, C.A., Freezer, L.J., Morton, K.E., Mavroukakis, S.A., Duray, P.H., Steinberg, S.M., Allison, J.P., Davis, T.A. & Rosenberg, S.A. (2003) Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A*, **100**, 8372-8377.
- Piersma, S.J., Jordanova, E.S., van Poelgeest, M.I., Kwappenberg, K.M., van der Hulst, J.M., Drijfhout, J.W., Melief, C.J., Kenter, G.G., Fleuren, G.J.,

- Offringa, R. & van der Burg, S.H. (2007) High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. *Cancer Res*, **67**, 354-361.
- Pierson, B.A. & Miller, J.S. (1996) CD56+bright and CD56+dim natural killer cells in patients with chronic myelogenous leukemia progressively decrease in number, respond less to stimuli that recruit clonogenic natural killer cells, and exhibit decreased proliferation on a per cell basis. *Blood*, **88**, 2279-2287.
- Pittet, M.J., Speiser, D.E., Valmori, D., Cerottini, J.C. & Romero, P. (2000) Cutting edge: cytolytic effector function in human circulating CD8+ T cells closely correlates with CD56 surface expression. *J Immunol*, **164**, 1148-1152.
- Porcelli, S., Yockey, C.E., Brenner, M.B. & Balk, S.P. (1993) Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8-alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med*, **178**, 1-16.
- Porrata, L.F., Litzow, M.R., Tefferi, A., Letendre, L., Kumar, S., Geyer, S.M. & Markovic, S.N. (2002) Early lymphocyte recovery is a predictive factor for prolonged survival after autologous hematopoietic stem cell transplantation for acute myelogenous leukemia. *Leukemia*, **16**, 1311-1318.
- Porter, D.L., Levine, B.L., Bunin, N., Stadtmauer, E.A., Luger, S.M., Goldstein, S., Loren, A., Phillips, J., Nasta, S., Perl, A., Schuster, S., Tsai, D., Sohal, A., Veloso, E., Emerson, S. & June, C.H. (2006) A phase 1 trial of donor lymphocyte infusions expanded and activated ex vivo via CD3/CD28 costimulation. *Blood*, **107**, 1325-1331.
- Powles, R.L., Russell, J., Lister, T.A., Oliver, T., Whitehouse, J.M., Malpas, J., Chapuis, B., Crowther, D. & Alexander, P. (1977) Immunotherapy for acute myelogenous leukaemia: a controlled clinical study 2 1/2 years after entry of the last patient. *Br J Cancer*, **35**, 265-272.
- Prussin, C. & Foster, B. (1997) TCR V alpha 24 and V beta 11 coexpression defines a human NK1 T cell analog containing a unique Th0 subpopulation. *J Immunol*, **159**, 5862-5870.
- Rabinovich, G.A., Daly, G., Dreja, H., Tailor, H., Riera, C.M., Hirabayashi, J. & Chernajovsky, Y. (1999) Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med*, **190**, 385-398.
- Rabinovich, G.A., Gabilovich, D. & Sotomayor, E.M. (2007) Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol*, **25**, 267-296.
- Ramsay, A.G., Clear, A.J., Fatah, R., Gribben, J.G. (2008b) Lenalidomide repairs suppressed T cell immunological synapse formation in follicular lymphoma. *Blood (ASH Annual Meeting Abstracts)*, **112**, Abstract #885
- Ramsay, A.G., Johnson, A.J., Lee, A.M., Gorgun, G., Le Dieu, R., Blum, W., Byrd, J.C. & Gribben, J.G. (2008a) Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. *J Clin Invest*, **118**, 2427-2437.
- Rang, H.P., Dale, M.M. *Pharmacology*. Churchill Livingstone.

- Raza, A., Jurcic, J.G., Roboz, G.J., Maris, M., Stephenson, J., Wood, B., Rege, B., Grove, L.E., Drachman, J.G., Sievers, E. (2007) Complete Remissions Observed in Acute Myeloid Leukemia Following Prolonged Exposure to SGN-33 (lintuzumab), a Humanized Monoclonal Antibody Targeting CD33. *Blood (ASH Annual Meeting Abstracts)*, **110**, Abstract #159.
- Recher, C., Beyne-Rauzy, O., Demur, C., Chicanne, G., Dos Santos, C., Mas, V.M., Benzaquen, D., Laurent, G., Huguet, F. & Payrastre, B. (2005) Antileukemic activity of rapamycin in acute myeloid leukemia. *Blood*, **105**, 2527-2534.
- Reichert, T.E., Rabinowich, H., Johnson, J.T. & Whiteside, T.L. (1998) Mechanisms responsible for signaling and functional defects. *J Immunother*, **21**, 295-306.
- Reis, E.A., Athanazio, D.A., Lima, I., NO, E.S., Andrade, J.C., Jesus, R.N., Barbosa, L.M., Reis, M.G. & Santiago, M.B. (2008) NK and NKT cell dynamics after rituximab therapy for systemic lupus erythematosus and rheumatoid arthritis. *Rheumatol Int*, **29**, 469-475.
- Rezvani, K., Yong, A.S., Tawab, A., Jafarpour, B., Eniafe, R., Mielke, S., Savani, B.N., Keyvanfar, K., Li, Y., Kurlander, R. & Barrett, A.J. (2008) Ex-vivo characterization of polyclonal memory CD8+ T-cell responses to PRAME-specific peptides in patients with acute lymphoblastic leukemia and acute and chronic myeloid leukemia. *Blood*.
- Ridway, J.C., Taylor, G.M., Freeman, C.B. & Harris, R. (1976) Receptors for human immunoglobulin on acute myeloid leukaemic leucocytes. *Br J Cancer*, **34**, 346-358.
- Roddie, H., Klammer, M., Thomas, C., Thomson, R., Atkinson, A., Sproul, A., Waterfall, M., Samuel, K., Yin, J., Johnson, P. & Turner, M. (2006) Phase I/II study of vaccination with dendritic-like leukaemia cells for the immunotherapy of acute myeloid leukaemia. *Br J Haematol*, **133**, 152-157.
- Rodriguez, M.W., Paquet, A.C., Yang, Y.H. & Erle, D.J. (2004) Differential gene expression by integrin beta 7+ and beta 7- memory T helper cells. *BMC Immunol*, **5**, 13.
- Roithmaier, S., Haydon, A.M., Loi, S., Esmore, D., Griffiths, A., Bergin, P., Williams, T.J. & Schwarz, M.A. (2007) Incidence of malignancies in heart and/or lung transplant recipients: a single-institution experience. *J Heart Lung Transplant*, **26**, 845-849.
- Rosenberg, S.A., Lotze, M.T., Muul, L.M., Leitman, S., Chang, A.E., Ettinghausen, S.E., Matory, Y.L., Skibber, J.M., Shiloni, E., Vetto, J.T. & et al. (1985) Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Engl J Med*, **313**, 1485-1492.
- Rosenberg, S.A., Yang, J.C. & Restifo, N.P. (2004) Cancer immunotherapy: moving beyond current vaccines. *Nat Med*, **10**, 909-915.
- Royer, P.J., Bougras, G., Ebstein, F., Leveque, L., Tanguy-Royer, S., Simon, T., Juge-Morineau, N., Chevallier, P., Harousseau, J.L. & Gregoire, M. (2008) Efficient monocyte-derived dendritic cell generation in patients with acute myeloid leukemia after chemotherapy treatment: application to active immunotherapy. *Exp Hematol*, **36**, 329-339.
- Ryningen, A., Wergeland, L., Glenjen, N., Gjertsen, B.T. & Bruserud, O. (2005) In vitro crosstalk between fibroblasts and native human acute

- myelogenous leukemia (AML) blasts via local cytokine networks results in increased proliferation and decreased apoptosis of AML cells as well as increased levels of proangiogenic Interleukin 8. *Leuk Res*, **29**, 185-196.
- Sahin, U., Tureci, O., Schmitt, H., Cochlovius, B., Johannes, T., Schmits, R., Stenner, F., Luo, G., Schobert, I. & Pfreundschuh, M. (1995) Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc Natl Acad Sci U S A*, **92**, 11810-11813.
- Sakaguchi, S. (2004) Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol*, **22**, 531-562.
- Sallusto, F., Geginat, J. & Lanzavecchia, A. (2004) Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol*, **22**, 745-763.
- Sandler, D.P. & Ross, J.A. (1997) Epidemiology of acute leukemia in children and adults. *Semin Oncol*, **24**, 3-16.
- Savoldo, B., Heslop, H.E. & Rooney, C.M. (2000) The use of cytotoxic t cells for the prevention and treatment of epstein-barr virus induced lymphoma in transplant recipients. *Leuk Lymphoma*, **39**, 455-464.
- Scheibenbogen, C., Letsch, A., Thiel, E., Schmittel, A., Mailaender, V., Baerwolf, S., Nagorsen, D. & Keilholz, U. (2002) CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia. *Blood*, **100**, 2132-2137.
- Schirrmann, T. & Pecher, G. (2005) Specific targeting of CD33(+) leukemia cells by a natural killer cell line modified with a chimeric receptor. *Leuk Res*, **29**, 301-306.
- Schmetzer, H.M., Braun, S., Wiesner, D., Duell, T., Gerhartz, H.H. & Mittermueller, J. (2000) Gene rearrangements in bone marrow cells of patients with acute myelogenous leukemia. *Acta Haematol*, **103**, 125-134.
- Schmidt-Wolf, I.G., Lefterova, P., Mehta, B.A., Fernandez, L.P., Huhn, D., Blume, K.G., Weissman, I.L. & Negrin, R.S. (1993) Phenotypic characterization and identification of effector cells involved in tumor cell recognition of cytokine-induced killer cells. *Exp Hematol*, **21**, 1673-1679.
- Schmidt-Wolf, I.G., Negrin, R.S., Kiem, H.P., Blume, K.G. & Weissman, I.L. (1991) Use of a SCID mouse/human lymphoma model to evaluate cytokine-induced killer cells with potent antitumor cell activity. *J Exp Med*, **174**, 139-149.
- Schmidt, R.E., Murray, C., Daley, J.F., Schlossman, S.F. & Ritz, J. (1986) A subset of natural killer cells in peripheral blood displays a mature T cell phenotype. *J Exp Med*, **164**, 351-356.
- Schmitt, M., Schmitt, A., Rojewski, M.T., Chen, J., Giannopoulos, K., Fei, F., Yu, Y., Gotz, M., Heyduk, M., Ritter, G., Speiser, D.E., Gnjatic, S., Guillaume, P., Ringhoffer, M., Schlenk, R.F., Liebisch, P., Bunjes, D., Shiku, H., Dohner, H. & Greiner, J. (2008) RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome, and multiple myeloma elicits immunologic and clinical responses. *Blood*, **111**, 1357-1365.
- Schwartz, R.H. (2003) T cell anergy. *Annu Rev Immunol*, **21**, 305-334.
- Scrivener, S., Goddard, R.V., Kaminski, E.R. & Prentice, A.G. (2003) Abnormal T-cell function in B-cell chronic lymphocytic leukaemia. *Leuk Lymphoma*, **44**, 383-389.

- Seddiki, N., Santner-Nanan, B., Martinson, J., Zauanders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S.I., Nanan, R., Kelleher, A. & Fazekas de St Groth, B. (2006) Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*, **203**, 1693-1700.
- Semple, J.W., Allen, D., Chang, W., Castaldi, P. & Freedman, J. (1993) Rapid separation of CD4+ and CD19+ lymphocyte populations from human peripheral blood by a magnetic activated cell sorter (MACS). *Cytometry*, **14**, 955-960.
- Shankaran, V., Ikeda, H., Bruce, A.T., White, J.M., Swanson, P.E., Old, L.J. & Schreiber, R.D. (2001) IFN $\gamma$  and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*, **410**, 1107-1111.
- Sharma, P., Shen, Y., Wen, S., Yamada, S., Jungbluth, A.A., Gnjatic, S., Bajorin, D.F., Reuter, V.E., Herr, H., Old, L.J. & Sato, E. (2007) CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma. *Proc Natl Acad Sci U S A*, **104**, 3967-3972.
- Shimizu, Y., Iwatsuki, S., Herberman, R.B. & Whiteside, T.L. (1990) Clonal analysis of tumor-infiltrating lymphocytes from human primary and metastatic liver tumors. *Int J Cancer*, **46**, 878-883.
- Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M. & et al. (1992) RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*, **68**, 855-867.
- Sievers, E.L., Larson, R.A., Stadtmauer, E.A., Estey, E., Lowenberg, B., Dombret, H., Karanes, C., Theobald, M., Bennett, J.M., Sherman, M.L., Berger, M.S., Eten, C.B., Loken, M.R., van Dongen, J.J., Bernstein, I.D. & Appelbaum, F.R. (2001) Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *J Clin Oncol*, **19**, 3244-3254.
- Smyth, M.J., Crowe, N.Y., Hayakawa, Y., Takeda, K., Yagita, H. & Godfrey, D.I. (2002) NKT cells - conductors of tumor immunity? *Curr Opin Immunol*, **14**, 165-171.
- Smyth, M.J., Thia, K.Y., Street, S.E., Cretney, E., Trapani, J.A., Taniguchi, M., Kawano, T., Pelikan, S.B., Crowe, N.Y. & Godfrey, D.I. (2000) Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med*, **191**, 661-668.
- Sotomayor, E.M., Borrello, I., Rattis, F.M., Cuenca, A.G., Abrams, J., Staveley-O'Carroll, K. & Levitsky, H.I. (2001) Cross-presentation of tumor antigens by bone marrow-derived antigen-presenting cells is the dominant mechanism in the induction of T-cell tolerance during B-cell lymphoma progression. *Blood*, **98**, 1070-1077.
- Spisek, R., Chevallier, P., Morineau, N., Milpied, N., Avet-Loiseau, H., Harousseau, J.L., Meflah, K. & Gregoire, M. (2002) Induction of leukemia-specific cytotoxic response by cross-presentation of late-apoptotic leukemic blasts by autologous dendritic cells of nonleukemic origin. *Cancer Res*, **62**, 2861-2868.
- Srivastava, R., Aggarwal, R., Bhagat, M.R., Chowdhury, A. & Naik, S. (2008) Alterations in natural killer cells and natural killer T cells during acute viral hepatitis E. *J Viral Hepat*, **15**, 910-916.

- Stanciu, L.A., Shute, J., Holgate, S.T. & Djukanovic, R. (1996) Production of IL-8 and IL-4 by positively and negatively selected CD4+ and CD8+ human T cells following a four-step cell separation method including magnetic cell sorting (MACS). *J Immunol Methods*, **189**, 107-115.
- Staveley-O'Carroll, K., Sotomayor, E., Montgomery, J., Borrello, I., Hwang, L., Fein, S., Pardoll, D. & Levitsky, H. (1998) Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proc Natl Acad Sci U S A*, **95**, 1178-1183.
- Steinman, R.M., Hawiger, D. & Nussenzweig, M.C. (2003) Tolerogenic dendritic cells. *Annu Rev Immunol*, **21**, 685-711.
- Stewart, S.A., Feuer, G., Jewett, A., Lee, F.V., Bonavida, B. & Chen, I.S. (1996) HTLV-1 gene expression in adult T-cell leukemia cells elicits an NK cell response in vitro and correlates with cell rejection in SCID mice. *Virology*, **226**, 167-175.
- Stripecke, R., Cardoso, A.A., Pepper, K.A., Skelton, D.C., Yu, X.J., Mascarenhas, L., Weinberg, K.I., Nadler, L.M. & Kohn, D.B. (2000) Lentiviral vectors for efficient delivery of CD80 and granulocyte-macrophage-colony-stimulating factor in human acute lymphoblastic leukemia and acute myeloid leukemia cells to induce antileukemic immune responses. *Blood*, **96**, 1317-1326.
- Stutman, O. (1974) Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science*, **183**, 534-536.
- Stutman, O. (1975) Immunodepression and malignancy. *Adv Cancer Res*, **22**, 261-422.
- Summers, K., Stevens, J., Kakkas, I., Smith, M., Smith, L.L., Macdougall, F., Cavenagh, J., Bonnet, D., Young, B.D., Lister, T.A. & Fitzgibbon, J. (2007) Wilms' tumour 1 mutations are associated with FLT3-ITD and failure of standard induction chemotherapy in patients with normal karyotype AML. *Leukemia*, **21**, 550-551; author reply 552.
- Suntharalingam, G., Perry, M.R., Ward, S., Brett, S.J., Castello-Cortes, A., Brunner, M.D. & Panoskaltsis, N. (2006) Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med*, **355**, 1018-1028.
- Tagami, S., Eguchi, Y., Kinoshita, M., Takeda, M. & Tsujimoto, Y. (2000) A novel protein, RTN-XS, interacts with both Bcl-XL and Bcl-2 on endoplasmic reticulum and reduces their anti-apoptotic activity. *Oncogene*, **19**, 5736-5746.
- Tahir, S.M., Cheng, O., Shaulov, A., Koezuka, Y., Buble, G.J., Wilson, S.B., Balk, S.P. & Exley, M.A. (2001) Loss of IFN-gamma production by invariant NK T cells in advanced cancer. *J Immunol*, **167**, 4046-4050.
- Tajima, F., Kawatani, T., Endo, A. & Kawasaki, H. (1996) Natural killer cell activity and cytokine production as prognostic factors in adult acute leukemia. *Leukemia*, **10**, 478-482.
- Takahashi, T., Haraguchi, K., Chiba, S., Yasukawa, M., Shibata, Y. & Hirai, H. (2003) Valpha24+ natural killer T-cell responses against T-acute lymphoblastic leukaemia cells: implications for immunotherapy. *Br J Haematol*, **122**, 231-239.
- Taussig, D.C., Pearce, D.J., Simpson, C., Rohatiner, A.Z., Lister, T.A., Kelly, G., Luongo, J.L., Danet-Desnoyers, G.A. & Bonnet, D. (2005) Hematopoietic

- stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. *Blood*, **106**, 4086-4092.
- Taylor, G.M., Fergusson, W.D. & Harris, R. (1979) Suppression of lymphoproliferative responses to alloantigens by autologous AML cells. *Clin Exp Immunol*, **35**, 53-61.
- Tenbrock, K., Juang, Y.T., Tolnay, M. & Tsokos, G.C. (2003) The cyclic adenosine 5'-monophosphate response element modulator suppresses IL-2 production in stimulated T cells by a chromatin-dependent mechanism. *J Immunol*, **170**, 2971-2976.
- Terabe, M., Matsui, S., Noben-Trauth, N., Chen, H., Watson, C., Donaldson, D.D., Carbone, D.P., Paul, W.E. & Berzofsky, J.A. (2000) NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol*, **1**, 515-520.
- Thiede, C., Studel, C., Mohr, B., Schaich, M., Schakel, U., Platzbecker, U., Wermke, M., Bornhauser, M., Ritter, M., Neubauer, A., Ehninger, G. & Illmer, T. (2002) Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood*, **99**, 4326-4335.
- Thiery, J.P., Brackenbury, R., Rutishauser, U. & Edelman, G.M. (1977) Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. *J Biol Chem*, **252**, 6841-6845.
- Thomas, L. (1959) *in Cellular and Humoral Aspects of the Hypersensitive States*. Hoeber-Harper, New York.
- Thomas, L. (1982) On immunosurveillance in human cancer. *Yale J Biol Med*, **55**, 329-333.
- Townsend, S.E. & Allison, J.P. (1993) Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science*, **259**, 368-370.
- Treleaven, J., Barrett, A.J. (2009) *Hematopoietic Stem Cell Transplantation in Clinical Practice*. Churchill Livingstone Elsevier.
- Trof, R.J., Beishuizen, A., Wondergem, M.J. & Strack van Schijndel, R.J. (2007) Spontaneous remission of acute myeloid leukaemia after recovery from sepsis. *Neth J Med*, **65**, 259-262.
- Troy, A.J., Summers, K.L., Davidson, P.J., Atkinson, C.H. & Hart, D.N. (1998) Minimal recruitment and activation of dendritic cells within renal cell carcinoma. *Clin Cancer Res*, **4**, 585-593.
- Tsuji, T., Yasukawa, M., Matsuzaki, J., Ohkuri, T., Chamoto, K., Wakita, D., Azuma, T., Niiya, H., Miyoshi, H., Kuzushima, K., Oka, Y., Sugiyama, H., Ikeda, H. & Nishimura, T. (2005) Generation of tumor-specific, HLA class I-restricted human Th1 and Tc1 cells by cell engineering with tumor peptide-specific T-cell receptor genes. *Blood*, **106**, 470-476.
- Uyttenhove, C., Pilotte, L., Theate, I., Stroobant, V., Colau, D., Parmentier, N., Boon, T. & Van den Eynde, B.J. (2003) Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat Med*, **9**, 1269-1274.
- Vaillant, F., Blyth, K., Andrew, L., Neil, J.C. & Cameron, E.R. (2002) Enforced expression of Runx2 perturbs T cell development at a stage coincident with beta-selection. *J Immunol*, **169**, 2866-2874.
- van den Heuvel, M.J., Peralta, C.G., Hatta, K., Han, V.K. & Clark, D.A. (2007) Decline in number of elevated blood CD3(+) CD56(+) NKT cells in

- response to intravenous immunoglobulin treatment correlates with successful pregnancy. *Am J Reprod Immunol*, **58**, 447-459.
- van der Vliet, H.J., Molling, J.W., Nishi, N., Masterson, A.J., Kolgen, W., Porcelli, S.A., van den Eertwegh, A.J., von Blomberg, B.M., Pinedo, H.M., Giaccone, G. & Scheper, R.J. (2003) Polarization of Valpha24+ Vbeta11+ natural killer T cells of healthy volunteers and cancer patients using alpha-galactosylceramide-loaded and environmentally instructed dendritic cells. *Cancer Res*, **63**, 4101-4106.
- van Dongen, J.J., Langerak, A.W., Bruggemann, M., Evans, P.A., Hummel, M., Lavender, F.L., Delabesse, E., Davi, F., Schuurings, E., Garcia-Sanz, R., van Krieken, J.H., Droese, J., Gonzalez, D., Bastard, C., White, H.E., Spaargaren, M., Gonzalez, M., Parreira, A., Smith, J.L., Morgan, G.J., Kneba, M. & Macintyre, E.A. (2003) Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*, **17**, 2257-2317.
- Velpeau, A. (1827) *Rev Med*, **2**.
- Verecque, R., Saudemont, A., Depil, S., Corm, S., Andrieux, J., Soenen-Cornu, V. & Quesnel, B. (2004a) Efficient generation of antileukemic autologous T cells by short-term culture and gamma-irradiation of myeloid leukemic cells. *Cancer Immunol Immunother*, **53**, 793-798.
- Verecque, R., Saudemont, A. & Quesnel, B. (2004b) Cytosine arabinoside induces costimulatory molecule expression in acute myeloid leukemia cells. *Leukemia*, **18**, 1223-1230.
- Vermi, W., Bonecchi, R., Facchetti, F., Bianchi, D., Sozzani, S., Festa, S., Berenzi, A., Cella, M. & Colonna, M. (2003) Recruitment of immature plasmacytoid dendritic cells (plasmacytoid monocytes) and myeloid dendritic cells in primary cutaneous melanomas. *J Pathol*, **200**, 255-268.
- Vidriales, M.B., Orfao, A., Lopez-Berges, M.C., Gonzalez, M., Hernandez, J.M., Ciudad, J., Lopez, A., Moro, M.J., Martinez, M. & San Miguel, J.F. (1993) Lymphoid subsets in acute myeloid leukemias: increased number of cells with NK phenotype and normal T-cell distribution. *Ann Hematol*, **67**, 217-222.
- Virappane, P., Gale, R., Hills, R., Kakkas, I., Summers, K., Stevens, J., Allen, C., Green, C., Quentmeier, H., Drexler, H., Burnett, A., Linch, D., Bonnet, D., Lister, T.A. & Fitzgibbon, J. (2008) Mutation of the Wilms' tumor 1 gene is a poor prognostic factor associated with chemotherapy resistance in normal karyotype acute myeloid leukemia: the United Kingdom Medical Research Council Adult Leukaemia Working Party. *J Clin Oncol*, **26**, 5429-5435.
- Virchow, R.L.K. (1847) Weisses Blut und Milztumoren. *Med Z*, **16**, 9.
- Vitale, A., Guarini, A., Latagliata, R., Cignetti, A. & Foa, R. (1998) Cytotoxic effectors activated by low-dose IL-2 plus IL-12 lyse IL-2-resistant autologous acute myeloid leukaemia blasts. *Br J Haematol*, **101**, 150-157.
- Vollmer, M., Li, L., Schmitt, A., Greiner, J., Reinhardt, P., Ringhoffer, M., Wiesneth, M., Dohner, H. & Schmitt, M. (2003) Expression of human leucocyte antigens and co-stimulatory molecules on blasts of patients with acute myeloid leukaemia. *Br J Haematol*, **120**, 1000-1008.

- von Andrian, U.H. & Mackay, C.R. (2000) T-cell function and migration. Two sides of the same coin. *N Engl J Med*, **343**, 1020-1034.
- Wang, M., Windgassen, D. & Papoutsakis, E.T. (2008) Comparative analysis of transcriptional profiling of CD3+, CD4+ and CD8+ T cells identifies novel immune response players in T-cell activation. *BMC Genomics*, **9**, 225.
- Wang, Q., Stanley, J., Kudoh, S., Myles, J., Kolenko, V., Yi, T., Tubbs, R., Bukowski, R. & Finke, J. (1995) T cells infiltrating non-Hodgkin's B cell lymphomas show altered tyrosine phosphorylation pattern even though T cell receptor/CD3-associated kinases are present. *J Immunol*, **155**, 1382-1392.
- Wang, X., Zheng, J., Liu, J., Yao, J., He, Y., Li, X., Yu, J., Yang, J., Liu, Z. & Huang, S. (2005) Increased population of CD4(+)CD25(high), regulatory T cells with their higher apoptotic and proliferating status in peripheral blood of acute myeloid leukemia patients. *Eur J Haematol*, **75**, 468-476.
- Wang, Z.Q., Dudhane, A., Orlikowsky, T., Clarke, K., Li, X., Darzynkiewicz, Z. & Hoffmann, M.K. (1994) CD4 engagement induces Fas antigen-dependent apoptosis of T cells in vivo. *Eur J Immunol*, **24**, 1549-1552.
- Wendelbo, O., Nesthus, I., Sjo, M., Paulsen, K., Ernst, P. & Bruserud, O. (2004) Functional characterization of T lymphocytes derived from patients with acute myelogenous leukemia and chemotherapy-induced leukopenia. *Cancer Immunol Immunother*, **53**, 740-747.
- Whiteway, A., Corbett, T., Anderson, R., Macdonald, I. & Prentice, H.G. (2003) Expression of co-stimulatory molecules on acute myeloid leukaemia blasts may effect duration of first remission. *Br J Haematol*, **120**, 442-451.
- Wu, H., Neilson, J.R., Kumar, P., Manocha, M., Shankar, P., Sharp, P.A. & Manjunath, N. (2007) miRNA profiling of naive, effector and memory CD8 T cells. *PLoS ONE*, **2**, e1020.
- Xue, S.A., Gao, L., Hart, D., Gillmore, R., Qasim, W., Thrasher, A., Apperley, J., Engels, B., Uckert, W., Morris, E. & Stauss, H. (2005) Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. *Blood*, **106**, 3062-3067.
- Yalcintepe, L., Frankel, A.E. & Hogge, D.E. (2006) Expression of interleukin-3 receptor subunits on defined subpopulations of acute myeloid leukemia blasts predicts the cytotoxicity of diphtheria toxin interleukin-3 fusion protein against malignant progenitors that engraft in immunodeficient mice. *Blood*, **108**, 3530-3537.
- Yanada, M., Matsuo, K., Emi, N. & Naoe, T. (2005) Efficacy of allogeneic hematopoietic stem cell transplantation depends on cytogenetic risk for acute myeloid leukemia in first disease remission: a metaanalysis. *Cancer*, **103**, 1652-1658.
- Yates, J.W., Wallace, H.J., Jr., Ellison, R.R. & Holland, J.F. (1973) Cytosine arabinoside (NSC-63878) and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia. *Cancer Chemother Rep*, **57**, 485-488.
- Yen, C.C., Liu, J.H., Wang, W.S., Chiou, T.J., Fan, F.S. & Chen, P.M. (1999) Prognostic significance of immunoglobulin and T cell receptor gene rearrangements in patients with acute myeloid leukemia: Taiwan experience. *Leuk Lymphoma*, **35**, 179-187.
- Yoneda, K., Morii, T., Nieda, M., Tsukaguchi, N., Amano, I., Tanaka, H., Yagi, H., Narita, N. & Kimura, H. (2005) The peripheral blood Valpha24+ NKT

- cell numbers decrease in patients with haematopoietic malignancy. *Leuk Res*, **29**, 147-152.
- Yoshino, I., Yano, T., Murata, M., Ishida, T., Sugimachi, K., Kimura, G. & Nomoto, K. (1992) Tumor-reactive T-cells accumulate in lung cancer tissues but fail to respond due to tumor cell-derived factor. *Cancer Res*, **52**, 775-781.
- Zhou, D., Mattner, J., Cantu, C., 3rd, Schrantz, N., Yin, N., Gao, Y., Sagiv, Y., Hudspeth, K., Wu, Y.P., Yamashita, T., Teneberg, S., Wang, D., Proia, R.L., Levery, S.B., Savage, P.B., Teyton, L. & Bendelac, A. (2004) Lysosomal glycosphingolipid recognition by NKT cells. *Science*, **306**, 1786-1789.
- Zhu, D., Corral, L.G., Fleming, Y.W. & Stein, B. (2008) Immunomodulatory drugs Revlimid (lenalidomide) and CC-4047 induce apoptosis of both hematological and solid tumor cells through NK cell activation. *Cancer Immunol Immunother*, **57**, 1849-1859.
- Zhu, J. & Paul, W.E. (2008) CD4 T cells: fates, functions, and faults. *Blood*, **112**, 1557-1569.
- Zittoun, R.A., Mandelli, F., Willemze, R., de Witte, T., Labar, B., Resegotti, L., Leoni, F., Damasio, E., Visani, G., Papa, G. & et al. (1995) Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. European Organization for Research and Treatment of Cancer (EORTC) and the Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto (GIMEMA) Leukemia Cooperative Groups. *N Engl J Med*, **332**, 217-223.
- Zlotnik, A., Godfrey, D.I., Fischer, M. & Suda, T. (1992) Cytokine production by mature and immature CD4-CD8- T cells. Alpha beta-T cell receptor+ CD4-CD8- T cells produce IL-4. *J Immunol*, **149**, 1211-1215.
- Zoll, B., Lefterova, P., Ebert, O., Huhn, D., Von Ruecker, A. & Schmidt-Wolf, I.G. (2000) Modulation of cell surface markers on NK-like T lymphocytes by using IL-2, IL-7 or IL-12 in vitro stimulation. *Cytokine*, **12**, 1385-1390.
- Zorn, E. & Hercend, T. (1999a) A MAGE-6-encoded peptide is recognized by expanded lymphocytes infiltrating a spontaneously regressing human primary melanoma lesion. *Eur J Immunol*, **29**, 602-607.
- Zorn, E. & Hercend, T. (1999b) A natural cytotoxic T cell response in a spontaneously regressing human melanoma targets a neoantigen resulting from a somatic point mutation. *Eur J Immunol*, **29**, 592-601.
- Zou, W., Machelon, V., Coulomb-L'Hermin, A., Borvak, J., Nome, F., Isaeva, T., Wei, S., Krzysiek, R., Durand-Gassel, I., Gordon, A., Pustilnik, T., Curiel, D.T., Galanaud, P., Capron, F., Emilie, D. & Curiel, T.J. (2001) Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. *Nat Med*, **7**, 1339-1346.