Natural Killer Cells and Acute Myeloid Leukaemia

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Submitted for the degree of PhD

December 2012

Abstract

Despite successful induction chemotherapy, most patients with acute myeloid leukemia (AML) will relapse. Immune surveillance by T cells or natural killer (NK) cells may play a role in preventing relapse. This thesis examines the potential of NK cells to control AML.

Study 1 explored in 248 patients with haematological malignancies from the US National Institutes of Health, the genetic diversity of NK killer immunoglobulin receptor (KIR) genes in patients and their stem cell donors and their impact on outcome after stem cell transplantation (SCT) for haematological malignancy. Individuals with AML receiving SCT from donors inheriting 3 particular B-haplotype KIRs were 4 times less likely to relapse than those with donors without these favourable KIRs.

Study 2 explored in samples obtained from 499 patients enrolled on UK MRC/NCRI AML trials, whether KIR genotype affects the risk of developing AML or the outcome of remission induction chemotherapy. While KIRs had no effect on the development or outcome of *de novo* AML, individuals with more activatory KIRs, in particular 2DS2, developed significantly less secondary AML, suggesting that activatory KIRs can protect against secondary AML. These studies support a role for NK-mediated immune surveillance in AML.

Study 3 investigated the phenotype and function of AML NK cells. In 32 prospectively collected samples from AML patients undergoing remission induction chemotherapy at the Hammersmith Hospital, AML patients were found to have reduced NK activatory receptors, increased NK inhibitory receptors, and reduced cytotoxic function towards leukaemia, compared to healthy donors. These abnormalities corresponded with failure to achieve remission and can be induced in normal NK incubated with AML blasts.

I conclude that KIR genetics have a limited influence on AML development and outcome but that AML itself can impair NK function, reducing the chance of achieving remission. These findings have implications for NK based immunotherapy for AML.

Acknowledgements

I am grateful to Dr John Barrett who inspired me to undertake this work and first introduced me to the idea of immune surveillance and natural killer cells. His love of science is contagious. He taught me the critical importance of asking the right questions, and always to enjoy the process of trying to answer them. His ideas, energy, immense knowledge and clear thinking have all been instrumental to this work.

I would also like to thank Professor Jane Apperley who has consistently encouraged me to do this work- as she has done with generations of clinician scientists in her department. She has taught me important research skills and provided the practical means for undertaking this work.

I am extremely grateful to Dr Katy Rezvani who has been a huge influence during the period of this PhD. She has supervised most of the laboratory work and provided much in the way of ideas and practical guidance. Her boundless enthusiasm for her work and her ability to nurture a productive team have been very important.

I would like to thank the other members of Dr Rezvani's lab for making my PhD an enjoyable and stimulating time, in particular Mr Takuya Sekine, Dr Ahmad Khoder, Mr Abdullah Alsuliman, Dr Hugues de Lavaillade and Dr Paula Garland.

I would also like to thank Professor Irene Roberts who first encouraged me to pursue a research path and directed me towards Dr Barrett. I am grateful to Professor David Linch for some helpful discussions and facilitating my access to the unique trial samples of the MRC.

I would like to thank my family for their loving support during this research period, particularly my parents Professor Brent and Moira Taylor who enabled me to finish writing this thesis in the months after my daughter was born, and my husband Dr Argyris Stringaris whose encouragement and discussions about the bigger questions of life and his own fascinating work continue to inspire me.

I am grateful to the bodies who funded this work, namely the leukaemia charity LEUKA, the British Society of Haematology who provided a start up grant, the NIH who funded my year in America and the NIHR BRC London as well as the charitable people who raised money and donated to LEUKA. The sponsors of this study are public or nonprofit organizations that support science in general. They had no role in gathering, analyzing, or interpreting the data.

Finally, I would like to thank all the patients who contributed samples for this work. Their faith that giving their blood for research will improve the lot of those who come after them, facilitates and inspires leukaemia research.

This thesis is my own work. All else is appropriately referenced.

Kate Stringaris

Table of Contents

Natural Killer Cells and Acute Myeloid Leukaemia 1

Abstract 2

Acknowledgements 3

Table of Contents 4

List of abbreviations 7

List of tables 12

List of figures 13

Chapter 1 Introduction 18

1.1 Leukaemia 18
1.2 Innate and adaptive immunity 20
1.3 Immune surveillance 20
1.4 Graft versus leukaemia 21
1.5 NK cells 22
1.6 Cytotoxic mechanisms of NK cells 22
1.6-i Granule dependent pathway-perforin/granzyme release (CD107a) 23
1.6-ii Death receptor pathways- Fas Ligand (FasL) and TNF related protein (TRAIL) 24
1.6-iii-Secretion of interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) 24
1.7 NK cell receptors 25
1.7-i-C-type lectin receptors 26
1.7-ii-Natural Cytotoxicity Receptors (NKp30, NKp44, NKp46) 27
1.7-iii-SLAM family receptors 28
1.7-iv-CD16 28
1.7-v-Killer-immunoglobulin-like-receptors (KIRs) 28
1.7-vi-The balance between NK activatory and inhibitory receptors 30
1.8 NK development and education/tolerance 30
1.8-i-CD56 30
1.8-ii-KIR ligands and NK education/ tolerance 31
1.9 Immune editing-the effect of leukaemia on the immune system 32
1.10 Immune escape- changes on leukaemia induced by the immune system 33
1.11 Structure of PhD project 34

Chapter 2 Materials and Methods 35

2.1 Sample collection 35
2.1.i- KIR genotype analysis in the setting of allogeneic stem cell transplantation 35
2.1.ii- KIR genotype analysis in the setting of chemotherapy for AML 35
2.1.iii- Functional studies of NK cells in AML patients and normal controls 35
2.2 Processing, freezing and thawing PBMC 36
2.3 DNA extraction 36
2.4 KIR genotyping 37
2.5 Growing cell lines to use as NK targets and controls 39
2.6 Phenotyping NK cell surface receptors and their ligands on AML blasts 39
2.6-i-Antibody staining method 39
2.6-ii-Rationale for NK surface receptor antibody panel 39

2.6-iii-Rationale for antibody panel characterising the expression of NK receptor ligands on the surface of AML blasts....44
2.7. Purification of NK cells- Miltenyi bead column negative selection method....45
2.8 Purification of PBMC and blasts from AML samples at diagnosis- Miltenyi columns....46
2.9 Stemsep custom antibody bead selection cocktail and selection on Robosep machine....47
2.10. NK cell cytotoxicity experiments....50
2.10.i- CD107a expression....51
2.10.ii- TNFα and IFNγ production....52
2.10.iii- Fas Ligand expression....52
2.10.iv- Incubation of normal NK cells with AML blasts prior to experiments....52
2.11. Completing and validating the transplant database at the NIH....56
2.12 Statistical methods....56

Chapter 3 KIR genotype in HLA matched allogeneic stem cell transplantation....58
3.1 Background....58
3.2 Hypotheses....58
3.3 Patterns of donor KIR inheritance....59
3.4 Patient characteristics....59
3.5 Results....61
3.5-i- Outcome after SCT....61
3.5-ii-Impact of KIR genes on transplant outcome in multivariate analysis....62
3.5-iii-Impact of donor KIR genes in individual diseases....63
3.6 Discussion....67

Chapter 4 KIR genotype in AML patients receiving induction chemotherapy....70
4.1 Background....70
4.2 Models for investigating KIR genetics in this study....71
4.2-i-KIR gene frequency....71
4.2-ii-Possession of 'favourable' and activatory KIRs....71
4.2-iii-Missing a ligand for inhibitory KIRs....72
4.2-iv-Missing a ligand for activatory KIRs....72
4.2-v-Statistical analysis....72
4.3 Aims of this chapter....73
4.4 Results....73
4.4-i-Patient Characteristics....73
4.4-ii-Does a particular KIR genotype predispose to developing AML?....73
4.4-iii-Do individuals with 'favourable' or haplotype B KIR genotypes, or those missing a ligand for their KIRs have better outcomes after chemotherapy for AML?....76
4.5 Discussion....81

Chapter 5 Phenotypic changes in surface receptors and cytotoxic function of NK cells in AML....85
5.1 Background....85
5.2 Aims of this chapter....86
5.3 Results....86
Patient characteristics....87
5.3-i- Is the NK surface receptor phenotype in AML different from that of healthy individuals?....88
5.3-ii-Do any surface receptor abnormalities seen in AML normalise in remission?....88
5.3-iii- Is NK cell cytotoxicity abnormal in AML patients?....92
5.3-iv-Do any cytotoxic abnormalities seen in AML normalise in remission?....97

5.3-v-What is the repertoire of NK ligands on AML blasts? 98
5.3-vi-Is expression of NK ligands on leukaemic blasts associated with changes in NK receptor phenotype or function? 99
5.3-vii-Do individuals with abnormal phenotype have worse cytotoxic function? 101
5.3-viii-Is it possible to induce abnormalities by exposing normal NK cells to AML blasts? 102
Summary of results 103
5.4 Discussion 105

Chapter 6 Using NK studies to predict clinical outcome in AML 111
6.1 Background 111
6.2 Aims of this chapter 111
6.3 Results 112
6.3-i-Clinical Outcomes 112
6.3-ii-Does having secondary AML impact upon NK immunity and outcome compared with *de novo* AML? 113
6.3-iii-Do NK surface receptor abnormalities affect response to treatment? 114
6.3-iv-Do cytotoxic and effector cytokine abnormalities in AML predict response to treatment? 115
6.4 Discussion 117

Chapter 7 Discussion 119
7.1 Summarised findings addressing aims of PhD 119
7.1-i-Do individual killer immunoglobulin receptor (KIR) genes (coding for specific NK surface receptors) affect the risk of developing leukaemia and the outcome of treatment aimed at cure, ie. chemotherapy and stem cell transplantation? 119
7.1-ii- Are NK cells abnormal in surface phenotype or cytotoxic function in AML patients and does the individual's NK cell receptor profile or cytotoxicity affect outcome of leukaemia remission induction treatment? 122
7.1-iii- Are abnormalities found in NK cells induced by the AML or do they occur because of genetic predisposition? 123
7.2 Limitations of this thesis 124
7.2-ii- Clinical constraints: 124
7.2-iii Statistical constraints: 124
7.3 Future directions 126
7.3-i- Clinical/therapeutic future directions 126
7.3-ii- Biological aspects - future directions 127
7.4. Immunotherapy for AML – concluding remarks 128

Appendix: Supplementary material 131
8.1 Consent form: functional study of NK cells in AML 131
8.2 Patient information sheet: functional study of NK cells in AML 132
8.3 Supplementary table 1. Optimising DNA extraction 135
8.4 Supplementary table 2. Univariate analyses in Cox models for factors affecting transplant outcome (n=261) 135
8.5 Supplementary table 3. Competing risk analysis 137
8.6 Supplementary figure 1. Patient representation for NIH cohort 137

References 138

List of abbreviations

ADCC	Antibody dependent cellular cytotoxicity
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
AML M3	Acute promyelocytic leukaemia
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATG	Anti thymocyte globulin
BCR-ABL	Abnormal fusion gene found in CML created by t(9;22)(q34;q11)
BD	Beckton Dickenson (Biosciences company)
BFA	Brefeldin A
CBFB-MYH11	Abnormal fusion gene found in AML M4eo created by inv(16)(p13q22)
CD	Cluster differentiation molecule
CEBP	CCAAT/enhancer binding protein (a transcription factor)
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CR	Complete remission
CSA	Cyclosporin
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DA	Daunorubicin and Cytarabine chemotherapy
DAP	DNA activating protein

DEK-NUP214	Abnormal fusion gene created by t(6;9)(p23;q34)
del(5q)	Deletion of part of the long arm of chromosome 5
dH20	Distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DR4	Death receptor 4
E:T	Ratio of effector cells to target cells
ELN	European Leukemia Net
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain protein
FasL	Fas Ligand
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Flag-Ida	Fludarabine, Cytarabine, GCSF and Idarubicin chemotherapy
FLT3-ITD	Fms-like tyrosine kinase3-Internal tandem duplication
GITRL	Glucocorticoid-induced tumour necrosis factor-related protein
GVHD	Graft versus host disease
GVL	Graft-versus-leukemia
Haplo	Haplotype
HCV	Hepatitis C
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLH	Haemophagocytic lymphohistiocytosis

HR	Hazard Ratio
IDO	Indoleamine 2, 3-dioxygenase
IFNγ	Interferon gamma
IL	Interleukin
IPSS	International Prognostic Scoring System for MDS
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer immunoglobulin receptor
LC30	Lymphocyte count at day thirty post transplant
LPS	Lipopolysaccaride
LSA	Leukaemia-specific antigens
MDR-1	Multi drug resistance-1
MDS	Myelodysplastic syndrome
MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
mHag	Minor histocompatibility antigens
MICA	MHC class-I-chain-related protein A
µl	Microlitre
MLL	Mixed lineage leukemia (a gene frequently rearranged in AML at 11q23)
MM	Multiple myeloma
MPA	Mycophenolic acid
MRC/NCRI	Medical Research Council/ National Cancer Research Institute
MSC	Mesenchymal stromal (or stem) cells
N-CAM	Neural cell adhesion molecule

NCR	Natural cytotoxicity receptor
NHL	Non Hodgkins lymphoma
NHLBI	National Heart Lung and Blood Institute (NIH, Maryland, USA)
NIH	National Institutes of Health
NK	Natural killer cell
NPM1	Nucleophosmin gene (frequently mutated in AML)
OS	Overall survival time
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PerCP	Peridinin-chlorophyll-protein complex
PI3K	Phosphatidylinositol 3-kinase
PMA	Phorbol ester
PRAME	Preferentially expressed antigen of melanoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute culture medium
RPN1-EVI1	Abnormal fusion gene resulting from a 3q chromosomal rearrangement
RUNX1	Runt-related transcription factor 1gene
Rx	Treatment
SCID	Severe Combined Immunodeficiency
SCT	Stem cell transplantation
SHIP	Src homology 2 domain-containing inositol-5-phosphatase
SHP-1	SH2-containing protein-tyrosine phosphatase-1
SLAM	Signal lymphocytic activating molecule

SSP	Sequence-specific priming
t-AML	Therapy related AML
TGFβ-1	Transforming growth factor beta-1
TNFR1	TNF receptor 1
TNFα	Tumour-necrosis factor alpha
TRADD	TNF R1 associated death domain protein
TRAIL	TNF related protein
Treg	Regulatory T cell
TRM	Transplant related mortality
TTR	Time to relapse
UCH	University College Hospital
ULBP	Ultra long-16 binding proteins
WHO	World Health Organization
WT1	Wilms tumour 1

List of tables

Table 1.1 Standardised reporting for correlation of cytogenetic and molecular genetic data in Acute Myeloid Leukaemia with clinical data according to the ELN Guideline[4] 19

Table 2.1 Antibody panel for characterising NK surface receptor phenotype 40

Table 2.2 Antibody panel for characterising the expression of NK receptor ligands on the surface of AML blasts 44

Table 2.3 Panel for NK cytotoxicity experiments 51

Table 3.1 Patient characteristics of NIH transplant cohort 60

Table 3.2 Transplant outcome 61

Table 3.3 Multivariate analysis of significant risk factors in Cox models 62

Table 3.4 Effect of favourable donor KIR genes on transplant outcome in different diseases 64

Table 5.1 AML patient characteristics 87

Table 6.1 Clinical outcomes of AML patients 112

List of figures

Figure 1.1 Morphological features of an NK cell under light microscopy (hematoxylin and eosin stain) 22

Figure 1.2 Different mechanisms of NK mediated cytotoxicity (derived from Frontiers in Bioscience 10, 1396-14, 2005).....23

Figure 1.3 NK receptors and their ligands (derived from Vivier et al.[72]).....26

Figure 1.4 KIR protein structure from Immunopolymorphism Database (IPD) http://www.ebi.ac.uk/ipd 29

Figure 1.5 KIR genes in the LRC on chromosome 19 (derived from Kulkarni et al[114]) 30

Figure 2.1 Example of gel and worksheet for KIR genotyping one individual. The ladder (L) is positioned far left. There is a positive control at 800bp in lanes 1-19 and at 200bp in lanes 20-21. Each lane corresponds to primers for a particular KIR gene or allele. The work sheet shows the molecular weight (5th row from bottom) of each KIR gene or allele (detailed in column 2) and the gel lane where it is expected to be (black squares). If present, the KIR gene shows up as a distinct band of the appropriate molecular weight along with the positive control (see lane 1). If the KIR gene is absent, only the control band will show in that lane (see lane 2). Some genes require two bands. Eg. Identification of KIR 2DL5A (see blue arrows) requires a product of 257bp in lane 5 and 1753bp in lane 6. 38

Figure 2.2 Example of FACS plots showing the gating strategy for NK surface receptor phenotyping. Cells were first gated on live lymphocytes using forward and side scatter (first column), then CD56+ cells which were negative for blast markers CD13, 33 and 34 were identified (second column). Next NK cells were separated from CD56+ T cells by gating on CD3-, CD56+ cells (third column) and finally the NK cells were assessed for their expression of the NK surface marker of interest (forth column). This figure also demonstrates a comparison of fresh (top row) and frozen (bottom row) cells from the same patient. 41

Figure 2.3 Example of titration experiment to find optimal antibody staining for KIR antibodies. Serially diluted volumes of antibody were used and the lowest concentration not resulting in loss of signal was used in experiments. These plots show no loss of signal when using half the volume recommended by the antibody company. Further dilutions would then be performed.....42

Figure 2.4 Example of FACS plots showing % expression of NK surface markers (on x axis): c-type lectins (top two rows), natural cytotoxicity receptors (third row) and KIR (bottom row). The NK marker CD56 is on the y axis. Positively staining cells were identified using PE conjugated isotypes to identify the border with the negative population (two plots top left). NKG2A was an IgG2A antibody, the rest were IgG1 antibodies.43

Figure 2.5 Example FACS plots showing the gating strategy for blast surface phenotype. Forward and side scatter was used to identify live cells, then blasts were identified using CD13, 33 and 34. The blasts were then assessed for surface expression of the NK ligand of interest (here HLA class I). 44

Figure 2.6 Example of FACS plots and histograms from one experiment showing expression of NK receptor ligands on the surface of a patient's AML blasts. Isotype and antibody staining of positive control cells (Hela or PBMC depending on ligand) was performed with each sample and are shown in the two columns to the left of the figure. The three columns to the right represent antibody staining of the patient's cells. Isotype controls were used to identify the negative population (middle column and red line on the histogram). The blue line on the histogram represents antibody staining of patient cells. This patient's blasts stained positive for the NK ligands DR4/5, HLA class 1, Fas and HLA E but not MICA/B.....45

Figure 2.7 Example of a FACS plot of the NK negative selection procedure from healthy donor PBMC using Miltenyi beads. The technique typically yields >95% NK cell purity. 46

Figure 2.8 Example of FACS plots showing results of a cell separation experiment using the Robosep cell separator machine. Cells collected at AML presentation were separated into blast and PBMC populations. The blast populations (left columns) were mostly positive for pan blast markers (CD13, 33, 34) with minimal contamination from NK (CD56+) and T (CD3+) lymphocytes. The PBMC populations were relatively free from AML blasts. Purity results are in brackets. AML patient 2 is an example of a patient whose AML blasts express the NK marker CD56. 49

Figure 2.9 Example of FACS plots showing gating strategy for NK cytotoxicity assay 53

Figure 2.10 Example of FACS plots showing reproducibility of cytotoxic data. Plots show data from 3 separate experiments performed on 3 separate days using cells collected from the same individual on one day and divided into a number of vials for freezing (mean average used). 53

Figure 2.11 Example of FACS plots showing one of a series of optimisation experiments. Peripheral blood mononuclear cells (PBMC) were incubated for 5 hours with leukaemia target cells at effector:target (E:T) ratios of 1:1. 3:1 and 10:1 (calculated using %NK in PBMC sample). This experiment and others showed NK degranulation (as measured by the number of NK cells positive for CD107a) was optimal at a 1:1 E:T ratio. Different numbers of leukaemia target cells were also tested: 100,00 cells was not enough for useful results. 250,000 targets were as effective as 1 million. A negative control helped to define the negative population and consisted of PBMC incubated in media without target cells. 54

Figure 2.12 Example FACS plots showing results from one experiment assessing a patient's NK cytotoxicity towards K562 (2nd and 3rd rows) and autologous blast targets (4^{th} and 5^{th} rows). Cells were incubated in media alone for the negative control and with PMA/Ionomycin for the positive control (top row). The y axis represents the NK marker CD56. The x axis represents the degranulation marker CD107a. NK cells from this patient showed very little cytotoxicity to autologous blasts. However, there was considerable response to K562 targets, with a greater response from KIR+ than KIR- NK cells. The columns represent different KIR antibodies (from left-right: 2DS1/L1, 2DS2/L2, 3DL1, Pan KIR). 55

Figure 3.1 Factors affecting SCT outcome in multivariate analysis of 261 patients. a) Lymphocyte count at day 30 post SCT and transplant related mortality b) Donor KIR genotype and relapse 63

Figure 3.2 Effect of donor KIR genes on relapse in AML patients (n=68). a) Stratified by donor KIR genes b) Stratified by donor KIR genes and risk group 65

Figure 3.3 Actuarial relapse rates for disease subgroups according to donor KIR genotype 66

Figure 4.1 The frequency of KIR genes in the AML population (left) and normal donors (right). There is no difference in KIR frequency between the two populations. 74

Figure 4.2 KIR gene prevalence looking at an individual's total number of activatory KIR. Patients with secondary AML had less activatory KIR than those with de novo AML or normal healthy controls. 74

Figure 4.3 The frequency of KIR genes in secondary AML compared to a) the normal donor population b) *de novo* AML 75

Figure 4.4 The role of 3 'favourable' KIR on AML outcome in MRC/NCRI trial patients. Presence of the 3 'favourable' KIR genes 2DS1, 3DS1, 2DL5a did not affect a) overall survival b) relapse risk in AML 77

Figure 4.5 The role of KIR haplotype on AML outcome in MRC/NCRI trial patients. Patient KIR haplotype did not affect a) overall survival or b) relapse risk in AML. Value 1=patients with haplotype B. 78

Figure 4.6 The role of inhibitory KIR ligands on AML outcome in MRC/NCRI trial patients. Missing a ligand for inhibitory KIRs did not affect a) overall survival or b) relapse risk in AML. Value 1= patients missing an HLA ligand for their inhibitory KIRs 79

Figure 4.7 The role of activatory KIR ligands on AML outcome in MRC/NCRI trial patients. Missing a ligand for activatory KIRs a) did not significantly affect overall survival but b) may be associated with a reduced relapse risk in AML 80

Figure 5.1 Examples of histograms showing NK surface phenotype in a representative AML patient and healthy control. Open histograms represent isotype-matched monoclonal antibody staining. Filled histograms represent staining with specific PE-congugated monoclonal antibodies 88

Figure 5.2 NK receptor expression in AML patients compared to normal controls. a) Proportions of CD56 bright and dim NK cells were not different between the groups b) There was no difference in surface expression of KIRs c) NKp46 expression was lower in AML but NKp30 and NKp44 were not different to normal controls d) AML patients showed an increase in NK expressing the inhibitory receptor NKG2A but no difference in NKG2D expression e) Gating strategy to identify NK from PBMC 89

Figure 5.3 NK phenotypic abnormalities in AML were partially restored in remission. (a) NKp46 expression was restored to normal levels (b) NKG2A expression remained elevated. P values denote significance of paired T tests comparing AML T1 with remission and unpaired T tests comparing AML with healthy controls. Horizontal bars denote mean expression. (c) NKp46 and NKG2A expression in a representative healthy control (left column) and AML patient at serial time points (right column): diagnosis (T0), following induction chemotherapy (T1), following consolidation chemotherapy #1 (T2), and following consolidation chemotherapy #2 (T3). Abbreviations: HC: healthy control 90

Figure 5.4 Expression at presentation and remission of NK receptors whose expression was not significantly altered at AML presentation a) NKG2D expression was unchanged in remission b) Expression of KIR2DL1/S1 and 2DL2/S2 were unchanged in remission. KIR3DL1/S1 was reduced c) NCRs tended to increase in AML remission compared to presentation (paired t tests). 91

Figure 5.5 NK cytotoxicity and effector function in AML patients at diagnosis compared to healthy controls a) Total NK (CD56+, CD3-) cytotoxicity (CD107a degranulation), TNFα and IFNγ production and Fas ligand expression against K562 leukaemia targets in 32 patients with AML and 15 healthy controls. Cytotoxicity towards K562 was significantly reduced in AML patients compared to normal controls in all measures except Fas ligand b) NK response at AML presentation to autologous blasts was as defective or more defective than towards K562 cells in all measures except Fas ligand. Horizontal bars denote mean expression. Abbreviations: NC: healthy control, AML: acute myeloid leukaemia (unpaired t tests) 92

Figure 5.6 KIR specific NK cytotoxicity and effector cytokine production in AML patients at diagnosis compared to healthy controls. a) comparison of NK cytotoxicity and effector cytokine function in KIR+ subsets; b) comparison of NK cytotoxicity and effector cytokine function in KIR- subsets. Figures show greater cytotoxicity and effector cytokine production from healthy control NK than AML patient NK cells and from KIR+ than KIR- NK cells irrespective of which particular KIR is expressed. Horizontal bars denote mean within group. Abbreviations: HC: healthy control, AML: acute myeloid leukaemia (unpaired t tests) ... 93

Figure 5.7 NK cells stimulated with PMA/Ionomycin maintain normal function in AML patients. NK cytotoxicity (CD107a degranulation), TNFα and IFNγ production in AML patients at diagnosis (T0), remission (CR) and healthy controls (HC) following stimulation with PMA/Ionomycin. Horizontal bars denote mean expression. Error bars denote standard deviation between individuals within group. Three groups compared using one way ANOVA tests. Abreviations: HC, healthy control; T0, AML presentation; CR, complete remission. 94

Figure 5.8 The role of inhibitory KIR ligands on NK cell (CD56+, CD3-) cytotoxicity towards K562 leukaemic target cells after 5 hour incubation. In each graph unpaired t tests compared those with a ligand for their inhibitory KIR (KIR2DL1+ C2+, first column; KIR2DL2/3+ C1+, second column, 3DL1+ Bw4+, third column) and those without in three groups: from left, healthy donors (NC), AML at presentation (AML) and AML at remission (AML R). Columns represent from left to right: degranulation, TNFα and IFN γ production. There was no beneficial effect of a) Missing a particular HLA ligand for inhibitory KIR or b) missing any HLA ligand for inhibitory KIRs on NK function. 95

Figure 5.9 The role of activatory KIRs on NK cell (CD56+, CD3-) cytotoxicity towards K562 leukaemic target cells after 5 hour incubation. In each graph unpaired t tests compared those with a ligand for their activatory KIR (KIR2DS1+ C2+ or KIR2DS2+ C1+) and those without in three groups: from left, healthy donors (NC), AML at presentation (AML) and AML at remission (AML R). Possessing a ligand for a) 2DS1 or b) 2DS2 does not affect NK cytotoxicity or effector cytokine production in any group. Columns represent from left to right: degranulation, TNFα and IFN γ production. 96

Figure 5.10 Partial normalisation of NK cytotoxic abnormalities in AML remission a) There was normalisation of degranulation and partial normalisation of IFNγ production but not TNFα towards K562 leukaemia cells. Paired t tests comparing D0 with remission, unpaired t tests comparing remission with healthy controls b) Aside from a few individuals, there was generally no improvement in cytotoxicity towards autologous blasts (paired t tests). Abbreviations: D0, AML presentation; CR, complete remission; NC, healthy controls 97

Figure 5.11 Expression of NK receptor ligands on the AML blasts of 32 patients. X axes represent the ligand of interest. Y axis represents percentage of AML blasts expressing that ligand. 98

Figure 5.12 Relationship between NKG2A expression and that of its ligand HLA E in AML a) While most had a raised proportion of NKG2A expressing NK cells, a subset of AML patients had low NKG2A (red circle) b) Low NKG2A expression was associated with high HLA E expression on blasts, although this correlation did not quite reach statistical significance c) HLA E/NKG2A ratio did not predict NK cytotoxicity. 100

Figure 5.13 The effects of abnormal NK surface phenotype on NK cytotoxicity and effector cytokine production. Individuals were separated into those with receptor levels above and below the median. Unpaired t tests compared the two groups a) High numbers of NK expressing surface NKG2A were associated with reduced TNFα b) Low NKp46 was associated with reduced CD107a and IFNγ production 101

Figure 5.14 AML cells induce impairments in NK effector function in vitro. The reduction in healthy donor NK CD107a degranulation, TNFα and IFNγ production against K562 leukaemia target after 24 hours of co-culture with primary AML blasts (ratio of 10:1) + IL-2 (200 iU/mL), is presented. The results are expressed as a normalised ratio against healthy donor NK. 102

Figure 5.15 Representative FACS plots showing the effect of co-incubating AML blasts from 2 different patients on healthy donor NK cell cytotoxicity and effector function are presented. After 24 hours of healthy donor NK co-culture with primary AML blasts (ratio of 10:1) + IL-2 (200 iU/mL), the effector function of NK cells was assessed against K562 leukaemia targets (ratio 1:1). Plots are gated on CD56+CD3- NK cells. 103

Figure 6.1 Secondary versus *de novo* AML: response to treatment and NK cytotoxicity. There is no significant difference between those with *de novo* AML (n=16) and secondary AML (n=16) with respect to a) response to first line treatment (Chi square test) and b) NK cytotoxicity (unpaired t tests). Grey bars represent proportion of patients achieving complete remission. 113

Figure 6.2 NK surface receptor phenotype and response to AML chemotherapy. Receptor expression in 32 AML patients was assessed. Patients were divided into those whose levels of NKG2A and NKp46 were above

or below the median. Figure shows that high NKG2A predicted poor response to treatment. Low NKp46 did not. 114

Figure 6.3 NKG2A and HLA E together helped predict response to treatment. a) Non-responders (in red) showed a pattern of higher NK NKG2A levels and lower blast HLA E expression than responders (in black) b) The HLA E/NKG2A ratio is significantly higher in those who respond to AML treatment. 115

Figure 6.4 NK cytotoxicity and response to treatment a) TNFα production at D0 predicted for achieving remission after initial chemotherapy (Chi^2 test) b) TNFα production from CD56+ CD3- cells against K562 leukaemic targets at AML presentation: Non-responders produced significantly less than normal controls and there appears to be a possible a dose effect with greater production of TNFα at presentation corresponding to earlier treatment response 116

Chapter 1 Introduction

Increasing evidence suggests that natural killer (NK) cells are an important part of the immune response against leukaemia, yet the mechanisms mediating this anti-leukaemia response remain mostly unclear. A better understanding of this response could help us exploit and/or augment NK immunity to improve outcome for patients. The aim of this PhD project is to investigate the innate immune response to acute myeloid leukaemia (AML) by asking three main questions:

1. Do individual killer immunoglobulin receptor (KIR) genes (coding for specific NK surface receptors) affect the risk of developing leukaemia and the outcome of treatment aimed at cure, ie. chemotherapy and stem cell transplantation?

2. Are NK cells abnormal in surface phenotype or cytotoxic function in AML patients and does the individual's NK cell receptor profile or cytotoxicity affect outcome of leukaemia remission induction treatment?

3. Are abnormalities found in NK cells induced by the AML or do they occur because of genetic predisposition?

1.1 Leukaemia

Leukaemia accounts for over 4000 deaths/year in the UK and is one of the few cancers to affect young people. Due to accumulation of malignant cells in the bone marrow, the production of normal blood cells is impaired and patients typically present with anaemia, infections and bleeding. Without treatment, patients with acute leukaemia usually die from their disease within a matter of weeks.

The last 40 years have brought great improvements for children with acute leukaemia but outcomes remain worse for adult patients. Current treatments with chemotherapy and/or stem cell transplantation bring remission to over 80% of adults with acute leukaemia. However, despite this improvement, more than 50% will relapse within the first three years.[1-3]

Prognostic factors predicting outcome of treatment for AML have evolved from morphological classifications to include cytogenetic and molecular characteristics of the disease. This enables us to categorise AML into three broad risk categories– favourable, intermediate and adverse risk (table 1.1).

However, since treatment success varies within these leukaemia-defined risk factors, other influences

both environmentally and genetically determined appear to play an important role in determining leukaemia treatment outcomes. One prognostic factor deserving further exploration is the role of the immune system in controlling the leukaemic process.

Table 1.1 Standardised reporting for correlation of cytogenetic and molecular genetic data in Acute Myeloid Leukaemia with clinical data according to the ELN Guideline[4]

ELN Genetic Risk Group	Subsets
Favourable	t(8;21)(q22;q22); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3-ITD* (normal karyotype) Mutated *CEBP*A (normal karyotype)
Intermediate-I	Mutated *NPM1* and *FLT3-ITD* (normal karyotype) Wild-type *NPM1* and *FLT3-ITD* (normal karyotype) Wild-type *NPM1* without *FLT3-ITD* (normal karyotype)
Intermediate-II	Cytogenetic abnormalities not classified as favourable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* t(6;9)(p23;q34); *DEK-NUP214* t(v;11)(v;q23); *MLL* rearranged -5 or del(5q); -7; abnl(17p); complex karyotype
Abbreviation:ELN=European LeukaemiaNet.	

It is increasingly recognised that interactions between the immune system and malignant disease are important at all stages from disease predisposition (immune regulation) to the success or failure of treatment (involving "immune editing " of the immune system by the malignancy and "immune

escape" where changes in the malignancy allow it to evade immune detection and destruction.) When considering the immune response to malignancy and leukaemia in particular there is evidence for both humoral and cellular immune interactions with leukaemia involving both innate and adaptive immunity.

1.2 Innate and adaptive immunity

The division of the immune system into innate and adaptive immunity can be a helpful way of describing two complementary sides to our immune armoury. Innate immune cells include dendritic cells and monocytes as well as natural killer cells, which can exert rapid effector function through a limited repertoire of germ-line encoded receptors, which are not antigen specific. While less specific, the innate system responds immediately to many common immune stimuli including infections and malignant cells. Adaptive immunity requires more time but can mount highly specific and repeatable responses to almost any foreign pathogen we might encounter. Adaptive immunity is mediated through T and B cells both of which express a large repertoire of antigen receptors, which are produced via somatic recombination following exposure to antigens in specialised lymphoid organs where they develop from naïve into mature effector cells.

1.3 Immune surveillance

The concept of immune surveillance of malignant disease (that is, the immune system constantly searching, detecting and eradicating emerging malignant cells) is widely accepted.[5-7] The process of immune surveillance involves two components (1) The role of the immune system in preventing the development of malignant disease and (2) The behavior of the immune system in the face of an established malignancy. Initial evidence for involvement of the immune system in the prevention of malignancy comes from the increased risk of malignant disease seen in immune deficient individuals. While there is abundant evidence for immunosuppression predisposing to lymphoid malignancy[8,9] the only evidence in man for the role of an intact immune system protecting against AML comes from a recent observation that patients receiving long term immunosuppression following solid organ transplants have a 2 and 5 fold higher risk of developing AML after renal or cardiac transplant respectively.[10]

In the face of an established AML there is evidence for both immune editing and immune escape (discussed further in sections 1.9 and 1.10). T cells and NK cells from patients with AML are unable to synapse normally with AML blasts.[11] Immunosuppressive regulatory T cells are increased in

AML,[12] and superior NK cytotoxicity against leukaemia targets (as assessed by % lysis of blasts) is associated with an improved leukaemia free survival.[13] There is also a growing body of literature associating robust lymphocyte recovery after remission induction for acute myeloid leukaemia (AML) with improved survival.[14,15] However, it is unclear which lymphocyte subset is implicated in this association. Certainly, both T cells and NK cells are implicated in the powerful graft-versus-leukaemia (GVL) effect observed after allogeneic stem cell transplantation (SCT),[16,17] a setting which, by transplanting a healthy immune system into an immunodeficient host provides an important model for the role of the immune system in controlling any residual or re-emerging malignant disease.

1.4 Graft versus leukaemia

Extensive evidence for T cells in the graft versus leukaemia effect[18] follows initial observations that SCTs depleted of T cells were associated with increased relapse rates[19] and that donor lymphocyte infusions could promote remission after relapse.[20] In order for T cell-mediated GVL to occur, T cells must recognise antigens on the leukaemia cell. These antigens can be either leukaemia-specific (LSA) or minor histocompatibility antigens (mHag): found on most tissues or restricted to haematopoetic tissues.[21] LSA range from aberrantly expressed proteins such as neutrophil elastase and proteinase 3, to tumour specific proteins such as BCR-ABL in chronic myeloid leukaemia, Wilms tumour 1 (WT1) and preferentially expressed antigen of melanoma (PRAME) which can act as antigens and stimulate a cytotoxic T cell response.[22] A third piece of evidence for T cell GVL is a fascinating mechanism of tumour evasion whereby relapsed leukaemia cells after haplo identical SCT can down regulate expression of the whole mismatched haplotype, therefore expressing only the matched haplotype and thus avoiding T cell (but not NK cell) recognition.[23]

Initial evidence for NK cell-mediated GVL came from data showing that SCTs mismatched for NK KIR ligands were associated with reduced relapse.[24-26] Further evidence of NK alloreactivity has also been documented in HLA identical SCT without inhibitory KIR-ligand mismatch suggesting a role for activatory KIRs in the process.[27-29] One particular benefit of the NK-mediated GVL effect post SCT is that unlike T cell responses, NK reactivity does not appear to be associated with an increased risk of graft versus host disease (GVHD), that is, an unwanted immune response targeting normal tissues such as skin, gut and liver. On the contrary, NK cells are thought to reduce the risk of GVHD by attacking host dendritic cells leaving fewer opportunities for antigen presentation to T cells from the incoming graft.[26]

The question of whether T cells or NK cells are primarily responsible for effective immune responses against AML is not yet resolved. It is possible that both NK cells and T cells exert some control over residual leukaemia after remission induction or that in different subsets of AML either T cells or NK cells predominate in their antileukaemic effect. Certainly much less is known about NK cell responses to leukaemia. The genetic KIR data above implicates NK cells in a useful graft versus leukaemia effect post SCT, indicating that an NK-mediated anti-leukaemic effect is present and therefore further knowledge is desirable. In this thesis I focus on the natural killer (NK) cell immune response to AML.

Below, I review current knowledge on the biology of NK cells and the mechanisms of cytotoxicity against leukaemia and discuss what we know about immune editing and immune escape in the context of both allogeneic and autologous NK cell interactions with AML.

1.5 NK cells

Natural killer (NK) cells are large granular lymphocytes, which normally make up 2-10% of the circulating lymphocyte population (fig. 1.1). Their main function appears to be to kill unwanted infected or malignant cells. They also play a role in maintaining the developing embryo in utero.[30]

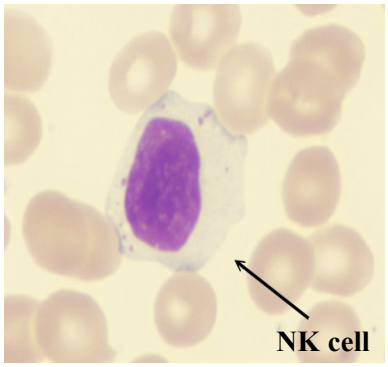


Figure 1.1 Morphological features of an NK cell under light microscopy (hematoxylin and eosin stain)

1.6 Cytotoxic mechanisms of NK cells

NK cells, by directly killing malignant cells, are believed to play an important role in the immune response against leukaemia. They kill by using perforin to deliver granzymes into target cells, and by using death receptor pathways such as TRAIL and Fas ligand. NK cells also secrete cytokines such as interferon gamma (IFNγ) and tumour-necrosis factor alpha (TNFα) (fig. 1.2).

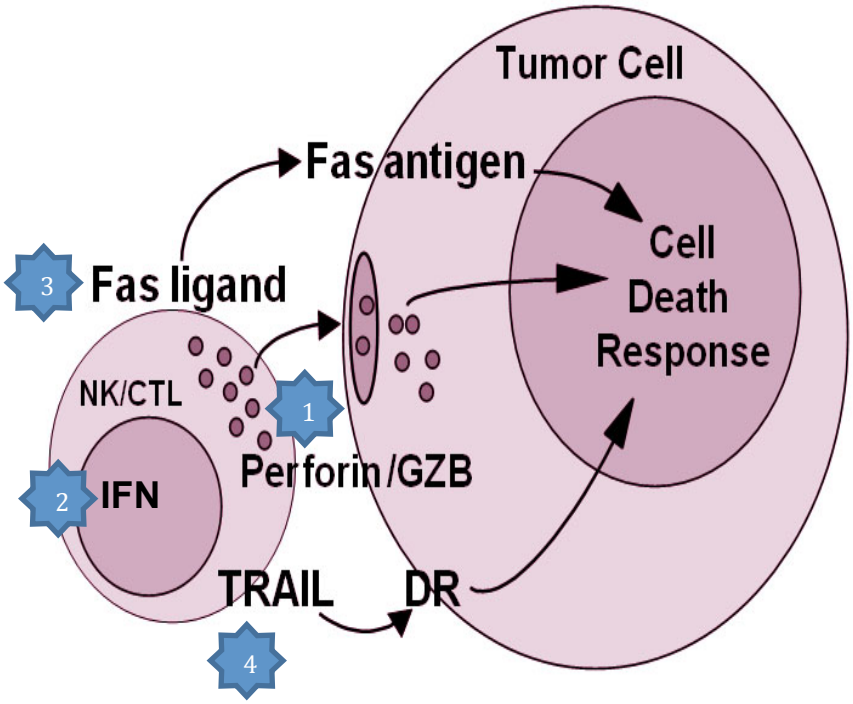


Figure 1.2 Different mechanisms of NK mediated cytotoxicity (derived from Frontiers in Bioscience 10, 1396-14, 2005).

1.6-i Granule dependent pathway-perforin/granzyme release (CD107a)

NK cells and CD8 T cells contain cytoplasmic vesicles or granules, which store the preformed lytic molecules perforin, granzymes and granulysin. Protease enzymes are made and stored in an inactive stage within the vesicle.[31] The wall of these granules contains a range of lysosomal-membrane-associated glycoproteins including CD107a, which protect the NK cell from the granule contents. When a target cell is recognised, the granule is mobilised along microtubules towards the synapse with the target cell. The microtubules then realign to ensure polarised delivery of the granules specifically to the synapse.[32] Degranulation then occurs when the lytic contents are released at the target by exocytosis.[33-35]

Released perforin anchors to the target cell membrane via its C terminal domain where, in the presence of calcium, polymerization occurs and its lytic N terminal domain begins to form 5-20nm cylindrical pores in the target cell membrane.[36-38] These pores allow entry to granzyme and granulysin and also ionic exchange causing an osmotic imbalance and cell death.[36] There is also evidence that granzymes enter the target cell independently of perforin.[39,40] The importance of perforin in the immune response is supported by studies showing that perforin-deficient mice develop lymphoma[41] as well as the familial haemophagocytic lymphohistiocytosis (HLH) syndrome in humans, where an inherited mutation in the perforin gene is associated with extensive immune suppression, particularly to infections caused by intracellular pathogens.[42] Granzymes, most abundantly granzyme A and granzyme B, once inside the target cell, trigger apoptosis via a cascade

of mechanisms including activation of the endoplasmic reticulum complex, an increase in reactive oxygen species, breaching of the cell nucleus and mitochondrial outer membrane and fragmentation and degradation of chromosomal DNA. Much of this activity is dependent on caspases found in the target cell cytoplasm.[43]

1.6-ii Death receptor pathways- Fas Ligand (FasL) and TNF related protein (TRAIL)

NK cells and other effector cells such as T cells, express surface FasL. Target cells (such as many AML blasts) which express Fas, are therefore susceptible to apoptosis via the Fas-FasL death receptor pathway. This pathway was first recognised in perforin-deficient mice as an important mechanism of killing independent to the perforin granzyme pathway, first in T cells and then NK cells.[44,45]

FasL is stored at high levels intracellularly but normally expressed at low levels on the surface of NK cells.[46] Preformed FasL is stored on the inner surface of NK granules, which also contain perforin and granzymes.[46] Upon activation of an NK cell via its activatory receptors, surface FasL is upregulated within 60 minutes.[47] Binding of FasL to Fas on a target cell leads to trimerisation and recruitment of Fas-associated death domain proteins (FADD). This leads to a cascade of caspase activation and cytochrome C production leading to DNA cleavage and cell death.[48] A similar mechanism leads to apoptosis when TRAIL binds its receptors DR4 or 5.[49-51] There is evidence that NK cells use different cytotoxic mechanisms depending on their degree of maturity and site of action. TRAIL is primarily used by more immature NK cells[52] and perforin-mediated cytotoxicity has been shown to be more effective in the spleen than the liver,[53] suggesting that NK cells may change with development and alter their mechanism of action depending on the environment.

Interestingly, high levels of FasL are constitutively expressed in immune privileged sites such as cells of the brain, testes and eye where it has been shown to be part of the immune tolerance mechanism at these sites.[54] Both the Fas and TRAIL death receptor pathways have been shown to be important in NK mediated killing of tumor cells.[55-58]

1.6-iii-Secretion of interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α)

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine which was named following the initial finding that it caused rapid haemorrhagic necrosis in tumours transplanted into mice.[59] Since then, as well as necrosis, it has been shown to play a role in a range of cellular activities including proliferation, differentiation, apoptosis and induction of other cytokines.[60-62] TNF-α is produced as a 26kDa trans-membrane precursor which is converted to its soluble form upon release via cleavage by ADAM-17 protease.[63] Its role in NK cell cytotoxicity is mediated by binding to TNF receptor 1 (TNFR1) on a target cell, leading to the recruitment of a number of proteins including TRADD (TNF

R1 associated death domain protein) and FADD/MORT1 (Fas-associated death domain protein), which induce apoptosis via a caspase cascade. Its inflammatory, immune regulatory and cytokine-inducing roles are mediated through the NF-κB and MAP kinase pathways after binding to TNF R1 and/or TNF R2.[64]

Interferon-gamma (IFN-γ) is produced by NK cells on target cell recognition, as well as in response to exogenous cytokines such as interleukin (IL)-2, 12, 15 and 18.[65] IFN-γ production by NK cells has a number of roles. As well as facilitating NK killing of tumour[66] and virally infected cells,[67] it also has an immunoregulatory role. In the latter capacity it has been shown to be important in dendritic cell homeostasis[68] and promoting priming of CD4 Th1 cells,[69] as well as having anti-inflammatory properties (in mouse arthritis models it has been shown to prevent neutrophil recruitment to affected joints.)[70] TNF-α and IFN-γ are produced *de novo* in the golgi body of the NK cell after contact with a target cell. They are then trafficked via a recycling endosome to the cell surface for release. These carriers transport the cytokines all over the cell surface, as well as to the target cell synapse,[71] as befits the multifunctional role of TNF-α and IFN-γ in triggering other immune cells as well as directly influencing target cell death.

1.7 NK cell receptors

NK cell receptors provide a means by which NK cells can recognise and differentiate potential targets from normal cells. Each NK cell has a range of receptors on its surface. Engagement of these receptors with their ligand on a target cell, will either result in the activation or inhibition of NK activity. The balance between activating and inhibitory receptors determines whether target cell killing occurs. Important families of NK receptors include the C-type lectin family (including NKG2A and D), the natural cytotoxicity receptor (NCR) family, which includes NKp46, NKp44 and NKp30, the signal lymphocytic activating molecule (SLAM) family which includes CD244, NTB-A, and CRACC, as well as CD16 and the killer immunoglobulin (KIR) receptor family. Known ligands for these receptors are shown in figure 1.3 below.

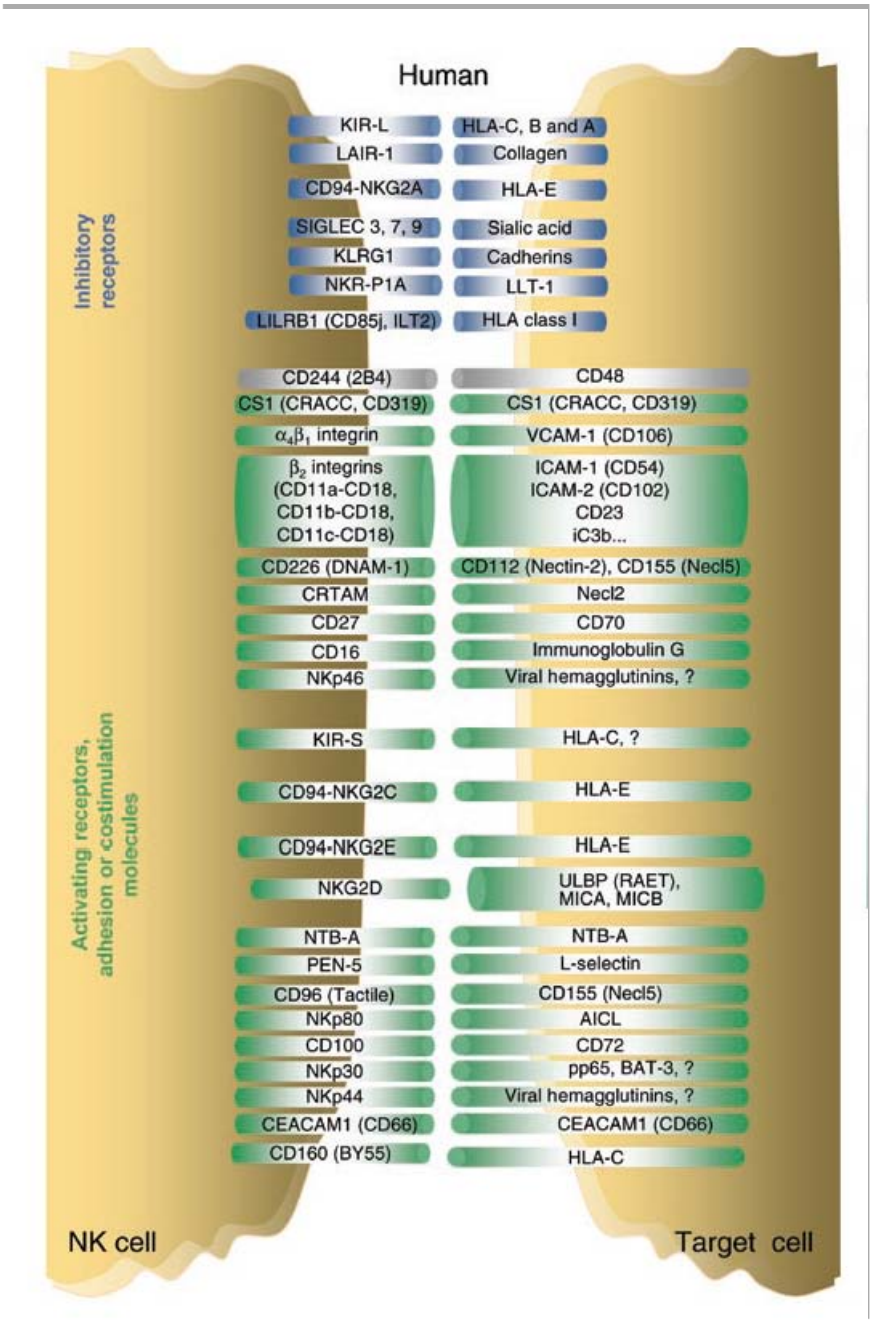


Figure 1.3 NK receptors and their ligands (derived from Vivier et al.[72])

1.7-i-C-type lectin receptors

The NKG2 family of NK receptors are type two glycoproteins encoded for by genes located in the NK complex on chromosome 12. The NKG2 genes (unlike, for example, KIR genes) have limited or no polymorphism and receptor function has been shown to be unaffected by the minor allelic variants.[73] NKG2-A, -B, -C, -E and -F form heterodimers with the CD94 molecule. They are either activatory or inhibitory depending on the molecules found in the cytoplasmic region of the NKG2 part of the complex.

The inhibitory receptor NKG2A has an immunoreceptor tyrosine-based inhibitory motif (ITIM) and inhibits NK killing when triggered.[74,75] Receptors containing an ITIM function via the phosphorylation of the tyrosine residue (probably by a Src family kinase), which results in recruitment of the lipid phosphatase SHIP (Src homology 2 domain-containing inositol-5-phosphatase) or the tyrosine phosphatases SHP-1 (SH2-containing protein-tyrosine phosphatase-1) or SHP-2.[76] These phosphatases, when recruited to the synapse between an NK and a target cell where activatory receptors are also located, inhibit NK responses by dephosphorylating the protein substrates of the tyrosine kinases linked to NK activatory receptors, thereby inhibiting calcium influx, degranulation, cytokine production and NK proliferation.[77] The ligand for NKG2A is HLA E.[78] NKG2A has also been shown to over-ride NK activatory signals by disrupting the formation of the normal actin cytoskeleton in the immune synapse of NK cells.[79]

In contrast to NKG2A-mediated inhibition, NKG2C, D and E lack ITIMs and transmit activating signals.[80] NKG2C has a 12kDa DNA activating protein (DAP-12) which contains an immunoreceptor tyrosine-based activating motif (ITAM).[81]

NKG2D does not form a dimer with CD94[82] and lacks an ITAM but forms an activating receptor complex with DAP-10.[83] This contains a binding site for the recruitment of the phosphatidylinositol 3-kinase (PI3K) through which activatory signal transduction occurs.[82,84] NKG2D recognises the cell surface glycoproteins MHC class-I-chain-related protein A (MICA), MICB and UL-16 binding proteins 1-4 (ULBP).[85,86] These molecules are structurally similar to MHC class 1 but do not have antigen presenting function. Expression of the ligands for NKG2D increases in 'stressed' cells[87] such as virally infected or tumour cells and modified expression of these ligands form part of the immune evasion technique employed by tumours (see sections 1.9 and 1.10 below).

1.7-ii-Natural Cytotoxicity Receptors (NKp30, NKp44, NKp46)

Natural cytotoxicity receptors (NCRs) are restricted to NK cells.[88] They are glycoprotein receptor members of the immunoglobulin superfamily whose names reflect their molecular size (30, 44 and 46 kDa respectively). The genes for the NCRs map either to the leukocyte receptor complex on chromosome 19 (NKp46) or to the TNF cluster of the MHC gene complex on chromosome 6 (NKp30 and p44). These activating receptors do not themselves contain ITAMs but couple to molecules such as CD3ζ, FcεRIγ and DAP12, which do.[89] This enables activatory signaling via phosphorylation of the ITAM tyrosine by Src family kinases and binding of the tyrosine kinases Syk and ZAP-70.[77] The ITAM activation results in actin cytoskeleton reorganization and NK granule release as well as transcription of cytokine and chemokine genes.

Despite the fact that the NCRs were discovered to be a separate group of NK activating receptors some years ago,[90-93] little is known about their ligands. There is evidence that viral haemagglutinins can trigger NCR-mediated activation[94-96] but their ability to activate killing of non-virally infected cells indicates cellular ligands which are still unknown. To date, only one tumour ligand has been found for NKp30, the stress-induced self molecule B7-H6.[97]

1.7-iii-SLAM family receptors

These include the immunoglobulin-related protein CD244, NTB-A and CRACC. They all contain Thr-x-Tyr-x-x-Leu/Ile motifs in their cytoplasmic tail and activation signals occur via SH2 containing adaptor proteins (Ma 07). The ligand for CD244 is CD48[98,99] which is highly expressed on EBV infected cells.

1.7-iv-CD16

CD16 was the first NK activating receptor to be identified.[100,101] It is a low affinity Fc receptor for IgG and mediates antibody dependent cellular cytotoxicity (ADCC). CD16 signals via its association with the ITAM- containing CD3ζ[102] and/or FcεRIγ[103] molecules.

1.7-v-Killer-immunoglobulin-like-receptors (KIRs)

One of the most important families of NK cell receptors is the KIRs. KIRs recognise class I HLA (A, B or C) ligands on normal cells. The names of KIR genes reflect the structure of the protein they encode. KIR proteins have either two or three immunoglobulin-like domains (therefore genes are named either KIR2D or KIR3D) as well as either short or long cytoplasmic tails (named KIR2DS or KIR2DL). The final part of the name numbers the genes within each structural subset (i.e.KIR2DS1, KIR2DS2 and KIR2DS3) (fig. 1.4). Long cytoplasmic tails contain two immune tyrosine-based inhibitory motifs (ITIM), which transduce inhibitory signals via SHP-1 to the NK cell. Short cytoplasmic tails contain a positively charged amino acid residue in the transmembrane region which allows them to associate with a DAP12 signalling molecule capable of generating an activation signal.[104] The activating KIR arose by gene duplication and conversion from inhibitory receptors[105,106] and therefore have very similar extracellular domains to their inhibitory counterparts, sometimes only differing in one amino acid. This fact makes finding monoclonal antibodies to distinguish between activating and inhibitory KIR extremely difficult, for most, only antibodies binding both are available.

The ligands for KIRs are class 1 HLA molecules. Each NK cell appears to have at least one MHC-specific inhibitory receptor, which recognises a ligand on every self cell, preventing killing of normal healthy cells.[107] Leukaemic and infected cells have been shown to down regulate these ligands, in an attempt to avoid T cell immunity, making them a particular target for NK killing.[108]

The 15 KIR genes and 2 pseudogenes are located on chromosome 19q13.4 (fig. 1.5). Certain 'framework' genes (3DL3, 3DP1, 2DL4 and 3DL2) are inherited by all individuals. Studies involving family segregation analysis have identified two KIR haplotypes. Haplotype A has a fixed combination of genes KIR2DS4, KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2, whereas haplotype B involves significant inter-individual variation and can include between one and five of the genes KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1.[109]

NK clones express particular KIRs randomly selected from available inherited genes so that an individual will have different subsets of circulating NK cells with specificity for different HLA ligands.[110] There is evidence that self tolerance is augmented by an 'education' process which occurs during NK development, during which NK cells require interaction with self MHC to become fully functional[111-113] as discussed below.

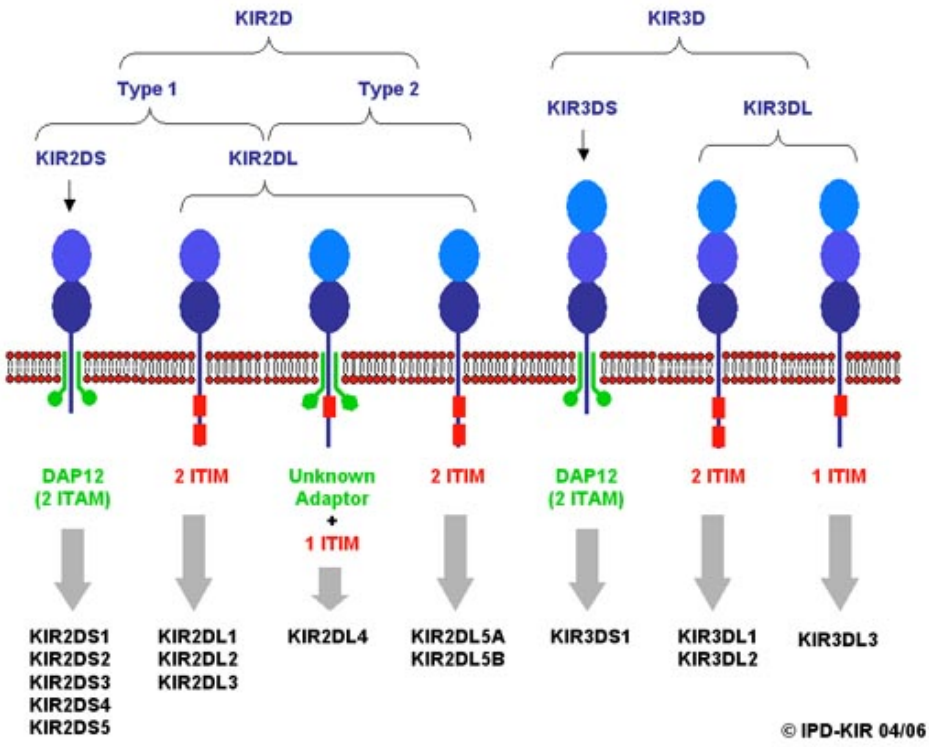


Figure 1.4 KIR protein structure from Immunopolymorphism Database (IPD) http://www.ebi.ac.uk/ipd

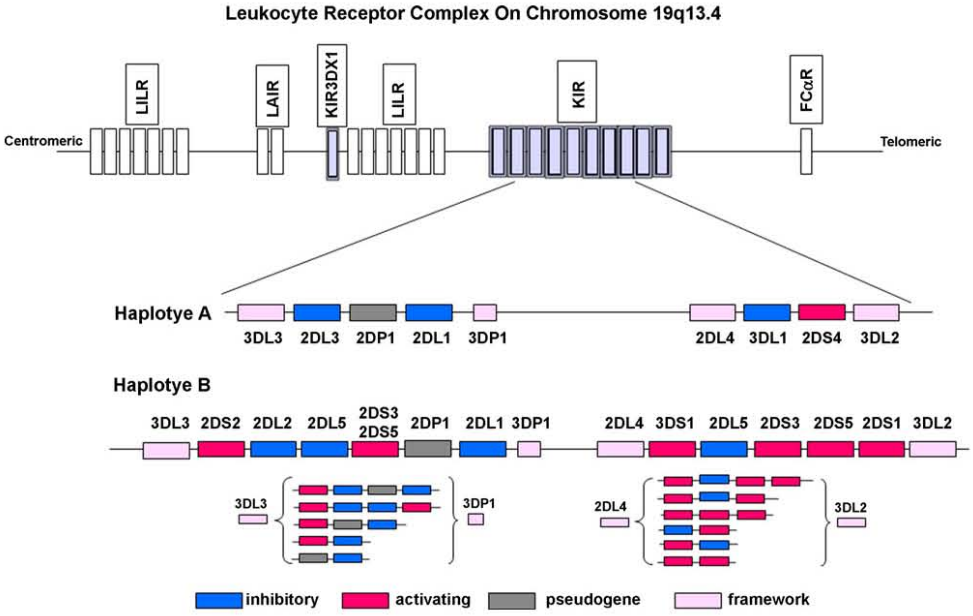


Figure 1.5 KIR genes in the LRC on chromosome 19 (derived from Kulkarni et al[114])

1.7-vi-The balance between NK activatory and inhibitory receptors

Importantly, the signaling for NK inhibitory receptors is localised and transient and if the NK cell subsequently encounters a new target without the ligands for inhibitory receptors, activation can occur.[77] Activatory receptors appear to work in combination, with signals often providing additive or synergistic effects[115] enabling a critical threshold of activation to occur in order to override any inhibitory signaling (which normally predominates in order to provide self tolerance).

1.8 NK development and education/tolerance

As with other lymphocyte subsets, NK cells are derived from CD34+ haematopoetic stem cells which are primarily found in the bone marrow.[116-118] However, in contrast to clear evidence for different stages of maturation of T cells in the thymus and of B cells in the bone marrow, the site and process of NK cell maturation is less clear. As well as bone marrow, studies isolating immature NK cells with high surface expression of CD56 from lymph nodes and tonsils have identified these sites as also likely to be important for NK maturation.[119-121] However, the details of the site and sequence of NK development are not, as yet, entirely clear.

1.8-i-CD56

Otherwise known as neural cell adhesion molecule (N-CAM) or Leu-19, CD56 is found on the surface of all NK cells although its precise function is unknown.[122] Some early studies indicated that it might be important in target cell recognition[123,124] although more recent work indicates that other receptors appear to be more important in this role (as detailed above). CD56 is, however a useful

marker in helping to categorise different subsets of NK cells. It is known that NK cells express surface CD56 in different densities and can be categorised as CD56 bright or dim. These two NK subsets differ in their localization[125] and in their functional ability.[126] CD56 bright cells encompass approximately 10% of peripheral blood NK, being mostly localised to the lymph nodes and tonsils. They do not express perforin[127] and are thought to be primarily cytokine secreting, producing IFNγ in response to stimulation with IL-12, IL-15 and IL-18.[125] In contrast, CD56 dim NK cells make up 90% of circulating NK cells and are cytotoxic, expressing perforin and producing IFNγ in response to tumour cells.[125] These features suggest that there is a gradient of maturity with high expression of CD56 denoting early NK cells which lose high expression of CD56 as they move into the peripheral blood and become better cytotoxic killers.[128-130] There is also evidence that the tonsils and lymph node tissues are the site for development of a CD56 bright population of NK cells from initial CD34+ stem cell NK precursors to mature CD56 bright NK.[121,131] Whether precursor NK cells migrate to the tonsils and lymph nodes from the bone marrow and peripheral blood for maturation or are produced here *de novo* is yet to be confirmed.

1.8-ii-KIR ligands and NK education/ tolerance

The role of major histocompatibility complex (MHC) molecules in NK function was first brought to light in a paper by Karre et al describing how NK cells kill cells that are lacking MHC molecules, in contrast to T cells which identify foreign proteins when they are bound to MHC.[132] This 'missing self' hypothesis, required all NK cells to possess an inhibitory receptor that could bind MHC class 1. As MHC are expressed on virtually all healthy cells, this prevents NK cells being activated under normal conditions.[108] These MHC class 1-recognising receptors were subsequently identified,[133,134] their genes encoded[135] and the human receptors labeled as KIRs.[136] That NK cells do not attack the few normal circulating cell types which express no or low levels of MHC (eg. red blood cells and neurons) was later explained by the fact that NK cells also require triggering of their activatory receptors to kill.[137-140] However, as discussed above, the exact ligand for many of these activatory receptors is still unknown. In a similar manner to the way in which T cells are educated, NK cells are also influenced by exposure of their activatory receptors to ubiquitously expressed viral or self ligands during development. Exposure downregulates receptor function, as well as partially deleting the autoreactive cells.[141-143] Interestingly, this 'tolerisation' process, when occurring via the NKG2D receptor has been shown to also induce reduction in function of other activatory receptors indicating 'cross tolerisation' between receptors.[144]

One area of NK biology which has had increasing attention in recent years, is the role of KIR ligands in NK education. These interesting studies, first in mice,[145,146] then in humans,[147] showed that KIR-expressing NK cells require exposure to the ligands of their inhibitory KIRs in order to become fully

functional, a process termed 'licensing'. Further studies looking at the activatory KIR 2DS1 (which binds HLA C2 as its ligand), have shown that exposure of an activatory KIR to its ligand during development also plays a role. KIR2DS1+ NK cells are hyporesponsive against targets in homozygous HLA C2 individuals,[148] indicating activatory KIRs also have educational value but in the opposite direction to that of inhibitory KIRs, towards maintaining tolerance to self rather than facilitating cytotoxicity.

The process of NK education is not uni-directional or restricted to the environment in which the NK cell develops. Studies transplanting normally functioning mature NK cells into MHC class 1-deficient hosts, show that they become anergic several days after transfer.[149] Conversely NK cells from MHC-deficient hosts (which were as expected, hyporesponsive, having never been 'licensed'), become responsive after transfer into MHC expressing hosts.[150] The exact location of this process is as yet unknown. The plasticity of NK maturity and the role of circulating 'unlicensed' NK cells, which are not destroyed as equivalent T cells are, may interestingly be of use in the response to viral infections. Murine work has shown recently that unlicensed NK cells are actually better effectors against murine CMV than licensed NK cells,[151] suggesting that licensing by inhibitory receptors, while important for tolerance and education, may inhibit response to viral infections.

1.9 Immune editing-the effect of leukaemia on the immune system

As introduced in section 1.2, tumour immune surveillance is likely to be important in leukaemia development: possessing an intact immune system to ensure that any emerging tumour cells are rapidly recognised and destroyed is an important part of remaining malignancy-free. It is perhaps expected therefore, that tumours might try to overcome this process and there is evidence to illustrate that tumour cells can induce changes to an otherwise healthy immune system, which facilitate their survival.

These include secretion of soluble factors by tumour cells which inhibit immune cells. For example, production of transforming growth factor beta (TGFβ) reduces NKG2D and NKp30 expression and consequently reduces killing of certain tumour cell lines.[152] AML cells secrete a soluble factor that inhibits T and NK cell proliferation but not cytolytic function.[153] AML can also alter the cytokine production of circulating lymphoid and myeloid cells, thereby changing normal immune interactions.[154]

Regulatory T cells are increased in AML.[12] There is evidence that AML cells may themselves be directly responsible for this via expression of the tyrosine converting enzyme indoleamine 2, 3-dioxygenase (IDO).[155] AML cells can also induce defects in the lymphocytes themselves: T cells from AML patients have abnormal phenotype and genotype and form defective immune synapses with AML blasts.[11] Defective thymic function in AML is suggested by the fact that these patients have reduced levels of recent thymic emigrants.[156] Furthermore, direct cellular contact between normal lymphocytes and AML blasts in mice results in reduced NK cytotoxicity and cytokine production via the down regulation of transcription factors T bet and eomesodermin, which, when forcibly expressed in these experiments reduces tumour size and improves survival.[157]

Using a different strategy, the malignant clone itself can generate dysfunctional immune cells which can then go on to alter immune function: for example, producing leukaemic dendritic cells which present antigen but are not normal[158] and inhibit induction of cytotoxic T cells inducing T cell anergy.[159-161] Leukaemia cells can also create changes in their microenvironment optimising their survival, for example, producing immunosuppressive mesenchymal stromal cells.[162]

1.10 Immune escape- changes on leukaemia induced by the immune system

Attempts to evade the immune system by altering phenotype, that is, mutating to facilitate survival, is a well-known strategy adopted by viruses and bacteria. There is ample evidence to suggest that tumour cells also have this capacity. Perhaps one of the more well-documented mechanisms for immune escape of leukaemia has been the down-regulation of surface ligands which normal immune cells recognise. These include AML blasts expressing reduced levels of HLA molecules both pre-treatment,[163] and after haplo-identical SCT when AML can down regulate the entire mismatched haplotype to avoid graft versus leukaemia from mismatched cytotoxic T lymphocytes.[23] Another example is the reduction in surface expression of MICA/B.[164] A number of mechanisms and consequences have been reported with regard to MICA/B expression, including alteration of DNA modifying enzymes such as HDAC leading to epigenetic regulation and repression of MICA/B expression in leukaemia,[165] as well as shedding of MICA/B which not only reduces ligands for NK cells to recognise but also, once shed, soluble MICA binds NKG2D on circulating NKs promoting NKG2D endocytosis and degradation and inhibiting further NK cytotoxicity.[166] As well as under-expressing ligands, leukaemia cells have been known to over express them, for example, in chronic myeloid leukaemia, the over expression of MICA/B induced by BCR/ ABL, causes sustained triggering of NKG2D and subsequent hypo-responsiveness with reduced cytotoxicity and IFNγ production.[167]

The important role of co stimulatory molecules in the immune response to leukaemia is suggested by evidence that a particular genotype of cytotoxic T-lymphocyte antigen 4 (CTLA-4), critical for maintaining T cell tolerance, is found with increased frequency in AML relapse after induction chemotherapy.[168] That AML can weakly express co stimulatory molecules and thereby favour escape from T cell-mediated killing, is suggested by evidence showing a higher probability of remaining in remission if blasts express both CD80 and CD86.[169] Altered expression of co-stimulatory molecules in patients relapsing with leukaemias post SCT was also associated with resistance to NK mediated lysis.[170] Furthermore, shedding of co stimulatory molecules may also allow leukaemia to block T cell attack via the binding of the soluble ligand to normal T cells.[171] A further mechanism for tumour evasion in leukaemia is to express molecules which directly inhibit immune function. For example, AML, in contrast to normal cells, can express the ligand for glucocorticoid-induced tumour necrosis factor-related protein (GITRL). Triggering through this ligand directly blocks NK function leading to impaired cytotoxicity and reduced IFNγ production.[172]

In the above sections 1.9 and 1.10 I have described immune editing in leukaemia, whereby malignant cells attempt to alter the immune system to maintain their survival, and also select or mutate to avoid immune-mediated attack. This provides a framework for my PhD, which focuses on the ways in which these processes occur in the natural killer cell response to leukaemia.

1.11 Structure of PhD project

This thesis is laid out around three large studies. The first addresses the role of NK cell receptor genes in a large (n=261) retrospective observational study of matched sibling allogeneic SCT patients who underwent treatment at the National Institutes of Health, Bethesda, USA. This explores the role of KIR genetics in predicting outcome (especially leukaemic relapse) after SCT for leukaemia, specifically identifying a special role of NK cells in control of AML (chapter 3). Having identified NK genetic characteristics that were favourable for sustained AML remission after SCT, I carried out an analysis of KIR genotypes to address the question of whether KIR genetics could determine the chance of developing AML and whether KIR genetics influenced outcome after chemotherapy treatment. This involved a large (n=499) retrospective observational study in AML patients recruited to the MRC/NCRI AML 10 and 15 trials receiving chemotherapy induction treatment. The results are presented in chapter 4. To better understand mechanisms of immune editing and leukaemia escape I then carried out a prospective analysis of the role of NK cell phenotype and function in 32 AML patients presenting at the Hammersmith Hospital between September 2009 and December 2011. These results are presented in chapters 5 and 6.

Chapter 2 Materials and Methods

2.1 Sample collection

2.1.i- KIR genotype analysis in the setting of allogeneic stem cell transplantation

These studies were performed in the Department of Stem Cell allo-transplantation at the National Institutes of Health (NIH), Bethesda, USA. All patients and donors had peripheral blood mononuclear cells (PBMC) collected and stored on institutionally reviewed and approved protocols for the purposes of research at the time of presentation. However, of the 261 patient-donor pairs transplanted and included in my project, 22 donors did not have cell samples available due to the usage of these samples in previous research projects. For completeness, I therefore contacted these donors to ask for blood or buccal swabs. This involved telephone calls (three-way with translators for Spanish-speaking patients) and letters. Of the 22, contact details were still valid for 11 and of these, 7 donors agreed to send us buccal swabs of their saliva. DNA was extracted from buccal swabs by the HLA laboratory using the Gentra Puregene buccal cell core kit (Qiagen, Gaithersburg, MD, USA). Being siblings, the patient and donor populations were ethnically matched, with the majority being of either Hispanic or Caucasian origin.

2.1.ii- KIR genotype analysis in the setting of chemotherapy for AML

All patients consented onto the AML MRC/NCRI trials have had blood samples collected by the patient's clinical team at the time of treatment and sent to University College Hospital (UCH) for molecular diagnostics. Professor David Linch and Dr Rosemary Gale have set up a Human Tissue Authority approved bio-bank of all DNA extracted from AML patients on the MRC trials since 1987. In collaboration with Professor Linch, we applied to the MRC/NCRI for ethical approval, which was granted. All patient samples were linked to comprehensive outcome data. The control population was the normal donor population described in section 2.1.ii. This had a higher proportion of Hispanics than the UK MRC patient population but as their KIR gene frequency did not differ from normal blood donor populations collected in the UK (http://www.allelefrequencies.net/kir6002a.asp.), this was deemed acceptable.

2.1.iii- Functional studies of NK cells in AML patients and normal controls

For my prospective study of AML patients in West London, I received approval from the local ethics board to collect blood and marrow from newly diagnosed AML patients at Imperial College Hospitals (NREC reference 10/H0711/16). I consented patients and controls according to local protocol (see 8.1 and 8.2 in appendix for consent form and patient information sheet) and the samples were collected either by the clinical staff looking after the patient or myself. Samples were given an anonymous code as per local guidelines and material linking that code to patient identity

and clinical outcome data was kept in a locked drawer to which only myself had access, within the secure haematology research department to which access was also restricted.

2.2 Processing, freezing and thawing PBMC

Separating of PBMC from whole blood: Fresh whole blood was collected in EDTA and mixed 1:1 with RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA). Lymphocyte separation medium (Ficoll-Hypaque, Organon-Teknika, Durham, NC, USA) was layered under the blood sample and the tube spun at 1800rpm for 30 minutes. The cell layer was then carefully removed with a sterile transfer pipette and washed in RPMI.

Freezing: PBMC were re-suspended in 20% foetal calf serum (FCS) in RPMI complete media (RPMI CM containing gentamicin, glutamine and hepes) and mixed on ice with an equal volume of freezing media (20% Dimethyl Sulfoxide (DMSO) in 20% FCS/RPMI CM) before being immediately transferred to a -80ºC freezer and then to a liquid nitrogen freezer (-196 ºC) within 7 days.

Thawing: Cell vials were quick-thawed in a 37ºC water-bath and washed in 10% FCS in RPMI before resting for 1-12 hours at 37ºC prior to experiments.

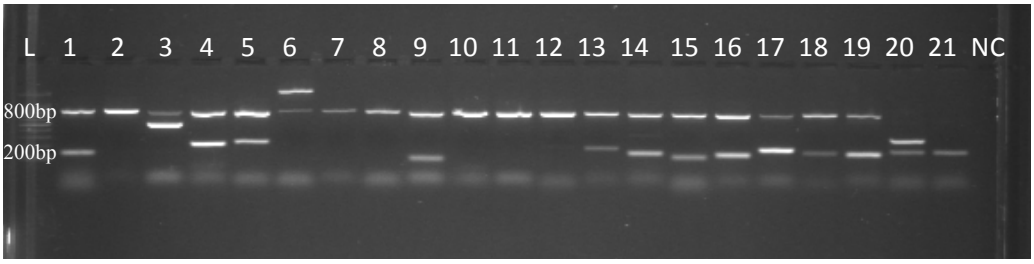
2.3 DNA extraction

Genomic DNA was extracted from frozen cell samples using the Qiagen column-based method (Qiamp midi kit, Qiagen, Gaithersburg, MD, USA). This involved lysing cells and then loading the lysate onto a spin column containing a silica membrane to which the DNA binds. Impurities were then washed away in two centrifugation steps before pure DNA was eluted from the membrane. Briefly, thawed PBMC were mixed with 100µl protease and 1.2ml lyzing buffer then the sample was vortexed vigorously for 1 min. Lyzed cells were then incubated for 10 min in a water-bath at 70ºC. To optimise DNA binding to the silica membrane, 1ml ethanol was added, the sample was mixed and then vigorously vortexed for 1minute before being passed through the Qiamp midi column. The column was then spun at 1850g (3000rpm) for 3 minutes, washed with 2ml AW1 buffer, spun, and washed again with 2ml AW2 buffer. DNA was then eluted from the column into a new tube by adding 150µl low-salt buffer, incubating at room temperature for 5 minutes and then spinning down at 4500g for 2 mins. DNA concentration and purity were measured using a nanodrop machine. The optimal cell number to use for this method was 5 $x10^6$ cells (see appendix, supplementary table 1) from which DNA purity was typically >90% with a concentration of between 50-200ng/µl.

2.4 KIR genotyping

KIR genotyping was performed using the Invitrogen KIR Genotyping SSP kit (Invitrogen, CA, cat. 78930-30). This identifies the presence or absence of each of the 15 known KIR genes and 2 pseudo genes as well as the common variants of 2DL5, 2DS4, and 3DP1 (fig.2.1). Briefly, 0.92µg DNA was added to the kits buffer mixed with 1.4µl taq polymerase (Invitrogen, CA,USA) and purified water. 8µl of this solution was added to each well, which contained a different set of sequence specific primers for each KIR gene. Plates were loaded onto the thermocycler (Gene amp PCR system 9700) and polymerase chain reaction was performed. This involved initialising for 1 minute at 95°C then 30 cycles of denaturation at 94°C for 20 seconds, annealing at 63°C for 20 seconds and elongation at 72°C for 90 seconds before the sample was cooled to 4°C. Each KIR gene was identified by the presence or absence of a band on 1.5% agarose electrophoresis gel stained with ethidium bromide (in USA) or SYBR safe DNA gel stain (in London) (Invitrogen, CA,USA).

Patient HLA typing for KIR ligands was performed by the HLA laboratory, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, USA (for the NIH cohort) and the HLA laboratory at the Hammersmith Hospital, London (for the UK patients). Typing was carried out for all known KIR HLA ligands: C1 (Cw*01,03,07,08,12,13,14), C2 (Cw*02,04,05,06,15,1602, 17,18), HLA G, HLA Bw4 (B*51,52,53,57,58, 08,13,27,37,44 and A*23,24,32), A*03 and A*11.



Typing Result +/-	Allele Specificity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
	2DL1*001/002/00301-00303/0040101/0040102/005/006	1																				
	2DL2*001-005		2																			
	2DL3*001-006			3																		
	2DL4*00101/00102/0010301/0010302/00104/00105/00201/00202/003-005/00601/00602/007/0080101-0080103/0080201/0080202/009-011				4																	
	2DL5A*001/005					5	6															
	2DL5B*002/004/007					5		7	8													
	2DL5B*003/006					5			8													
	2DS1*001-004									9												
	2DS2*00101/00102/00103/002-005										10											
	2DS3*00101-00103/002											11										
	2DS4*0010101-0010103/00102/002												12									
	2DS4*003/004/006/007													13								
	2DS5*001-005														14							
	3DL1*00101/00102/002/00401/00402/00501/006-009/01501/01502/016-020															15						
	3DL2*001-008/00901/00902/010-016																16					
	3DL3*001/00201-00205/003/00401/00402/005/00601/007/00801/00802/009/010/01101/012/01301/01401																	17				
	3DS1*010-01301/01302/014																		18			
	2DP1*00101/00102																			19		
	3DP1*001/002/004 (975bp)																				20	21
	3DP1*00301/00302 (344bp)																				20	
Product Size (bp)		145	145	510	230	257	1753	1883	1772	100	207	162	215	200	160	129	150	203	170	171	344/975	975
Lane Number		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Failed Controls																						
False Positive																						
False Negative																						

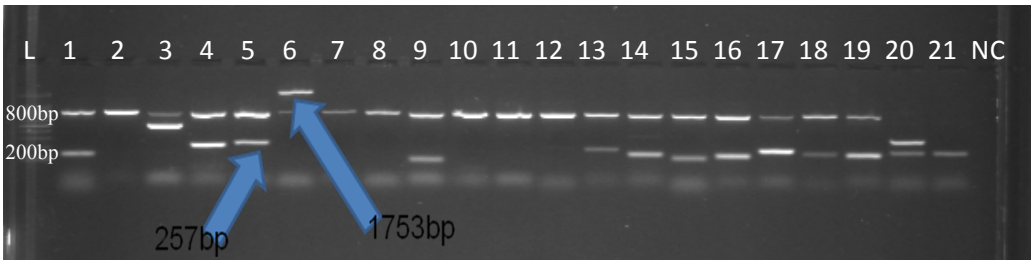


Figure 2.1 Example of gel and worksheet for KIR genotyping one individual. The ladder (L) is positioned far left. There is a positive control at 800bp in lanes 1-19 and at 200bp in lanes 20-21. Each lane corresponds to primers for a particular KIR gene or allele. The work sheet shows the molecular weight (5th row from bottom) of each KIR gene or allele (detailed in column 2) and the gel lane where it is expected to be (black squares). If present, the KIR gene shows up as a distinct band of the appropriate molecular weight along with the positive control (see lane 1). If the KIR gene is absent, only the control band will show in that lane (see lane 2). Some genes require two bands. Eg. Identification of KIR 2DL5A (see blue arrows) requires a product of 257bp in lane 5 and 1753bp in lane 6.

2.5 Growing cell lines to use as NK targets and controls

K562 is an erythroleukaemia cell line obtained from a chronic myeloid leukaemia patient in blast crisis. It is particularly useful as a target for NK cells as it does not express MHC class I molecules and therefore has no ligand for inhibitory KIR. It contains the translocation (9;22).

Jurkat cells come from an immortalised T lymphocyte cell line, originally from a patient with T cell leukaemia. Unlike K562, they express Fas and can therefore be used as a control for cytotoxicity experiments involving Fas ligand.

Hela cells are an adherent ovarian cancer cell line which consistently expresses the NK ligands DR4/5, HLA class 1 and MICA/MICB. It was used as a positive control for immunophenotyping.

All cell lines were kindly donated from the laboratories of Dr A. Karadimitris and Dr Junia Melo. K562 and Jurkat cells were cultured in RPMI 1640 media with 10% FCS in culture flasks, and incubated at 37 ºC, 5% CO2. Hela cells were cultured in DMEM media (Gibco, UK) and harvested from the flask using 0.05% trypsin (Invitrogen, CA). Media was supplemented with penicillin and streptomycin and changed every 2-5 days adjusting cell concentration according to growth.

2.6 Phenotyping NK cell surface receptors and their ligands on AML blasts

2.6-i-Antibody staining method

Fluorochrome-labelled, monoclonal antibodies were used to identify the expression of activating and inhibitory receptors on the surface of NK cells (table 2.1). Briefly, PBMC were washed with phosphate buffered saline (PBS) and stained with antibodies as detailed in the panels below. Stained cells were incubated for 20 minutes in the dark at room temperature before being washed again. The pellet was re-suspended in PBS and cells analyzed by 4 colour Facs Caliber Flow cytometry (BD, UK). Where cells were available, experiments were performed in triplicate to ensure reproducibility. As patient sample collection began before experiments were optimised, all our results were taken from frozen samples to ensure we were comparing like with like. Even so, as a precaution, a comparison between fresh and frozen cells for all experiments was performed to ensure that the freeze-thaw process did not significantly distort results (an example is shown in fig. 2.2).

2.6-ii-Rationale for NK surface receptor antibody panel

APC conjugated CD56 and PerCP conjugated CD3 (BD Biosciences, UK) were used to identify the NK cell (CD56+, CD3-) subset. A cocktail containing FITC conjugated CD33, CD34 and CD13 (Biolegend, Cambridge Biosciences, UK) was used in every tube to efficiently separate the AML blast population from the NK population (fig.2.2). This step was necessary as up to 40% of AML

blasts express CD56[173] and therefore these cells would contaminate an 'NK' population gated as CD56+ CD3- , if they were not separately gated out. This cocktail also enabled me to accurately identify the AML blast population to phenotype this as detailed below. Optimal concentrations of antibodies were decided upon using titration experiments (fig 2.3). Gating for the receptor positive population was set using isotype controls (fig.2.4). Where positive populations were not distinct as was the case with the NCRs, mean fluorescent intensity was used.[174] Analysis was performed using Flo-jo software (© Tree Star, Inc, 1997-2012).

Table 2.1 Antibody panel for characterising NK surface receptor phenotype

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9	Tube 10
FITC	CD33, CD34, CD13									
PE	NKp44	NKp46	NKp30	NKG2D	NKG2A	KIR3DL1	KIR2DL1/S1	KIR2DL2/S2	IgG1 isotype	IgG2a isotype
PERCP	CD3									
APC	CD56									

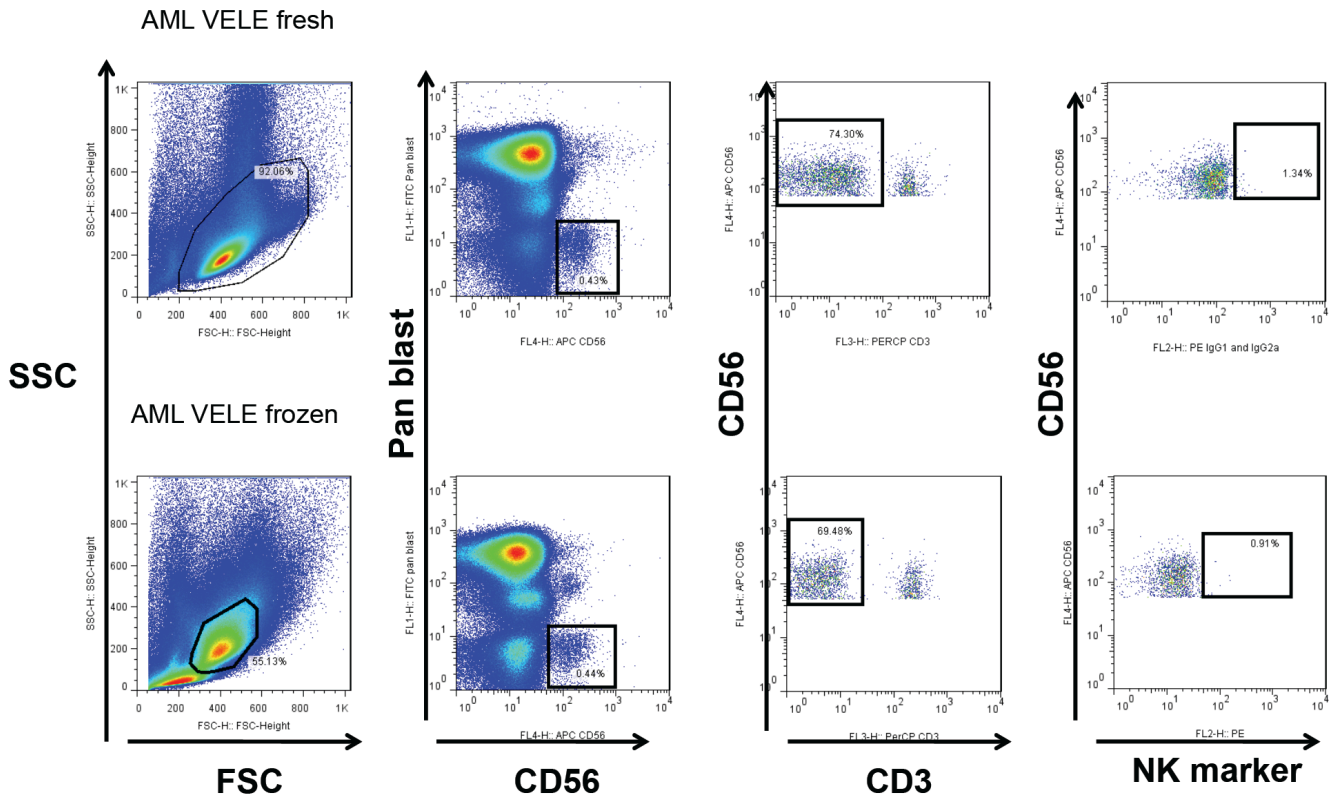


Figure 2.2 Example of FACS plots showing the gating strategy for NK surface receptor phenotyping. Cells were first gated on live lymphocytes using forward and side scatter (first column), then CD56+ cells which were negative for blast markers CD13, 33 and 34 were identified (second column). Next NK cells were separated from CD56+ T cells by gating on CD3-, CD56+ cells (third column) and finally the NK cells were assessed for their expression of the NK surface marker of interest (forth column). This figure also demonstrates a comparison of fresh (top row) and frozen (bottom row) cells from the same patient.

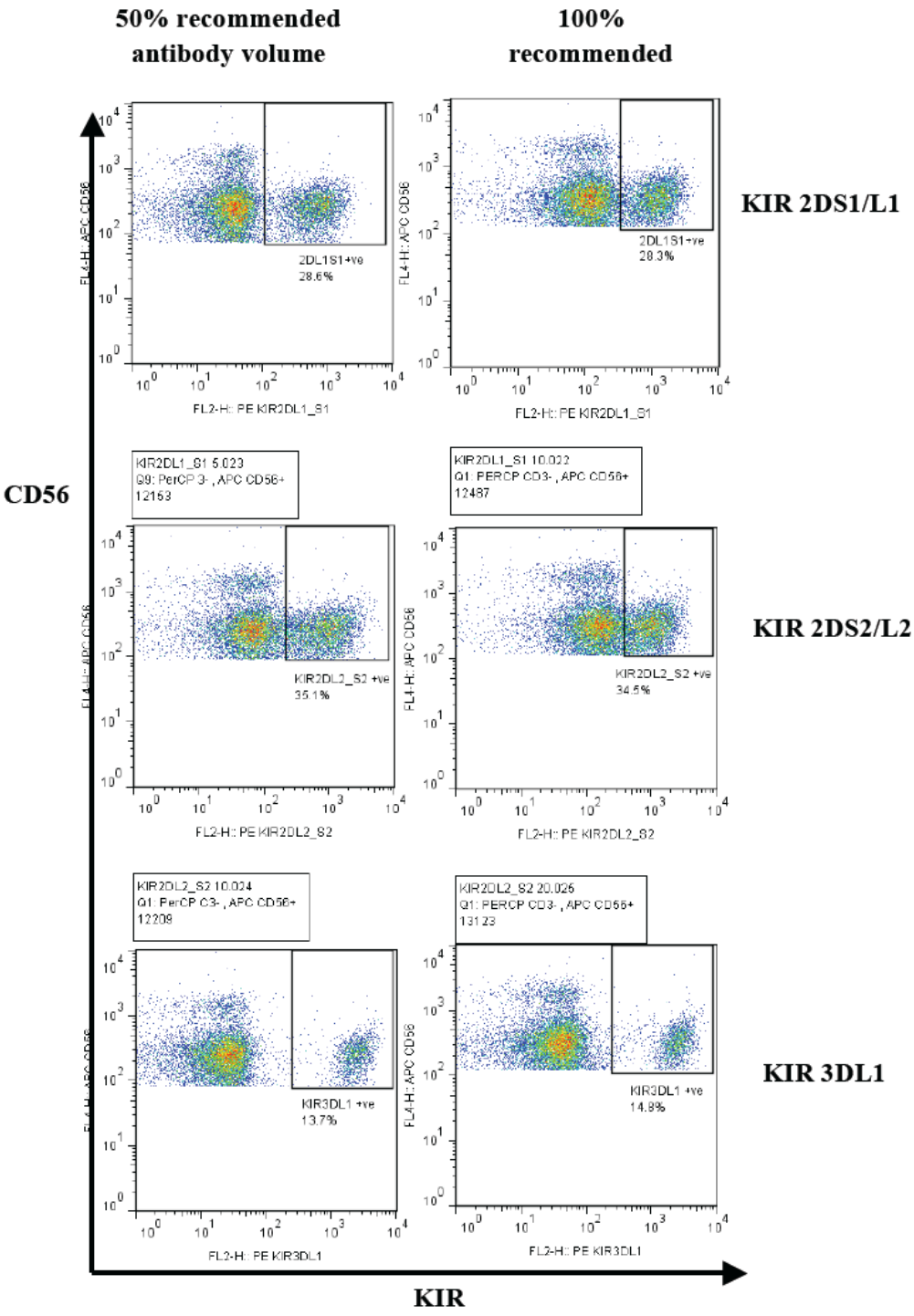


Figure 2.3 Example of titration experiment to find optimal antibody staining for KIR antibodies. Serially diluted volumes of antibody were used and the lowest concentration not resulting in loss of signal was used in experiments. These plots show no loss of signal when using half the volume recommended by the antibody company. Further dilutions would then be performed.

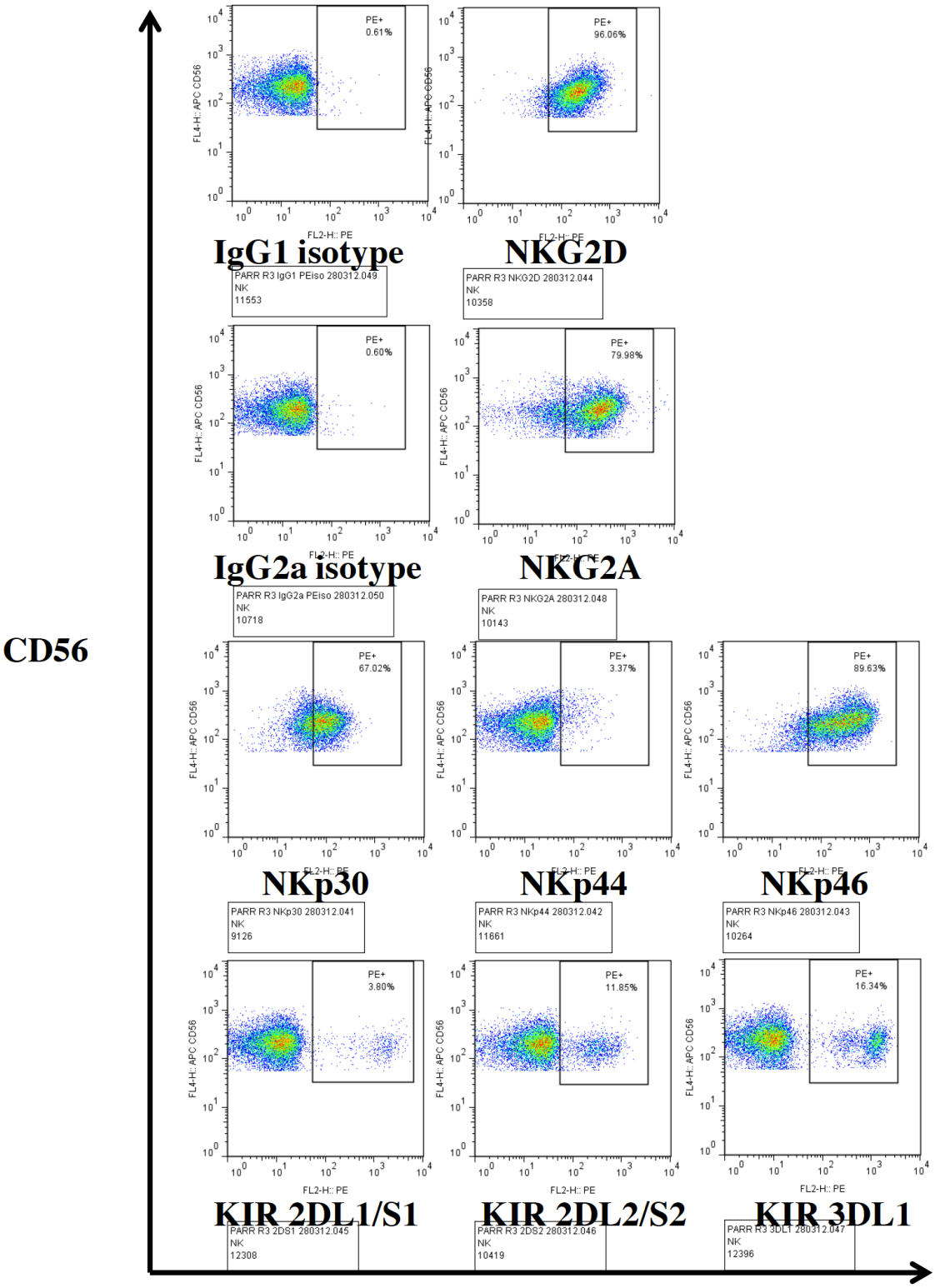


Figure 2.4 Example of FACS plots showing % expression of NK surface markers (on x axis): c-type lectins (top two rows), natural cytotoxicity receptors (third row) and KIR (bottom row). The NK marker CD56 is on the y axis. Positively staining cells were identified using PE conjugated isotypes to identify the border with the negative population (two plots top left). NKG2A was an IgG2A antibody, the rest were IgG1 antibodies.

2.6-iii-Rationale for antibody panel characterising the expression of NK receptor ligands on the surface of AML blasts

This panel (table 2.2) was used to study the expression of some of the relevant NK receptor ligands including Class 1 HLA (ligands for KIRs), MICA and B (ligands for NKG2D) and HLA E (ligand for NKG2A). I also studied the target ligands for the two death receptor pathways used by NK cells; namely Fas for the Fas-Fas ligand pathway and death receptors 4 and 5 (DR4/5), the ligands for TRAIL. The ovarian cancer cell line Hela was used as a positive control for DR4/5, MICA/B and HLA expression, and normal human PBMC was used as a positive control for HLA E and Fas expression (fig. 2.6).

Table 2.2 Antibody panel for characterising the expression of NK receptor ligands on the surface of AML blasts

FITC	CD33 CD34 CD13	Positive control	Isotype
PE	DR4/5	Hela cells	IgG1
PerCP	HLA-A,B,C	Hela cells	IgG2a
APC	MICA/B	Hela cells	IgG2a

FITC	CD33 CD34 CD13	Positive control	Isotype
PE	HLA E	Normal PBMC	IgG1
Alexa Fluor647	Fas	Normal PBMC	IgG1

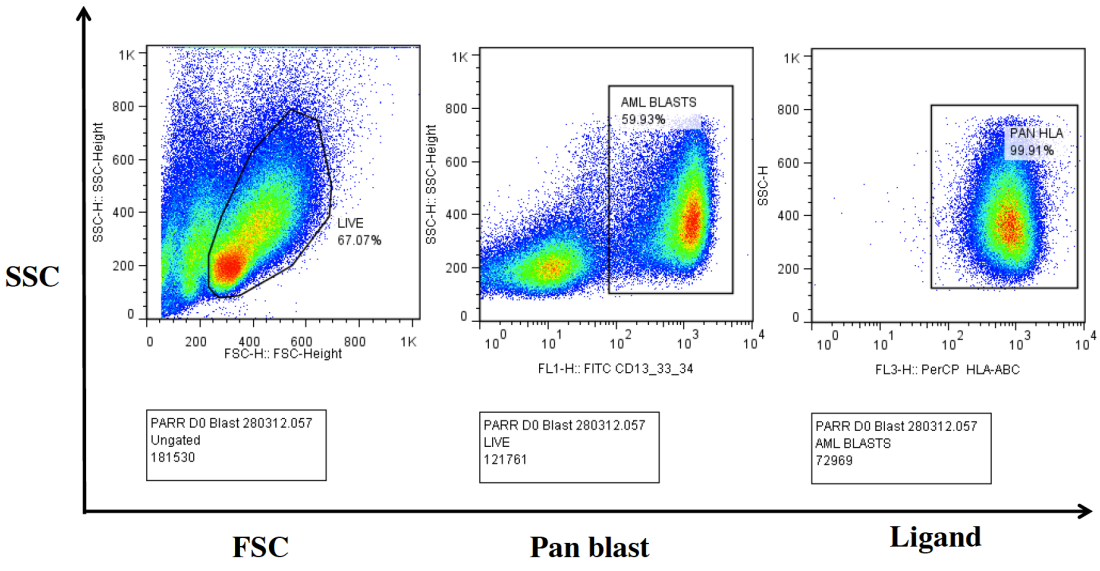


Figure 2.5 Example FACS plots showing the gating strategy for blast surface phenotype. Forward and side scatter was used to identify live cells, then blasts were identified using CD13, 33 and 34. The blasts were then assessed for surface expression of the NK ligand of interest (here HLA class I).

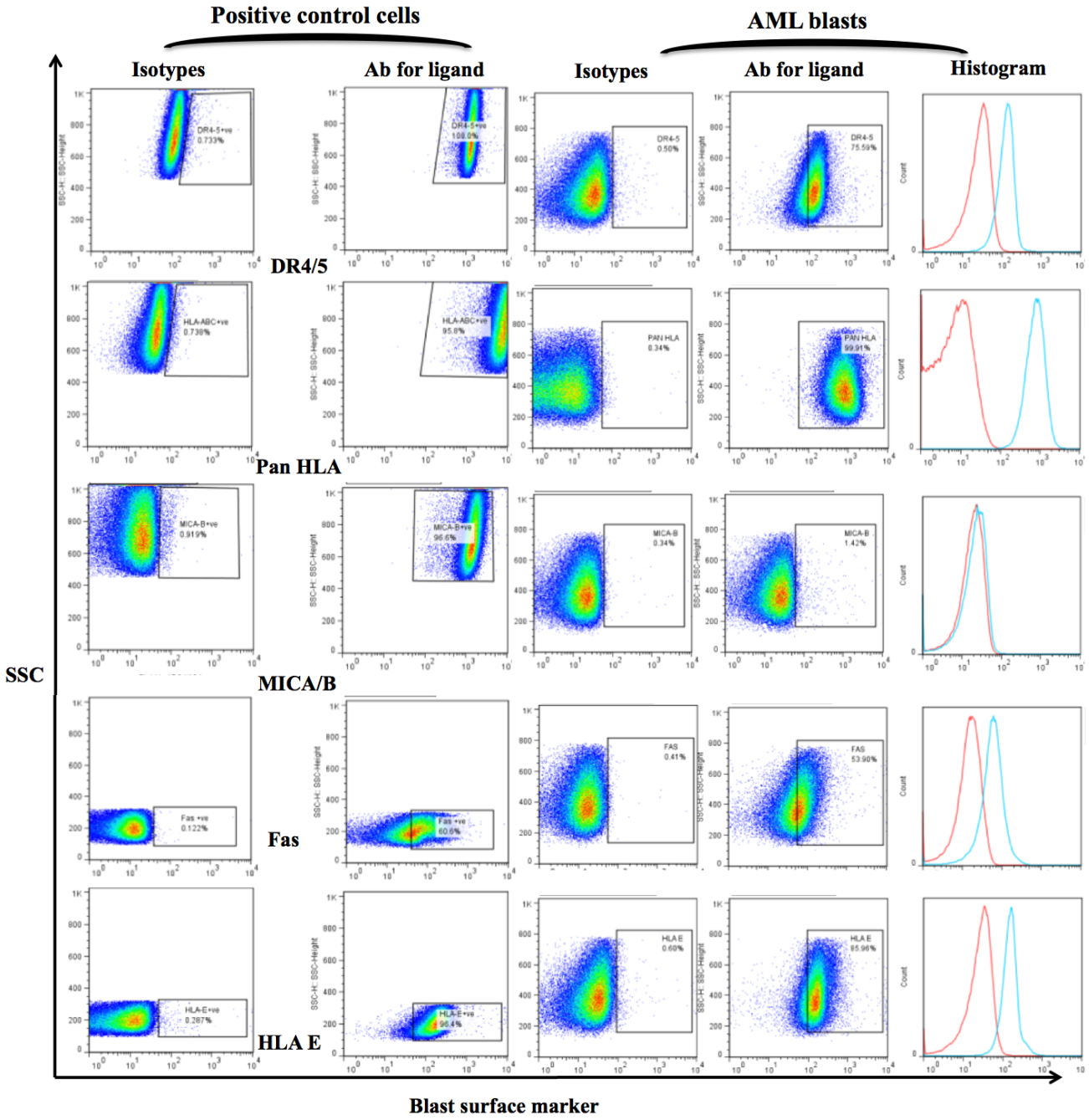


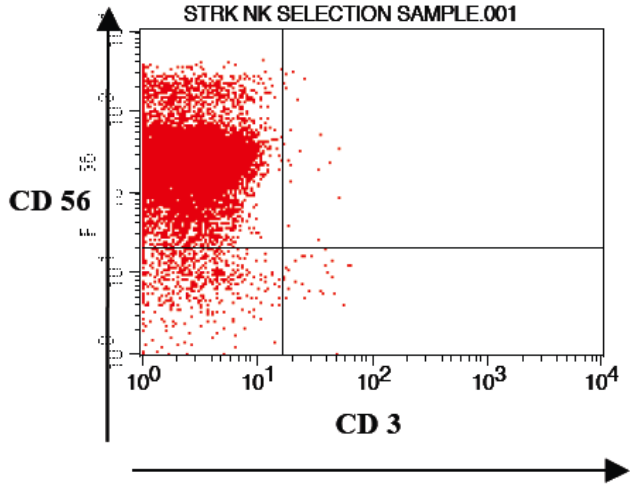
Figure 2.6 Example of FACS plots and histograms from one experiment showing expression of NK receptor ligands on the surface of a patient's AML blasts. Isotype and antibody staining of positive control cells (Hela or PBMC depending on ligand) was performed with each sample and are shown in the two columns to the left of the figure. The three columns to the right represent antibody staining of the patient's cells. Isotype controls were used to identify the negative population (middle column and red line on the histogram). The blue line on the histogram represents antibody staining of patient cells. This patient's blasts stained positive for the NK ligands DR4/5, HLA class 1, Fas and HLA E but not MICA/B.

2.7. Purification of NK cells- Miltenyi bead column negative selection method

This method selected NK cells from a mixed cell suspension using a cocktail of antibodies to identify non-NK cells (CD3, CD8, CD14 and CD4) and labeled these antibody-bound cells with metallic beads (NK cell isolation kit, Miltenyi Biotec GmbH, Germany). The cell suspension was then passed

through a magnetic column, which retained the antibody-bound cells and enabled the non-bound, NK cells to pass through into a tube at the bottom.

Briefly, after thawing and performing a cell count, PBMC were re-suspended in 4 µl buffer /10^6 cells. NK Cell Biotin-Antibody Cocktail was then mixed with cells at a dose of 1µl /10^6 cells, which were then incubated in the fridge for 10 minutes. Cells were then washed in 3µl buffer /10^6 cells and then 2 µl of NK Cell Micro-bead Cocktail was added/10^6 cells. Cells were washed in 200 µl buffer /10^6 cells before being spun and re-suspended in buffer to be passed through the negative selection column. CD56 +ve, CD3 –ve NK cells were collected in a falcon tube, washed, spun and counted for use in experiments. Typically, this technique yielded a purity of >95% NK cells (fig.2.7).



Quadrant Statistics

File: STRK NK SELECTION SAMPLE.001 | Log Data Units: Linear Values
Sample ID: STRK NK SELECTION SAMPLE | Patient ID:
Tube: Untitled | Panel: Untitled Acquisition Tube Lis
Acquisition Date: 29-Jan-10 | Gate: G1
Gated Events: 33405 | Total Events: 40965
X Parameter: PerCP-CD3 (Log) | Y Parameter: PE-CD56 (Log)
Quad Location: 17, 21

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	32224	96.46	78.66	2.76	2.23	374.04	259.23
UR	39	0.12	0.10	35.37	30.44	642.67	293.46
LL	1094	3.27	2.67	3.41	2.71	9.83	8.15
LR	48	0.14	0.12	33.76	31.21	8.30	6.69

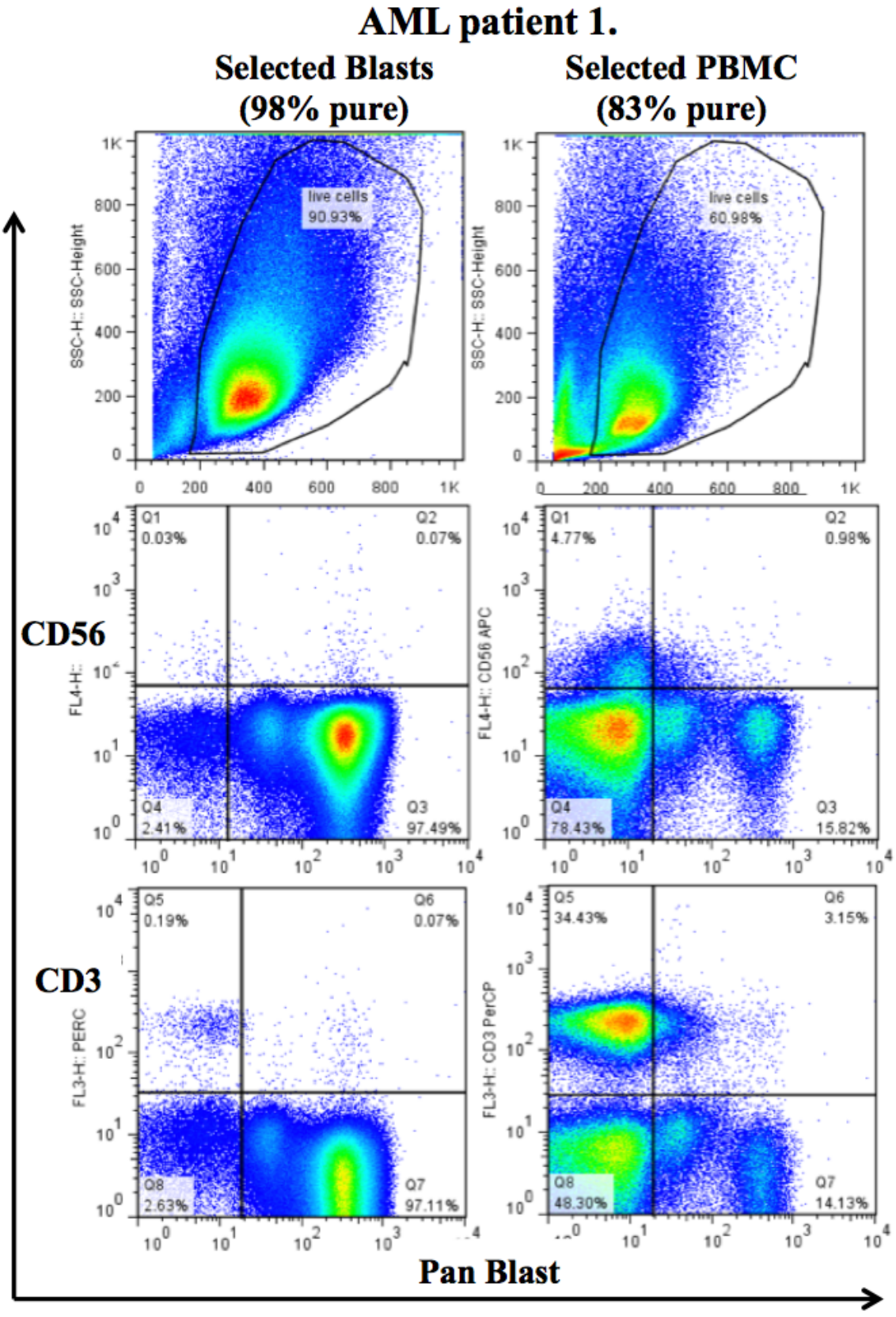
Figure 2.7 Example of a FACS plot of the NK negative selection procedure from healthy donor PBMC using Miltenyi beads. The technique typically yields >95% NK cell purity.

2.8 Purification of PBMC and blasts from AML samples at diagnosis- Miltenyi columns

For the cytotoxicity experiments, PBMC from AML patients were co-incubated with leukaemia target cells. In order to use primary autologous AML blasts as targets for NK cytotoxicity, blasts were positively selected from PBMC collected from AML patients at presentation. The yield and purity using the Miltenyi selection column method described above (section 2.7) was suboptimal and the technique was not consistent enough to yield sufficient number of cells for the experiments. In order to overcome these problems, I investigated a second method, using a custom made antibody cocktail optimised and published by John Gribben's group at St. Bartholemew's hospital in London.[175]

2.9 Stemsep custom antibody bead selection cocktail and selection on Robosep machine

This method allowed for positive selection of AML blasts using a cocktail of antibodies, leaving a population of PBMC unbound to beads or antibody. I modified the published cocktail, (which was initially used in experiments to select T cells) by omitting the CD11c marker, which, while found on a proportion of AML blasts is also found on normal NK cells. The cocktail included CD33, CD34, CD123 and CD36 (StemSep, Grenoble, France). The majority of AML blasts will express at least one and usually 2 or more of these markers. This cocktail also targets some monocytes and dendritic cells, making our PBMC population depleted of these cells too. In order to minimise the problems of high blast counts blocking the selection columns, I used a Robosep cell separator machine (STEMCELL technologies Inc, Grenoble, France), which has the benefit of a constant cool temperature and faster selection time, facilitating a greater yield of pure cell populations. This method consistently yielded populations of PBMC and blasts with 85- 90% purity (fig.2.8).


AML patient 1.
Selected Blasts
(98% pure)
Selected PBMC
(83% pure)
live cells
90.93%
live cells
60.98%
SSC-H:: SSC-Height
CD56
FL4-H::
FL4-H:: CD56 APC
Q1
0.03%
Q2
0.07%
Q4
2.41%
Q3
97.49%
Q1
4.77%
Q2
0.98%
Q4
78.43%
Q3
15.82%
CD3
FL3-H:: PERC
FL3-H:: CD3 PerCP
Q5
0.19%
Q6
0.07%
Q8
2.63%
Q7
97.11%
Q5
34.43%
Q6
3.15%
Q8
48.30%
Q7
14.13%
Pan Blast

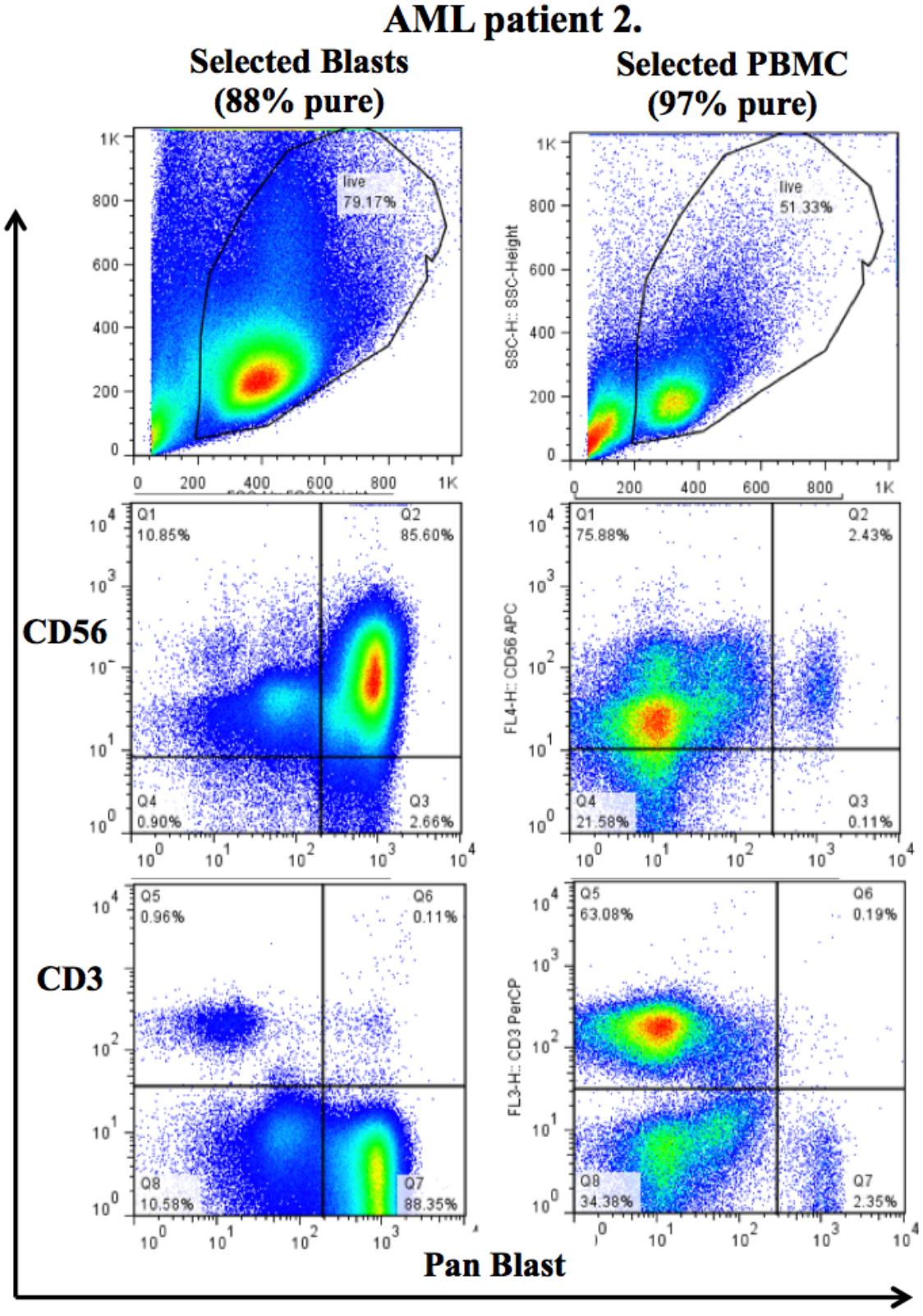


Figure 2.8 Example of FACS plots showing results of a cell separation experiment using the Robosep cell separator machine. Cells collected at AML presentation were separated into blast and PBMC populations. The blast populations (left columns) were mostly positive for pan blast markers (CD13, 33, 34) with minimal contamination from NK (CD56+) and T (CD3+) lymphocytes. The PBMC populations were relatively free from AML blasts. Purity results are in brackets. AML patient 2 is an example of a patient whose AML blasts express the NK marker CD56.

2.10. NK cell cytotoxicity experiments

PBMC were incubated with leukaemia target cells for 5 hours. NK-mediated cytotoxicity was then assessed by measuring the activity of the perforin/granzyme pathway (CD107a expression), cytokine production (intracellular IFNγ and TNFα production) and death receptor pathways (surface expression of Fas Ligand). I have optimised these methods as described below and in figures 2.9 to 2.12.

Briefly, PBMC were incubated on round bottomed 96 well plates at 37ºC, 5% CO_2 in RPMI media supplemented with 20% foetal calf serum (final volume 200µl/well) with target cells at effector: target ratios of 1:1. Targets include K562 erythroleukaemia cells and autologous blasts where available. Effector cells were incubated without targets as the negative control and stimulated with PMA and ionomycin as the positive control.

Prior to incubation, 5µl FITC conjugated CD107a monoclonal antibody (BD, UK) was added to wells to ensure that cell surface, as well as internalised CD107a were captured. Five µl of Monensin (BD GolgiStop^{TM} Cat#554724, diluted 1:10 in PBS) was also added to ensure the vesicle PH remains suitable for the fluorochome and to prevent cytokine leakage. Ten µl BFA (Brefeldin A, Sigma Cat#B-7651, stock solution 5mg in 1mL DMSO, diluted 1:10 in PBS) was added to wells to prevent release of the intracellular cytokines prior to detection by the assay. After incubation, cells were harvested from plates and washed in PBS before surface staining with antibodies for CD3, CD56 and KIRs.

After surface staining, the intracellular cytokine assay for NK production of IFNγ and TNFα was performed. Briefly, cells were mixed with 2ml lysing solution (BD FACS lysing solution Cat# 349202 diluted 1:10 in dH20) and incubated in the dark for 10 minutes. Cells were then washed in 2ml PBS before 500µl permealising solution (BD Cat# 340973 diluted 1:10 in dH20) was added and cells were again incubated for 10 minutes in the dark. Cells were then washed in PBS and stained with 2.5µl IFNγ and TNFα monoclonal antibodies (BD Biosciences, UK) before being incubated for 30 minutes in the dark. Finally, cells were washed again and FACS analysis was performed on a BD LSRFortessa cell analyzer to assess the percentage of NK cells exhibiting cytotoxicity as evidenced by expression of CD107a, IFNγ, TNFα or FasL. The rationale for choosing these is detailed below.

Table 2.3 Panel for NK cytotoxicity experiments

Laser Fluorochrom e	**Tube 1 1:1 PBMC K562**	**Tube 2 1:1 PBMC K562**	**Tube 3 1:1 PBMC K562**	**Tube 4 1:1 PBMC K562**	**Tube 5 1:1 PBMC K562**	**Tube 6 Neg C No K562**	**Tube 7 Pos C PMA/Io**
488 -530 -30 FITC BD Cat# 555800	107a 5µl	107a 5µl	107a 5µl	107a 5µl	107a 5µl	107a 5µl	107a 5µl
488 -710 -50 Percp cy5.5 BD cat# 641397	TNF 2.5µl	TNF 2.5µl	TNF 2.5µl	TNF 2.5µl	TNF 2.5µl	TNF 2.5µl	TNF 2.5µl
561 -582 -15 PE	KIR 2DL1/S1 5µl	KIR 2DL2/S2 5µl	KIR 3DL1 DX9 5µl	Pan KIR 5µl	IgG1 iso For KIR 5µl	Pan KIR 5µl	Pan KIR 5µl
561 -780 -60 PE Cy7 BD	CD56 3µl	CD56 3µl	CD56 3µl	CD56 3µl	CD56 3µl	CD56 3µl	CD56 3µl
640 -670 -14 Alexafl 647 AbD serotec Cat#MCA240 9A647T	FasL 5µl	FasL 5µl	KIR 3DL1 158e1/2 (Z27) APC 5µl	FasL 5µl	FasL 5µl	FasL 5µl	FasL 5µl
640 -780 -60 APCcy7 BD cat# 641397	CD3 3µl	CD3 3µl	CD3 3µl	CD3 3µl	CD3 3µl	CD3 3µl	CD3 3µl
405 -450 -50 VG450 BD 560371	IFNg 2.5µl	IFNg 2.5µl	IFNg 2.5µl	IFNg 2.5µl	IFNg 2.5µl	IFNg 2.5µl	IFNg 2.5µl

2.10.i- CD107a expression

Measurement of surface CD107a (lysosomal-associated membrane protein-1 or LAMP-1), while originally developed with CD8 T cells,[176] is now an established method for measuring NK cytotoxicity, and CD107a expression has been shown to correlate with NK-mediated target cell killing.[177] Measuring CD107a has an advantage over standard cytotoxicity assays such as chromium release/ CFSE fluorescent dye release as these methods only look at death of target cells ie. the aftermath of NK effector function, whereas the CD107a assay enables us to identify and quantify effector cells. CD107a measures the granule dependent pathway of NK cytotoxicity. This pathway does not require *de novo* synthesis of proteins by NK cells (unlike IFNγ which is synthesised *de novo*) but uses pre-formed granules located in the cytoplasm. When cells are stimulated, either by encountering a target cell or by phorbol-12-myristate-13-acetate (PMA) and ionomycin,[178] microtubules transport the vesicle to the cell surface where the synapse with the target cell is located.

The NK granule core (containing proteins such as perforin and granzymes) is surrounded by a lipid bilayer containing lysosomal associated membrane glycoproteins which include 107a (LAMP1),

107b (LAMP2) and CD69 (LAMP3).[34,179] These proteins are also found in the lysosomal/endosomal membranes of other cells and are ubiquitously expressed on the surface of platelets, which may explain why there is usually a higher degree of CD107a background staining when PBMC are used as effectors rather than selected NK cells. When NK degranulation occurs, CD107a is expressed on the cell surface where it can be bound by fluorochrome-conjugated antibody. Re-internalization rapidly occurs[180] but as long as antibody is added to media at the start of effector-target incubation, cells with even transient expression will bind antibody and this will fluoresce when re-internalised within the vesicle. These vesicles are acidic and could therefore potentially neutralise fluorochromes such as FITC; this can however, be avoided by using monensin to help neutralise the ph of the vesicles (as well as inhibiting the secretion of intracellular cytokines).[176]

2.10.ii- TNFα and IFNγ production

CD107a expression by NK cells is closely associated with IFNγ secretion; the majority of NK that produce IFNγ will also express CD107a. There is however, a separate subset of cells which do not secrete IFNγ but do express CD107a[177] and different triggers can result in different modes of cytotoxicity, making the combination of these markers a more sensitive method of assessing NK function than one alone. As TNFα and IFNγ are intracellular cytokines, the NK cell membranes need to be lysed and permeabilysed to allow binding of fluorochrome labeled antibody with cytokine. This method therefore measures the *de novo* production of these cytokines in response to target cell stimulation over a 5 hour period (the peak period of cytokine production).[181] The addition of BFA prevents the cytokine from being released from the cell prior to measurement.

2.10.iii- Fas Ligand expression

FasL is stored in NK granules and transported to the cell surface upon NK cell stimulation. As with CD107a, the FasL antibody is added to the culture media at the start of incubation to ensure that all FasL expressed on the cell surface during incubation with target cells is stained with antibody even if re-internalised after expression. For both CD107a and Fas L, I confirmed this by comparing different times for adding antibody and found that prior to incubation was optimal.

2.10.iv- Incubation of normal NK cells with AML blasts prior to experiments

Healthy donor NK cells were negatively selected using a NK cell negative isolation kit, (Miltenyi Biotec GmbH, Germany). NK cells were then cultured in 96-well plates at 250,000 cells/well with 100 IU/mL rhIL-2 (PROLEUKIN, Chiron Corporation, Emeryville, CA.) for 24 hours, in the presence or absence of primary AML blasts. An NK: blast ratio of 10:1 was chosen as the most clinically relevant, reflecting a state of minimal residual disease. In two experiments where enough

cells were available, I also tested other E:T ratios and found a dose effect with higher numbers of blasts associated with more NK inhibition. NK were cultured for 24 hours with autologous mononuclear cells as another control that is not expected to alter functional activity of target cells. NK cells were then analysed for cell surface receptor expression, and for cytotoxicity and effector cytokine production against K562 leukaemia cell targets as described above.

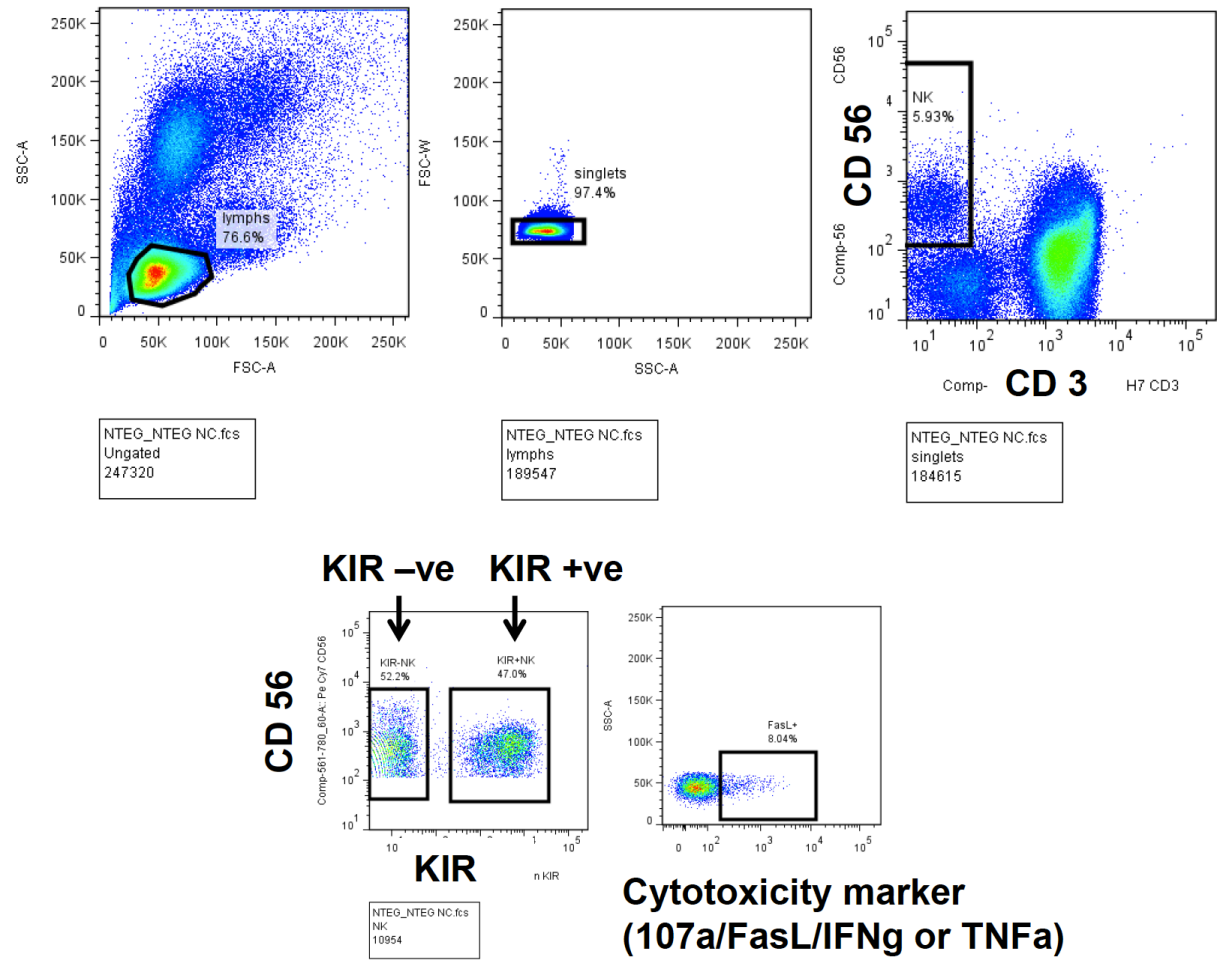


Figure 2.9 Example of FACS plots showing gating strategy for NK cytotoxicity assay

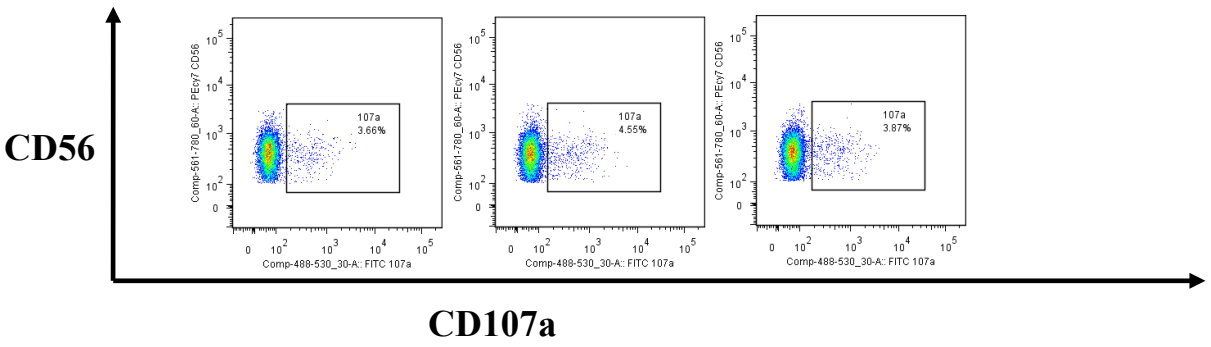


Figure 2.10 Example of FACS plots showing reproducibility of cytotoxic data. Plots show data from 3 separate experiments performed on 3 separate days using cells collected from the same individual on one day and divided into a number of vials for freezing (mean average used).

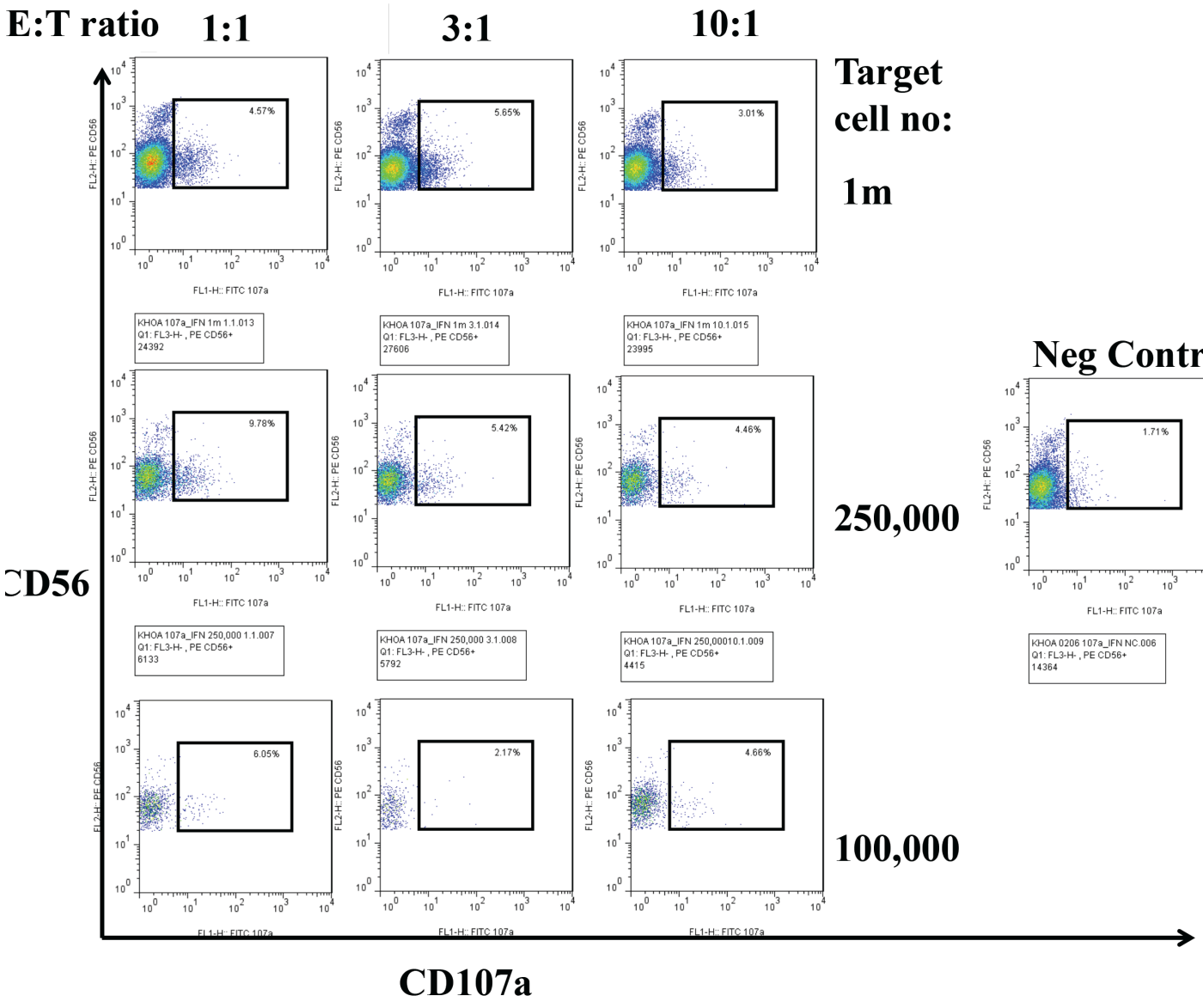


Figure 2.11 Example of FACS plots showing one of a series of optimisation experiments. Peripheral blood mononuclear cells (PBMC) were incubated for 5 hours with leukaemia target cells at effector:target (E:T) ratios of 1:1. 3:1 and 10:1 (calculated using %NK in PBMC sample). This experiment and others showed NK degranulation (as measured by the number of NK cells positive for CD107a) was optimal at a 1:1 E:T ratio. Different numbers of leukaemia target cells were also tested: 100,00 cells was not enough for useful results. 250,000 targets were as effective as 1 million. A negative control helped to define the negative population and consisted of PBMC incubated in media without target cells.

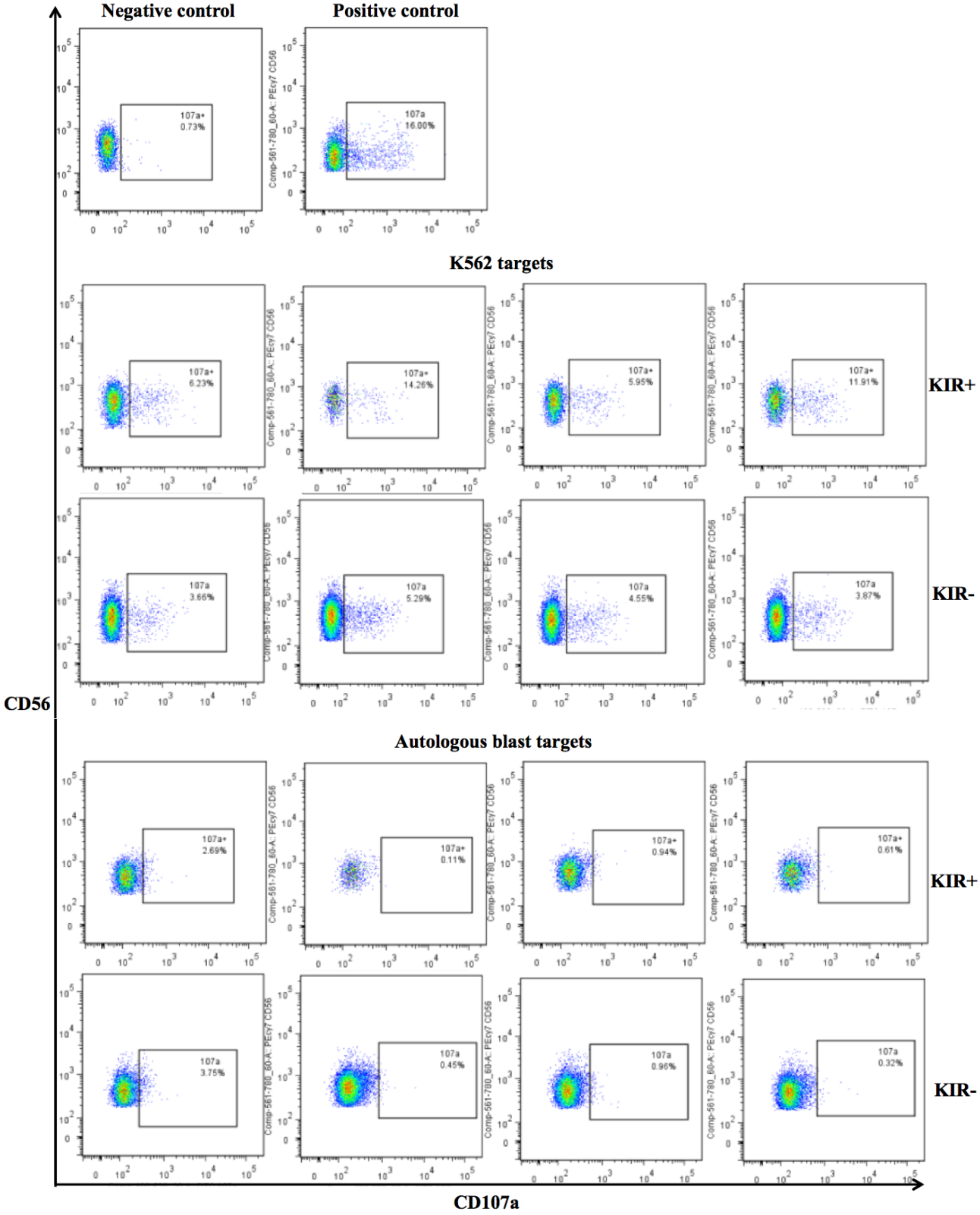


Figure 2.12 Example FACS plots showing results from one experiment assessing a patient's NK cytotoxicity towards K562 (2nd and 3rd rows) and autologous blast targets (4th and 5th rows). Cells were incubated in media alone for the negative control and with PMA/Ionomycin for the positive control (top row). The y axis represents the NK marker CD56. The x axis represents the degranulation marker CD107a. NK cells from this patient showed very little cytotoxicity to autologous blasts. However, there was considerable response to K562 targets, with a greater response from KIR+ than KIR- NK cells. The columns represent different KIR antibodies (from left-right: 2DS1/L1, 2DS2/L2, 3DL1, Pan KIR).

2.11. Completing and validating the transplant database at the NIH

Demographic and clinical details of patients undergoing SCT at the NIH were entered into a comprehensive computerised database by clinical staff at the time of treatment, which was further updated by me.

2.12 Statistical methods

For the transplant cohort, baseline patient characteristics and transplant outcomes were described using summary statistics, including means, medians, percents, standard deviations and 95% confidence intervals. Univariate and multivariate Cox Proportional Hazard Models were used to evaluate the effects of KIR genes, disease, and other factors on the cumulative distribution functions of time to transplant related mortality (TRM), overall survival time (OS), and time to relapse (TTR). Because there were multiple events per patient, our analyses included both single-event Cox Proportional Hazard Models and multiple-event competing risk models. For the single-event analysis, we considered TRM, OS and TTR separately, and used three right censoring schemes: (a) for the analysis of time to TRM, patients who did not die from TRM were treated as censored; (b) for the analysis of time to OS, patients who were alive at the last visits were treated as censored; (c) for the analysis of time to relapse, patients who did not relapse before death or last visit were treated as censored. Statistical inferences for the effects of KIR genes and other covariates on the distribution functions of TRM, OS and TTR were summarised using the likelihood ratio tests, the log-rank tests and their corresponding p-values. Cumulative distributions of TRM, OS and TTR over days since transplant were estimated by the Kaplan-Meier method for patients stratified by KIR genotype, disease and other factors. For the multiple-event competing risk analysis (supplementary table 2), we included relapse, TRM and non-TRM death as competing risks, and considered the marginal competing risk models with event time defined to be the "time to first event" (Terry M. Therneau and Patricia M. Grambsch, Section 8.4, "Modelling Survival Data, Extending the Cox Model", 2000, Springer-Verlag, New York). These models were selected because of their simple and clinically meaningful interpretations. Statistical inferences for the effects of KIR genes and covariates were summarised using the likelihood ratio tests and their p-values. The correlation between KIR genes was analyzed using Pearson's correlation coefficient. Analyses were carried out using the S-Plus 8 (TIBCO Software Inc., Palo Alto, CA) and SPSS (SPSS Inc., Chicago, Illinois) statistical packages. Aside from the competing risk model (supplementary table 2.), I performed the statistics myself on SPSS with the help and guidance of statistician Dr Colin Woo at the NIH who checked all my statistical work.

For the MRC/NCRI non-transplant AML cohort, I collaborated during the analysis with the trial statistician Dr Robert Hills who performed most of the statistical work. This was a necessary part of

using the samples from this trial as open access to outcome data was not available to individual researchers. I conducted power calculations to estimate the sample sizes required for the effects hypothesised using my NIH transplant results (presented in chapter 3) extrapolated to the AML population receiving chemotherapy. For analysis of outcome, logistic regression was used, adjusted for age, trial and performance status.

For the prospective cohort of AML patients I performed statistics using SPSS software (SPSS Inc., Chicago, Illinois) and Graphpad Prism software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Normally distributed groups were compared using either a paired or unpaired T test as appropriate, as well as a three-way ANOVA test when comparing three groups.

Chapter 3 KIR genotype in HLA matched allogeneic stem cell transplantation

3.1 Background

Although outcomes for allogeneic stem cell transplantation (SCT) are improving, better survival is largely related to reduced transplant-related mortality (TRM) and not to reduction in leukaemic relapse, which still accounts for about one third of treatment failures. Relapse represents a failure of the conditioning regimen or the alloimmune graft-versus-leukaemia (GVL) effect. Both T-lymphocytes and natural killer (NK) cells have GVL effects, but the exact mechanism by which these two cell types contribute to leukaemia cure after SCT is not known and probably varies according to disease type, transplant conditions and donor-recipient match.

Several studies have drawn attention to the predictive value for relapse and survival of the lymphocyte count approximately one month after HLA identical SCT for leukaemia.[182-187] A previous small series from the NHLBI of 54 patients receiving T-cell depleted allogeneic SCT from HLA identical donors, showed that outcome and especially relapse risk, was linked to NK cell and not T cell recovery.[188] Since NK alloreactivity is determined by mismatches between recipient HLA class I group and NK cell killer immunoglobulin-like receptor (KIR) expression,[108] patterns of NK-donor mismatching and KIR genetics in the recipient and donor were examined in order to determine whether allo-disparity or specific KIR genotypes were predictive of relapse. In the multivariate analysis, only NK count at day thirty post-transplant emerged as a significant factor affecting outcome, but in the univariate analysis, a number of donor KIR factors were significant. In particular, donor KIR genes 2DL5A, 2DS1 and 3DS1 (which are highly correlated, i.e. usually inherited together), were associated with a significantly reduced relapse rate and improved survival.[188] Similar results have been reported in recipients of unrelated donor SCT: in a group of AML recipients, donors with KIR haplotype B (which includes 2DL5A/2DS1/3DS1) had less relapse and more favourable outcome after SCT than recipients of donors without these genes.[189] It therefore appears that donor KIR genetics may influence the risk of relapse, at least in patients with myeloid leukaemia.

In this study I set out to better define the contribution of KIR genotype to transplant outcome in specific disease types by analyzing 261 related donor transplants for leukaemia performed at NIH in the fifteen-year period 1993 to 2008. KIR genotype, KIR haplotype and inheritance of genes 2DL5A, 2DS1 and 3DS1 were included in univariate and multivariate analysis of transplant variables.

3.2 Hypotheses

a. Donor KIR genotype is associated with improved outcome after allo-SCT

b. Donor KIR genotype is associated with robust lymphocyte recovery post allo-SCT

3.3 Patterns of donor KIR inheritance

Firstly I examined the 'missing KIR ligand' model,[26,28,190] by identifying patients missing an HLA A, Bw or C ligand for which their donor had an inhibitory KIR (a situation possible in the HLA matched setting due to the fact that HLA and KIR genes segregate separately on different chromosomes). I did not evaluate the reverse situation, where donors were missing a ligand for patient KIRs as no cases of graft rejection were seen and my focus was the ability of donor NK to detect leukaemia cells rather than residual patient immunity against the incoming graft. We then assessed the donor KIR genes separately to identify whether individual KIRs are associated with differences in outcome measures in univariate analysis. The presence in the donor of three favourable KIR genes 2DL5A, 2DS1 and 3DS1 together was then examined. The impact of donor haplotype B (defined by the presence of at least one of the genes 2DL5A, 2DS1, 3DS1, 2DS2, 2DS3 or 2DS5), as well as the total number of activating and inhibitory KIRs,[191] were also assessed.

Definitions

Donors possessing the three KIR genes: 2DL5A, 2DS1 and 3DS1, (co-inherited with correlation coefficients of 0·86-0·96), were termed to have favourable KIRs. Standard patient risk group was defined as AML or ALL in 1st complete remission, CML in first chronic phase, and MDS intermediate-1 by WHO classification; all other patients were categorised as high risk. Relapse was defined as haematological relapse.

3.4 Patient characteristics

The study comprised 261 consecutive patients receiving HLA identical sibling allografts at the NHLBI between 1993 and May 2008. The cohort in which donor samples were available for KIR genotyping, encompassed 95% of all HLA sibling transplants in the unit since 1993. Patients received conditioning with Cyclophosphamide 120mg/kg and fractionated total body irradiation (400-1300cGy) with or without Fludarabine 125 mg /m^2 followed by T-cell depleted CD34 cell selected SCT and cyclosporin as the single post-transplant prophylaxis for graft-versus-host disease (GVHD). Donor samples for KIR DNA typing consisted of 239 cryopreserved donor cell samples and 7 buccal swabs. All patients and donors were enrolled onto National Institutes of Health treatment protocols and provided written informed consent. Follow up ranged from 6 months to 15 years. Patient characteristics are described in table 3.1.

Table 3.1 Patient characteristics of NIH transplant cohort

Age	Median	36yrs (range 9-66)
Sex	Male	146 (56%)
	Female	115 (44%)
Race	Asian	35 (13%)
	African/American	25 (10%)
	Hispanic	131 (50%)
	Caucasian	70 (27%)
Disease	ALL	40 (15%)
	AML	70 (27%)
	CML	99 (38%)
	MDS	39 (15%)
	CLL/NHL/MM	13 (5%)
Disease risk group	Standard Risk	105 (40%)
	High Risk	156 (60%)
Graft type	Bone Marrow	41 (16%)
	Peripheral Blood Stem	220 (84%)
CD34+ dose	Median	5 x 10^{6}/kg (range 0·5-16)
CD3+ dose	Median	0.5 x10^{5}/kg (range 0·005-3)
Acute GVHD	Grade 0-1	107 (57%)
	Grade 2-4	81 (43%)
	No GVHD data	72

3.5 Results

3.5-i- Outcome after SCT

The cohort consisted of 261 patients. The majority of patients (85%) had myeloid diseases, of whom seventy (27%) had AML. The outcomes of the 246 for which I had donor samples available were representative of the whole cohort of 261 (appendix: supplementary fig. 1). There were significant differences between diseases in relapse and OS with the highest relapse rates in AML and ALL (table 3.2.)

Table 3.2 Transplant outcome

	No pts (%)	**TRM**	**Overall Survival**	**Relapse**	**GVHD gd 2-4**
Disease					
AML	n=70 (27%)	16 (23%)	30 (43%)	24 (34%)	31 (53%)
MDS	n=39 (15%)	6 (16%)	21 (54%)	12 (31%)	4 (36%)
CML	n=99 (38%)	23 (23%)	61 (61%)	21 (21%)	33 (40%)
CLL/NHL	n= 13 (5%)	2 (15%)	6 (46%)	4 (31%)	1 (25%)
ALL	n=40 (15%)	8 (20%)	16 (40%)	17 (43%)	17 (43%)
Cox model		P= 0.8	**P= 0.022***	**P= 0.03***	P=0.520
All patients	n= 261	55 (21%)	134 (51%)	78 (30%)	85 (44%)

NB. GVHD data was available in 188 patients. Cox model used to compare difference in outcomes between diseases.

Univariate analysis of variables affecting transplant outcome showed that, as expected, high risk patients had more relapse, more TRM and lower survival rates (appendix: supplementary table 2). CD34 doses above 5 x10 ^{6}/kg were associated with less relapse, less TRM and better survival. Peripheral blood graft type, higher lymphocyte count at day thirty post transplant (LC30) and later transplant era, were all associated with reduced TRM but did not affect survival or relapse rates. The only KIR-related variable to affect outcome was the possession by the donor of favourable KIR genes 2DL5a, 2DS1, 3DS1 which were associated with reduced relapse and improved OS. No other KIR-related variable affected transplant outcome, including missing donor KIR ligand, presence of donor haplotype B, total number of inhibitory KIRs and total number of activatory KIRs.

In multivariate analysis (table 3.3), high risk disease was associated with more relapse, more TRM and lower survival rates. Higher LC30 was associated with less TRM (p=0.015) (fig. 3.1a) but did not independently affect risk of relapse or OS. Better OS was seen in recipients of a CD34 cell dose $> 5 \times 10^{6}$/kg.

Table 3.3 Multivariate analysis of significant risk factors in Cox models

	Hazard Ratio	95% CI	P value
Relapse			
High risk disease	4.65	2.6-8.4	**<0.001***
CD34 > 5 x10 ^{6}/kg	0.64	0.4-1.0	0.64
Donor favourable KIRs	0.54	0.3-0.9	**0.02***
Overall Survival			
High risk disease	3.1	2.0-4.7	**<0.001***
CD34 > 5 x10 ^{6}/kg	0.54	0.3-0.8	**0.001***
Donor favourable KIRs	0.64	0.43-0.94	**0.024***
TRM			
High risk disease	2.36	1.3-4.3	**0.004***
CD34 > 5 x10 ^{6}/kg	0.55	0.3-1.0	0.057
PBSC graft	0.68	0.3-1.4	0.276
LC30 >600 x10 ^{7}/l	0.42	0.2-0.9	**0.015***
Recent transplant era	0.95	0.5-1.8	0.866

3.5-ii-Impact of KIR genes on transplant outcome in multivariate analysis

KIR gene frequency among the donor population studied was similar to that of published data in the normal population.[27,192,193] In this sample of 246 patients for whom donor KIR results were available, the presence of the three favourable donor KIR genes 2DL5A, 2DS1 and 3DS1 occurred in eighty-nine cases (35% of donors). In multivariate analysis, patients transplanted from donors with favourable KIRs had significantly less relapse (p=0.02) (fig. 3.1b).

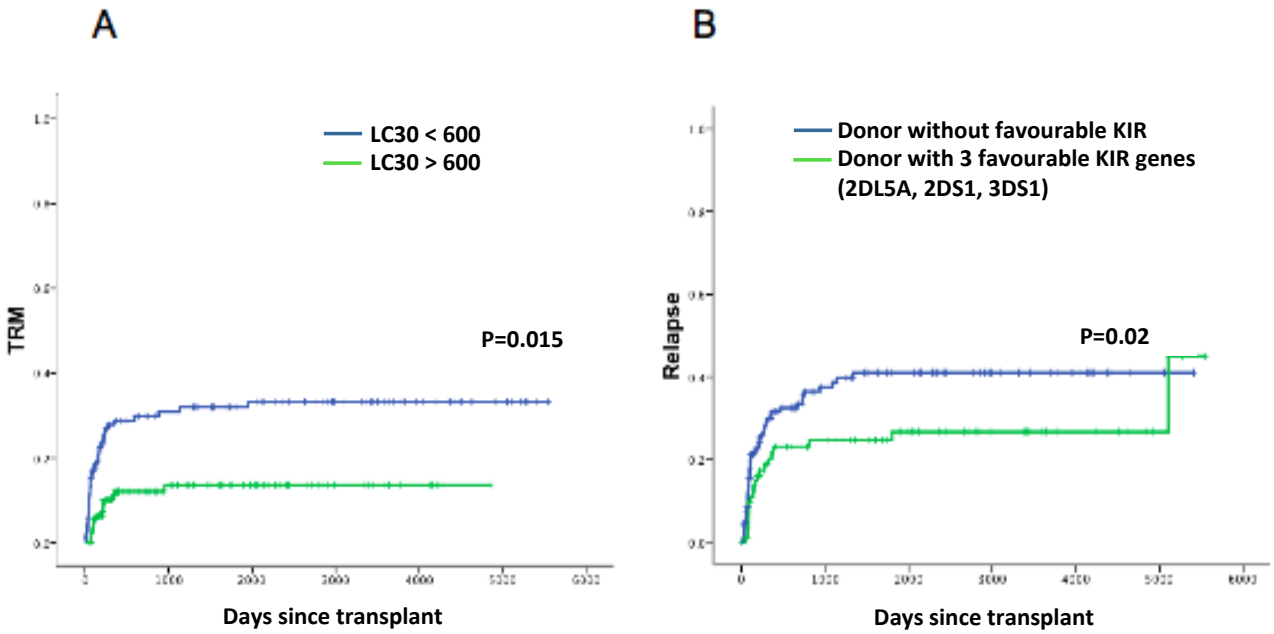


Figure 3.1 Factors affecting SCT outcome in multivariate analysis of 261 patients. a) Lymphocyte count at day 30 post SCT and transplant related mortality b) Donor KIR genotype and relapse

3.5-iii-Impact of donor KIR genes in individual diseases

Relapse: Because relapse rates were different in different diseases, I selected factors that significantly affected relapse rates in multivariate analysis, and studied them in disease subsets (table 3.4). The protective effect of favourable donor KIR genes was restricted to patients with AML. AML patients receiving transplants from donors with favourable KIRs were four times less likely to relapse than recipients of donors not possessing favourable KIRs (p=0.02, Hazard Ratio =0.24) (fig. 3.2a).

The protective effect of favourable KIRs on relapse was observed in both standard and high risk AML patients (fig. 3.2b). None of 13 patients with standard risk AML receiving SCT from favourable KIR donors relapsed, whereas 19 standard risk patients receiving SCT from donors without favourable KIRs had a 32% relapse rate. Similarly, patients with high risk AML receiving

SCT from favourable KIR donors had a relapse rate of 30%, whereas high risk patient recipients of SCT from donors without favourable KIRs had a 70% relapse rate. In diseases other than AML, neither favourable KIRs, nor any other KIR-related variable, affected relapse (fig. 3.3), survival, or TRM (data not shown).

Table 3.4 Effect of favourable donor KIR genes on transplant outcome in different diseases

Patient group with donor KIR group	**TRM**	**p**	**HR**	**Survival**	**HR**	**p**	**Relapse**	**HR**	**p**
AML + Fav KIR donor (n=23)	6 (26%)	0.8	1.14	13 (57%)	0.59	0.151	3 (13%)	0.24	**0.02***
Not fav (n=45)	9 (20%)			17 (38%)			20 (57%)		
AML + haplo B donor (n=46)	10 (22%)	0.92	0.95	20 (44%)	1.07	0.86	15 (33%)	0.86	0.73
Not haplo B donor (n=22)	5 (23%)			10 (46%)			8 (36%)		
MDS + Fav (n=16)	1 (6%)	0.53	0.46	10 (63%)	0.56	0.28	6 (37%)	0.61	0.412
Not fav (n=17)	2 (12%)			8 (47%)			6 (35%)		
CML + Fav (n=32)	6 (19%)	0.52	0.73	21 (66%)	0.78	0.47	6 (19%)	0.75	0.55
Not fav (n=63)	15 (24%)			38 (60%)			14 (22%)		
CLL/NHL + Fav (n=4)	0 (0%)	0.56	0.03	3 (75%)	0.01	0.22	0 (0%)	0.02	0.37
Not fav (n=9)	2 (22%)			3 (33%)			4 (44%)		
ALL + Fav (n=12)	2 (17%)	0.82	0.83	4 (33%)	1.21	0.66	6 (50%)		0.417
Not fav (n=12)	5 (20%)			11 (44%)			9 (36%)		
High Risk + Fav(n=50)	8 (16%)	0.1	0.51	24 (48%)	0.59	**0.02***	19 (38%)	0.63	0.1
Not Fav (n=94)	25 (27%)			31 (33%)			41 (44%)		
Std Risk Fav (n=37)	7 (19%)	0.43	1.51	27 (73%)	0.88	0.74	2 (5%)	0.27	0.09
Not Fav (n=65)	8 (12%)			46 (71%)			12 (18%)		

TRM indicates treatment related mortality; HR, hazard ratio; Fav indicates SCT donor with 3 favourable KIR genes 2DL5A/2DS1/3DS1; Not Fav, SCT donor without all 3 genes; Standard Risk, AML or ALL in 1st complete remission, CML in first chronic phase, and MDS intermediate-1 by WHO classification; High Risk = all other patients. 246 patients had donor samples available for KIR typing. Bold type with asterix indicates a p value significant at the level of 0.05 or below.

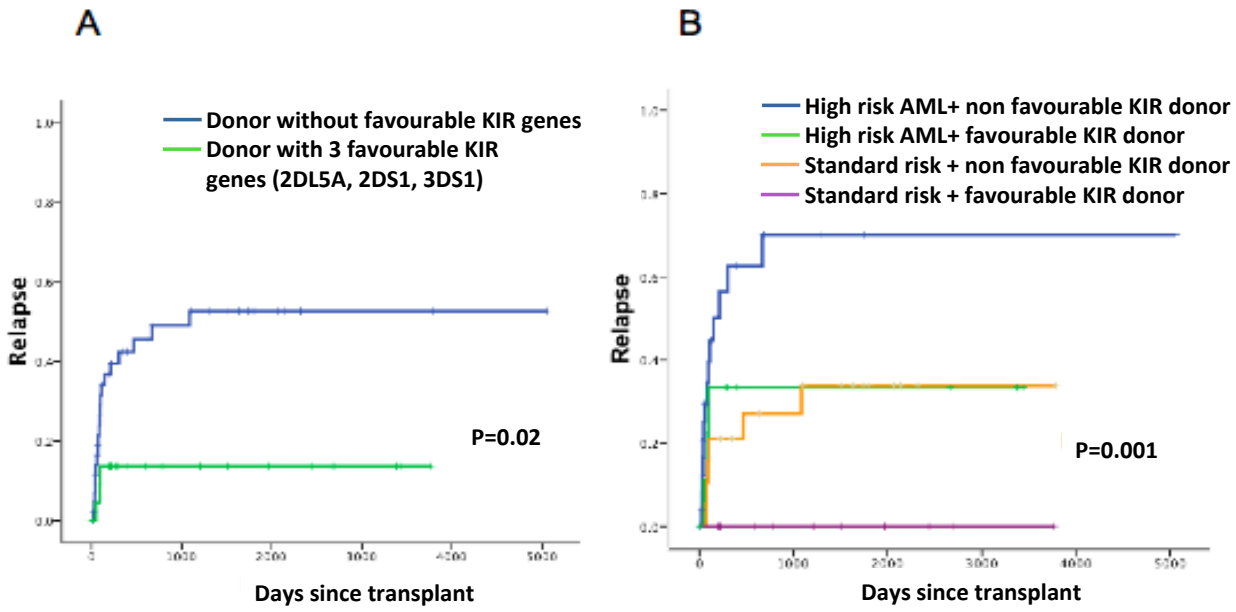


Figure 3.2 Effect of donor KIR genes on relapse in AML patients (n=68). a) Stratified by donor KIR genes b) Stratified by donor KIR genes and risk group

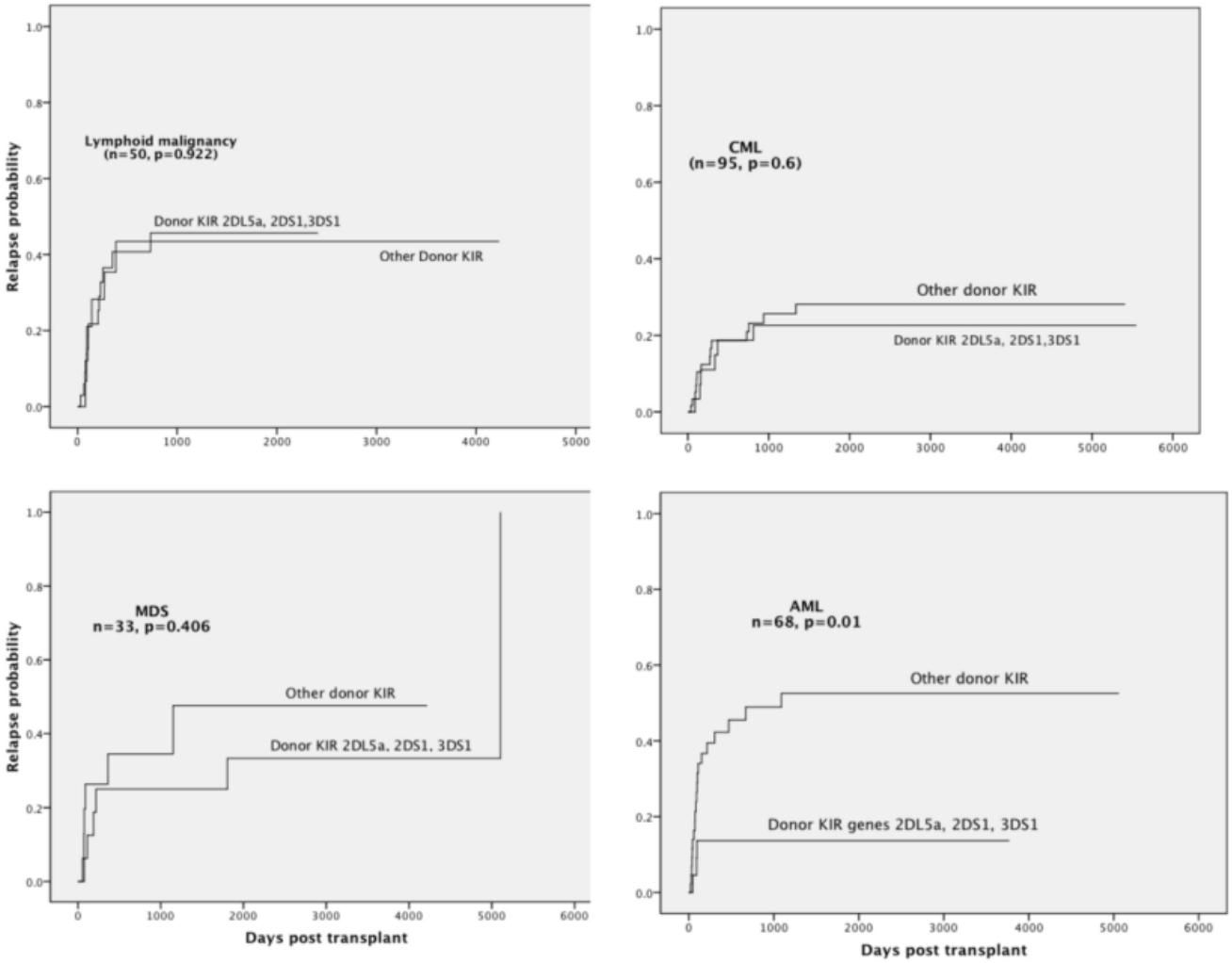


Figure 3.3 Actuarial relapse rates for disease subgroups according to donor KIR genotype

Transplant Related Mortality: In the entire cohort of 261 patients, LC30 and high risk disease were significant for TRM in multivariate analysis. In disease subsets, LC30 below 600 x10^7/l was again significant for TRM in AML (45% vs 9.5% for LC30 > than the median; p 0·04) and MDS (28% vs 4.8% respectively; p=0·047) but not in CML. In CML, TRM was affected by risk group with high risk patients having a higher TRM (39% vs 14%; p=0·005) and by CD34 count with higher TRM for CD34 counts below the median (32% vs 12%; p=0·022). In ALL, only bone marrow as the stem cell source was a significant risk factor for higher TRM (p=0·043).

Overall Survival: Disease risk, CD34 cell dose and a donor with favourable KIRs significantly affected OS in the entire cohort. In disease subsets, disease risk and CD34 cell dose were still significant in AML (for risk group: 63% survival for low risk vs 26% for high, p=0·004; for CD34 dose: 21% below vs 64% above median dose, p 0·001). CD34 dose was also significant for survival in CML (28% below vs 79% above median dose).

3.6 Discussion

In this study, I analysed factors affecting allogeneic transplant outcome in a cohort of 261 patients with haematological malignancies undergoing T depleted myeloablative SCT from HLA matched sibling donors. I identified three donor KIR genes (2DL5A, 2DS1 and 3DS1) associated with a reduced AML relapse rate post SCT. My findings suggest that an NK-mediated GVL effect in AML may be modulated by specific KIR genes in the donor.

Studying the determinants of allo-SCT outcome can identify risk factors that can be used to optimise transplant schedule design and donor selection. Furthermore, clinical results can point to biological mechanisms underlying success or failure of allo-SCT. In addition to well recognised disease-related factors predicting outcome, my colleagues and others have identified CD34+ cell dose and lymphocyte counts thirty days after SCT as predictive factors for transplant outcome.[184,194,195] In a previous analysis on a smaller cohort of 54 patients, they found that donor KIR genotype was also a strong predictor of survival and relapse after SCT. In particular, three closely linked donor genes 2DL5A, 2DS1 and 3DS1, of the B haplotype were predictive for outcome and relapse.[188] I therefore extended this observation in the present study where there were sufficient numbers of patients to assess the impact of donor KIR group on outcome in different diseases. Here I show that, in addition to known risk factors for outcome (disease risk, disease type, CD34+ cell dose and day thirty lymphocyte counts), donor KIR genes 2DL5A, 2DS1 and 3DS1 were associated with significantly reduced relapse rates after T depleted SCT. This effect was restricted to patients with de-novo AML and had no effect in CML, MDS or lymphoid malignancies.

The impact of donor KIRs on relapse in AML was striking: relapse occurred in 13% of recipients of transplants from donors with favourable KIRs and in 57% of recipients whose donors lacked these genes (p=0·02). The effect of donor KIRs was seen in both standard and high risk AML: no standard risk patient receiving a transplant from a donor with favourable KIRs relapsed compared with a relapse rate of 32% for standard risk recipients of donors with unfavourable KIRs. Similarly relapse rates for high risk patients were affected by donor KIR group: 30% for recipients with favourable KIR donors and 70% for recipients with unfavourable KIR donors.

My results support a recent analysis in AML patients receiving unrelated donor SCT, where SCT from donors of the B haplotype (which contains the favourable KIR genes) were associated with less relapse.[189] There is strong linkage equilibrium among B haplotype genes and it is difficult to be certain which genes are associated with the anti-leukemic effect. The statistical problems associated with multiple comparisons mean the results must be viewed cautiously. However, in this study, the

strongest association with reduced AML relapse was with the KIR genes 2DL5A, 2DS1 and 3DS1 (p=0.02, HR 0.24) and analysis of B haplotype itself was not found to be significant (p=0.73, HR 0.86). My findings contrast with data by Hsu et al, who studied the role of donor KIRs in a mixed disease population of patients receiving HLA identical sibling, T-cell depleted SCT similar to mine, and found no benefit for the presence of any particular donor KIR on outcome.[28] Although I found that missing a ligand for donor KIRs reduced TRM in AML patients, I could not replicate their findings of less relapse and improved survival in these patients. The difference may be explained by the fact that patients in Dr Hsu's study all received bone marrow grafts, whereas the majority of my patients received blood derived stem cells. A separate analysis of the thirty nine bone marrow recipients in my series did not show any impact of donor KIR groups, while the association between donor KIRs and relapse was of greater significance when the PBSCT were analysed separately (p <0.029). Further analysis of larger patient series receiving different types of transplant regimens is required to clarify these differences. The disease specificity of my finding, with the impact of KIR group genetics mainly affecting AML, is consistent with findings from other centres where NK-KIR mediated effects have been found to be strongest in AML,[26,28] as well as studies showing that alloreactive NK cells do not kill ALL cells in vitro.[25]

The mechanism whereby KIR genes 2DL5A, 2DS1 and 3DS1 mediate a protective effect against relapse is unclear. The previous small study linked these genes to higher NK cell counts post transplant suggesting a genetic control of NK cell recovery.[188] Interestingly, in my larger sample, I did not find an association of these genes with higher lymphocyte counts post transplant, but in the small group of patients who did not achieve lymphocyte counts above 0.2 x 10^9/l, the positive effect of these KIR genes was not seen, suggesting that a minimum level of NK cells need to be present for the effect of KIR genes to be facilitated. The association between favourable KIRs and lower relapse rates was also stronger in PBSCT where higher circulating levels of all cells including NK occur earlier after transplant, than with BM grafts.

Since the three favourable genes are usually inherited together, it is impossible to determine whether all three, or only one KIR gene acts as a protective factor. Since 1 of the 3 genes occurs in only 5% of donors, very large patient cohorts would be required to explore the contribution of specific favourable KIR.

These results suggest that there may be specific ligands on the surface of AML cells that make the interaction between favourable KIR receptors and AML cells particularly effective at causing NK-mediated AML-cell death. Alternatively these favourable KIR genes may simply represent a marker

linked to an as yet undefined GVL pathway. Further studies are required to investigate this and determine whether the beneficial KIR-AML interaction is restricted to specific AML subtypes and reproduced in HLA matched but unrelated SCT.

These findings have practical implications and support the concept of selecting donors by KIR genotype in order to optimise GVL effects after SCT for AML. In the absence of a favourable donor, patients could be considered for experimental treatments to boost GVL, for example using NK cell infusions or lower doses of immunosuppression. Recent trials using allogeneic NK cell infusions to boost immunity post transplant are promising, with small numbers showing that this treatment modality is safe and feasible and can lead to reduction in tumour load in some patients.[196-198] Selecting donors with favourable KIRs could potentially increase the ability of such infusions to control leukaemia and prevent relapse. This would be a practical possibility since donors with favourable KIR genes represent up to 40% of the population.

Chapter 4 KIR genotype in AML patients receiving induction chemotherapy

4.1 Background

While there are clearly major differences between normal donor NK cells engrafting after transplant and the NK cells of a leukaemic patient regenerating after chemotherapy, there are some precedents that suggest observations seen in the allograft setting may translate to non-transplant patients. First, in both settings, at the time of cellular regeneration, NK cells account for a higher proportion of the normal lymphocyte population than normal with a marked increase in both relative and absolute numbers of NK cells,[195,199,200] suggesting the importance of the NK-mediated immune response in both. Second, in both settings, patients who achieve higher absolute lymphocyte counts one month after treatment have improved outcome.[14,187] Third, there is evidence that patients with greater NK-mediated cytotoxicity towards leukaemic cells post treatment do better following both SCT and chemotherapy.[13,26] Gathering further evidence to support the hypothesis that variability in NK cell genes may account for variability in disease and relapse risk is an important step forward in understanding anti-leukaemia biology and in helping identify those chemotherapy patients who may be at higher risk of AML relapse. If the role of KIR genes is not supported in the autologous setting, this information will enable us to re-focus our investigation onto the characteristics of the leukaemic blasts in inducing or escaping the humoral or T cell anti-leukaemia control.

Although we cannot conclude from data in chapter 3 that the improved outcome of transplant for AML is a direct consequence of the donor KIR type, the multivariate analysis showing reduced relapse in patients receiving transplants from donors with 3 particular KIR genes at least suggests a role for donor NK cell activity in this disease. Recent studies to identify risk factors in AML have focused on new cytogenetic and molecular markers. This has brought significant advances in our ability to stratify treatment intensity according to cytogenetic or molecular risk and enables poor risk leukaemias to be selected for stem cell transplant or exploratory treatments while avoiding unnecessary toxicity in good risk leukaemias.

These developments have improved our understanding of AML relapse but focus on identifying factors intrinsic to the leukaemia. For example: leukaemic cells which highly express the multi drug resistance-1 (MDR-1) gene extrude chemotherapeutic drugs thereby facilitating cell survival;[201-204] mutations of the FMS-like tyrosine kinase 3 (FLT-3) lead to constitutive activation of the tyrosine kinase and downstream activation of signalling pathways that mediate cell survival and proliferation.[205] Less attention has been given to host factors influencing the immune system's ability to prevent the initial occurrence of leukaemia or maintain remission, that is, the competence of the immune systems tumour surveillance. The importance of NK cells in killing leukaemic cells is

increasingly recognised and further studies into factors which influence an NK cell's ability to detect and destroy a leukaemic target, such as KIRs, are justified.

The role of KIRs and KIR ligands in predicting disease and outcome for AML has not been investigated outside the allogeneic transplant setting. As non-transplant treatments are given to the majority of AML patients, it is relevant to explore the role of KIR genetics in these individuals. There are indications that KIR-KIR ligand interactions may be important in the setting of autologous disease from studies of autologous stem cell transplantation for neuroblastoma,[206] lymphoma and solid tumours,[190] where patients missing a ligand for their inhibitory KIRs had improved survival rates. Data in support of an autologous role for KIR-KIR ligands in modifying risk of disease and disease outcome has also been produced in the setting of autoimmune and infective disease.[114,207] In this chapter, I have investigated the role of KIR-KIR ligand interactions in a large cohort of AML patients receiving only chemotherapy for their disease. Our normal donors were siblings of US patients with haematological malignancies. Their KIR gene frequency was not significantly different to the UK donor populations registered at http://www.allelefrequencies.net/kir6002a.asp who are demographically matched to the MRC patient cohort.

4.2 Models for investigating KIR genetics in this study

The distinct variation in KIR gene inheritance means that NK cells between individuals could have differing abilities to identify and interact with leukaemic target cells. Here I investigate whether particular KIR genotypes thereby affect (1) predisposition to developing AML and (2) outcome after AML chemotherapy.

4.2-i-KIR gene frequency

I compared the frequency of KIR genes in individuals with AML and in a normal donor population without AML and correlated the presence of individual KIR genes with outcome after AML chemotherapy. As discussed in the introduction, although there are 17 different KIR genes which can be inherited, some (KIR 2DP1 and 3DP1) are pseudo genes, and do not encode for surface receptors, and some (3DL3, 3DP1, 2DL4 and 3DL2) are 'framework genes' and inherited by all individuals. I therefore restricted my analysis to the 11 KIR genes, which could potentially be of use in predicting variation in disease risk and outcome between individuals.

4.2-ii-Possession of 'favourable' and activatory KIRs

Following the results presented in chapter 3, a primary aim of this study was to investigate whether possession of the three 'favourable' KIR genes, 2DS1, 3DS1 and 2DL5a also conferred benefit on AML patients outside the transplant setting. A recent model from the transplant setting, reported that individuals with the more 'activatory' haplotype B (containing at least one of the activatory genes

KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1)[189] had improved outcome post SCT. This variable compares those with at least one B haplotype to those with A/A haplotype.

4.2-iii-Missing a ligand for inhibitory KIRs

Clinical data from unrelated SCT indicates that patients missing a ligand for their inhibitory KIRs have better outcome.[26,208] An example of an individual missing a KIR ligand would be a patient possessing the gene for KIR2DL2 and/or 2DL3 but missing the C1 ligand for these KIRs by virtue of being homozygous for HLA C2. Conversely, individuals possessing KIR2DL1 but being homozygous for C1 are missing the C2 ligand for their inhibitory KIR. As all individuals express KIR2DL2/3, and most express KIR2DL1, the vast majority of HLA C homozygotes are missing a ligand for their inhibitory KIRs. The third ligand in this model is that for KIR3DL1, which recognises HLA-B Bw4 molecules with an isoleucine at position 80. Therefore individuals possessing this KIR but also homozygous for Bw6 and not possessing any of the HLA A alleles which are also recognised by KIR3DL1 (HLA A23, A24, A32) are also missing a KIR ligand.

4.2-iv-Missing a ligand for activatory KIRs

A second concept associated with KIR ligands, is that HLA ligands for activatory KIRs are also important for educating the NK response. This is of particular interest considering my results in chapter 3 linking a combination of largely activatory KIRs with better outcome in AML patients. Although strength of binding between most activatory KIRs and HLA class 1 is typically weaker than for inhibitory KIRs,[209] making it likely that there are other as yet unknown ligands which are more specific, this is not the case for 2DS1, where there is good evidence that HLA C2 is indeed its main ligand.[209-211] NK cells expressing activatory KIR2DS1 from C2 homozygotes are down-regulated in their cytotoxic ability towards targets whereas in C1 homozygotes they are not.[148] This suggests a mechanism of tolerance during NK education whereby exposure of activatory KIRs to their ligand 'tunes down' responsiveness and is complementary to the opposite effect seen when inhibitory KIRs interact with their HLA ligand and become 'licensed' to kill.

If it follows that those missing a ligand for their activatory KIRs do not undergo this 'tuning down' process, (as seen in the 2DS1+ cells from C1 homozygotes who are missing the 2DS1 ligand C2), the model could identify a group as potentially more able to clear leukaemic cells by virtue of their KIR-KIR ligand genetics. To create this variable, I looked at individuals possessing the activatory KIR2DS1 and 2DS2 and assessed whether the corresponding HLA C2 or C1 ligand was present.

4.2-v-Statistical analysis

Statistical analysis for this cohort was performed by Dr Robert Hills at Cardiff University in collaboration with myself. As stipulated by the MRC trials unit, access to outcome data is not

routinely available to individual researchers and working with a designated MRC statistician is part of the agreement when accessing samples. The results have been adjusted to take into account factors known to affect outcome in this cohort (age and performance status as well as trial).

4.3 Aims of this chapter

a. Does a particular KIR genotype predispose to developing AML?

b. Do individuals with the activatory 'favourable' or haplotype B KIR genotypes or those missing a ligand for their KIRs have better outcome after chemotherapy for AML?

4.4 Results

4.4-i-Patient Characteristics

I obtained access to a unique cohort of patients from the largest and most comprehensive AML trials in the UK. 500 AML patients consecutively enrolled onto NCRI (MRC) AML trials 10 and 15 prior to 2006 were included in this study. All patients were aged between 16 and 60 and received four courses of intensive chemotherapy, as per protocol. Patients had a minimum follow-up of 3 years, incorporating the period at which they are at maximum risk of relapse. Each sample was linked to comprehensive cytogenetic, molecular and outcome data collected by the MRC enabling me to ask questions about the role of KIRs in AML cytogenetic and molecular subgroups in this project. I have KIR genotyping results on 499 patients. One patient aged 9 years was excluded from the analysis.

4.4-ii-Does a particular KIR genotype predispose to developing AML?

The KIR gene frequencies of *de novo* AML did not differ significantly from those of the normal population (fig. 4.1). Frequency of KIR2DS2 was significantly lower in secondary AML compared to *de novo* AML and normal controls (26% (10/37) vs. 51% (234/462) for *de novo* AML, p=0.004 and 44% (111/246) for normal controls) as were total number of activatory KIR (fig. 4.2 and 4.3). There was some evidence that KIR2DL2, which shows linkage disequilibrium with KIR2DS2, was also reduced in secondary AML (32% v 54%, p=0.009). Rates of KIR2DS2 and KIR2DL2 were lower in both secondary groups: therapy-related AML (t-AML) and those with antecedent haematological disorders. Patients with t-AML had lower rates of KIR Haplotype B than those without t-AML (27% v 67%, p=0.005). However, analyses of the AML cohort adjusted for known prognostic factors showed no significant prognostic effect of any single KIR group, or KIR B haplotype. These results suggest that inheritance of KIR2DS2 may be protective for the development of secondary AML and that individuals lacking haplotype B KIR genes are more prone to develop t-AML. These observations raise the possibility that KIR gene inheritance determines the efficiency of immunosurveillance of AML by NK cells in the context of a pre-leukemic disease phase, which characterises secondary AML.

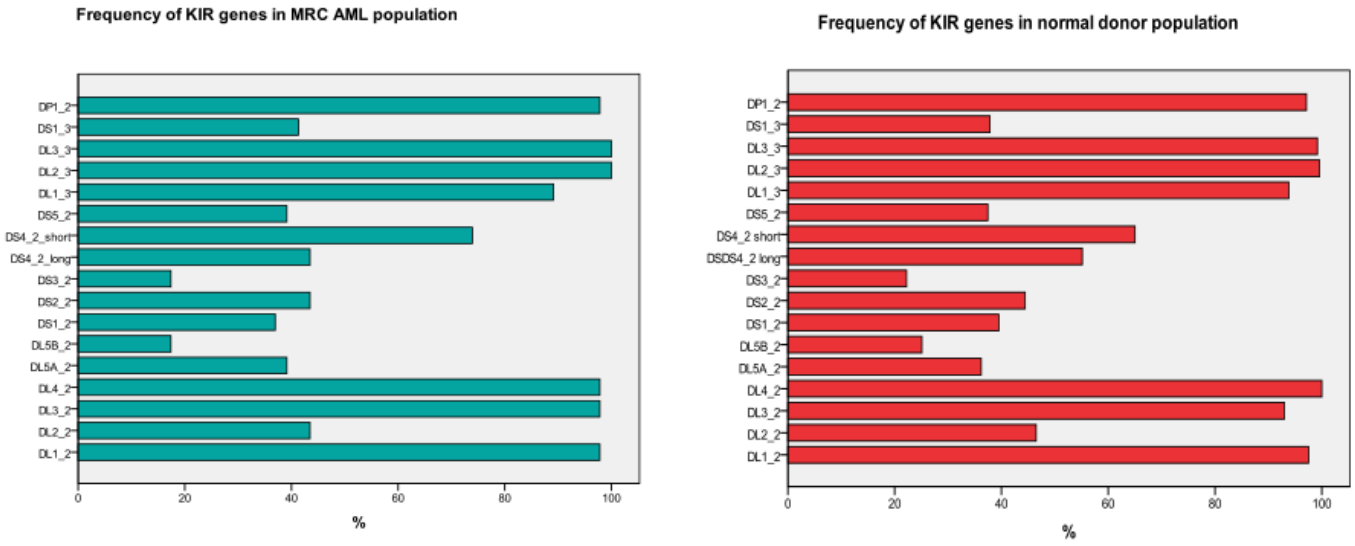


Figure 4.1 The frequency of KIR genes in the AML population (left) and normal donors (right). There is no difference in KIR frequency between the two populations.

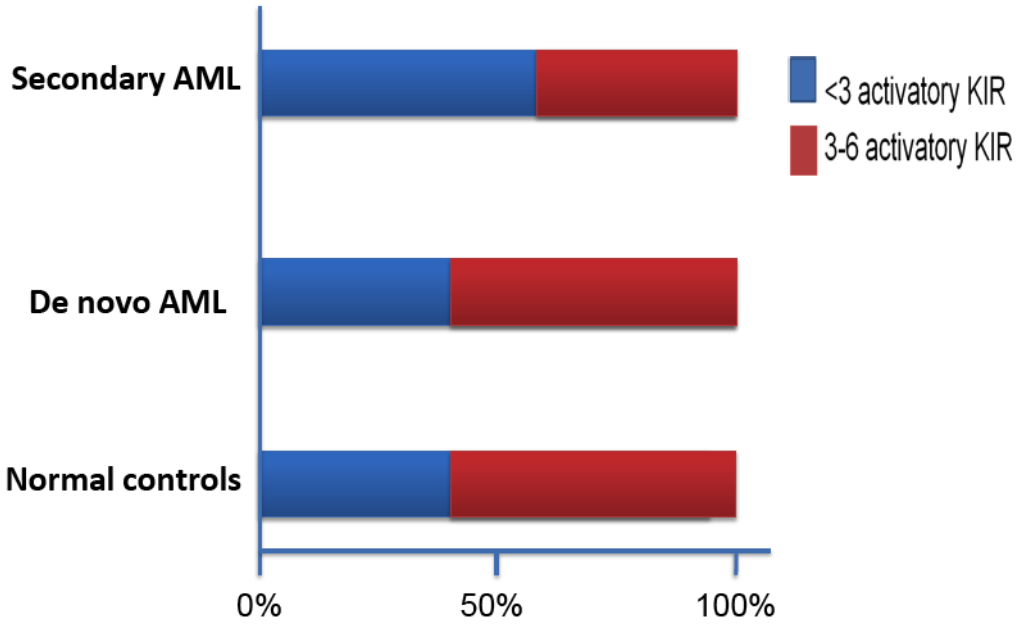


Figure 4.2 KIR gene prevalence looking at an individual's total number of activatory KIR. Patients with secondary AML had less activatory KIR than those with de novo AML or normal healthy controls.

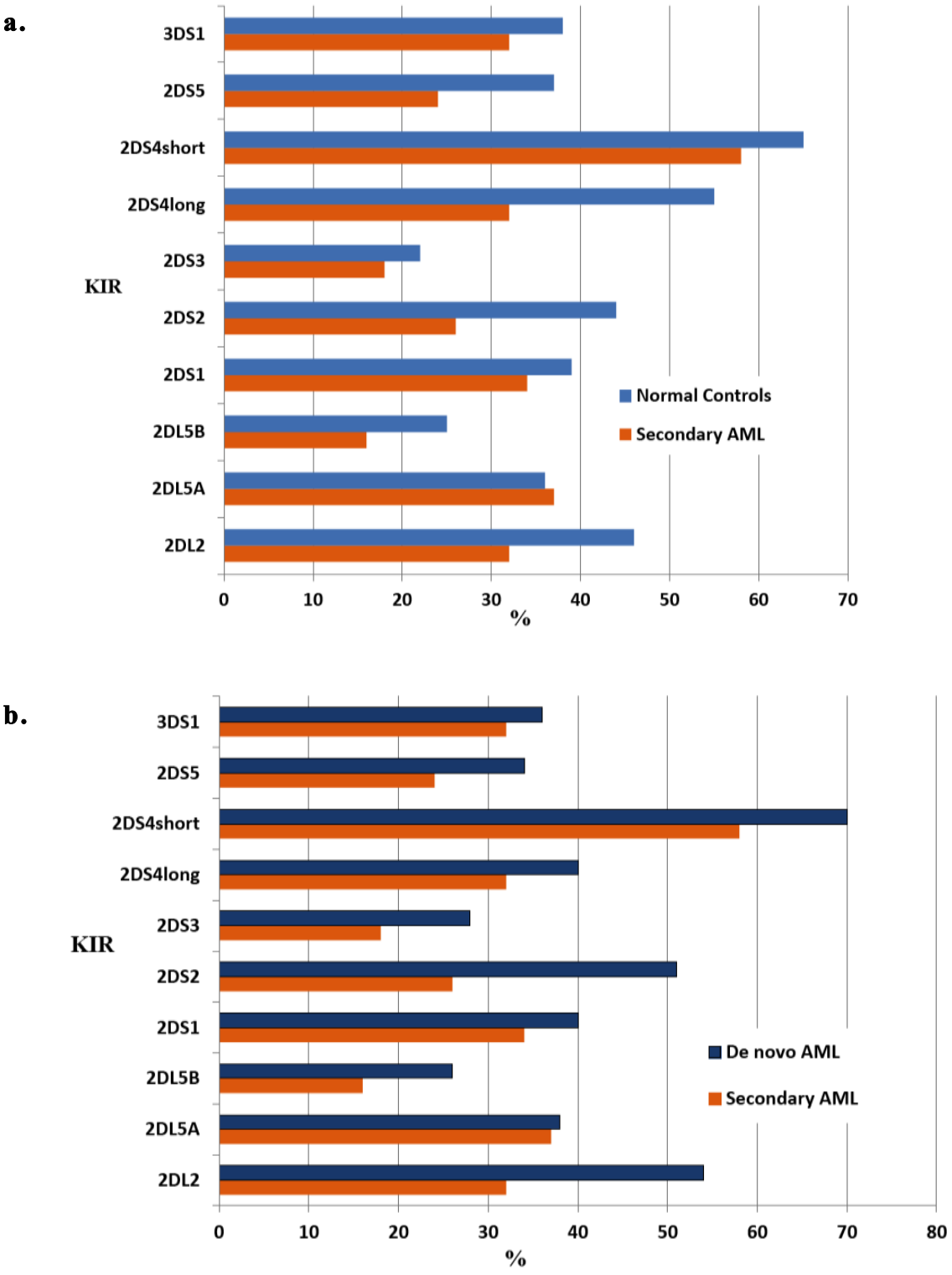
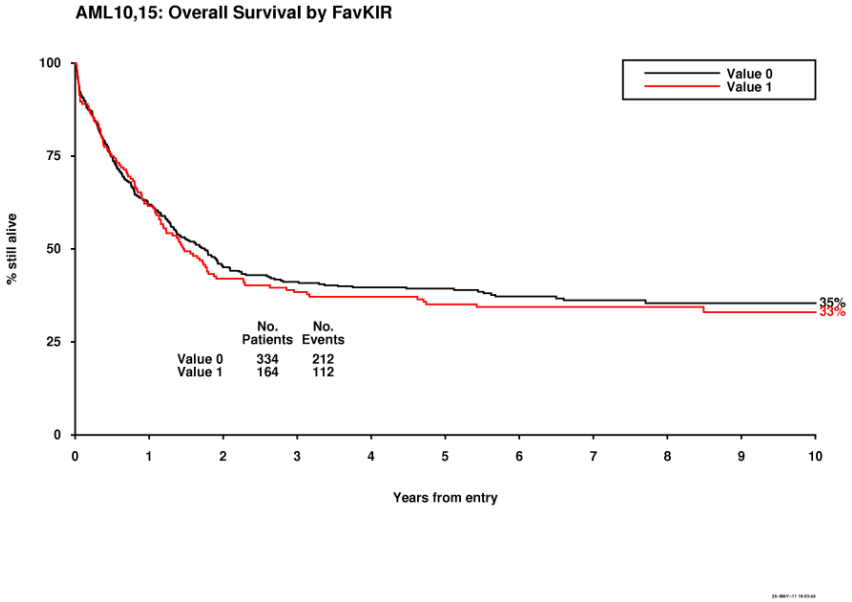


Figure 4.3 The frequency of KIR genes in secondary AML compared to a) the normal donor population b) *de novo* AML

4.4-iii-Do individuals with 'favourable' or haplotype B KIR genotypes, or those missing a ligand for their KIRs have better outcomes after chemotherapy for AML?

I did not find a favourable effect either for possession of the three favourable KIR genes discussed in chapter 3, or for haplotype B in predicting response to AML patients receiving chemotherapy (fig. 4.4 and 4.5). I did not find that missing a ligand for inhibitory KIRs had any impact in this setting either (fig. 4.6). My results combining activatory KIRs and their ligands are more complicated. While missing a ligand for activatory KIRs had no statistically significant effect on overall survival in AML (p=0.3) there is possibly a trend (fig. 4.7a), and this variable did appear to affect relapse risk with individuals missing a ligand for their activatory KIRs having reduced relapse risk compared to those not missing a ligand (45% (8/19) for those missing C1 and 41% (19/52) for those missing C2 versus 56% (110/219) relapse in those not missing a C ligand, p=0.01) (fig.4.7b). These data would suggest that individuals missing a C ligand for their activatory KIRs may have greater cytotoxic NK activity and therefore supports the model put forward by Fauriat et al[148] discussed in section 4.2-iv where it is suggested that NK expressing activatory KIRs are tuned down in responsiveness when exposed to their HLA C ligand.

a.



b.

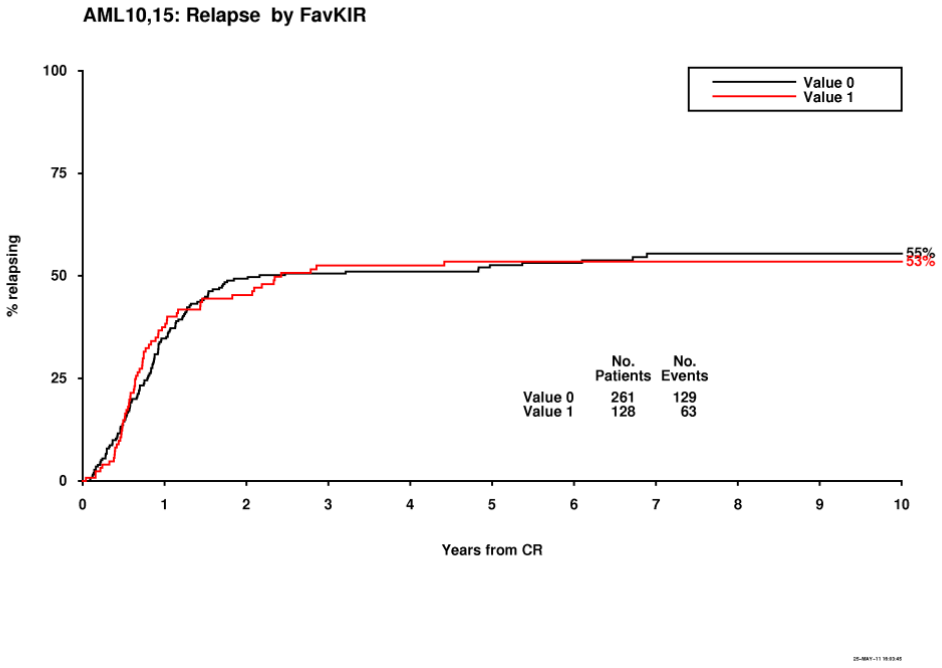
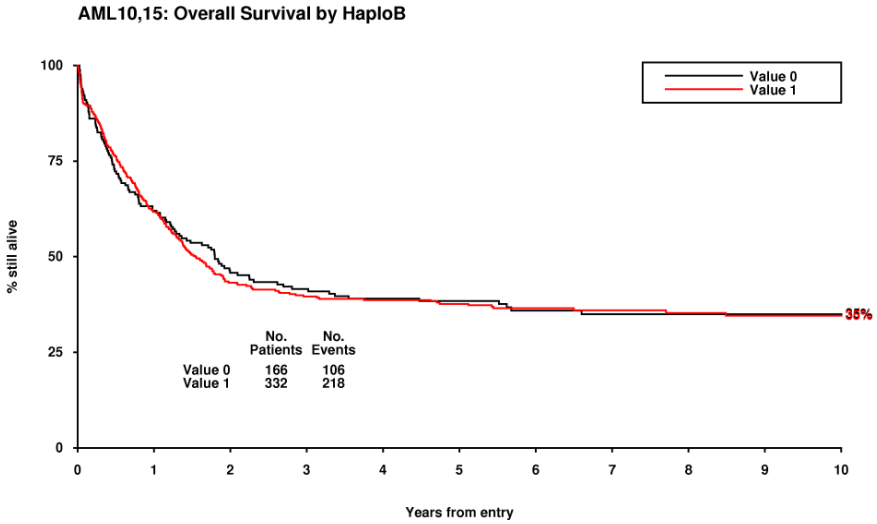


Figure 4.4 The role of 3 'favourable' KIR on AML outcome in MRC/NCRI trial patients. Presence of the 3 'favourable' KIR genes 2DS1, 3DS1, 2DL5a did not affect a) overall survival b) relapse risk in AML

a.



b.

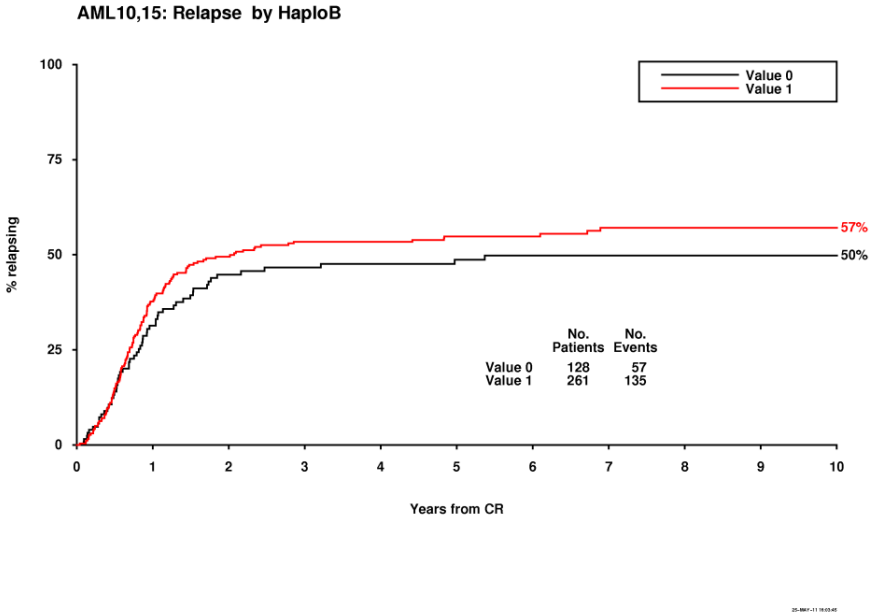
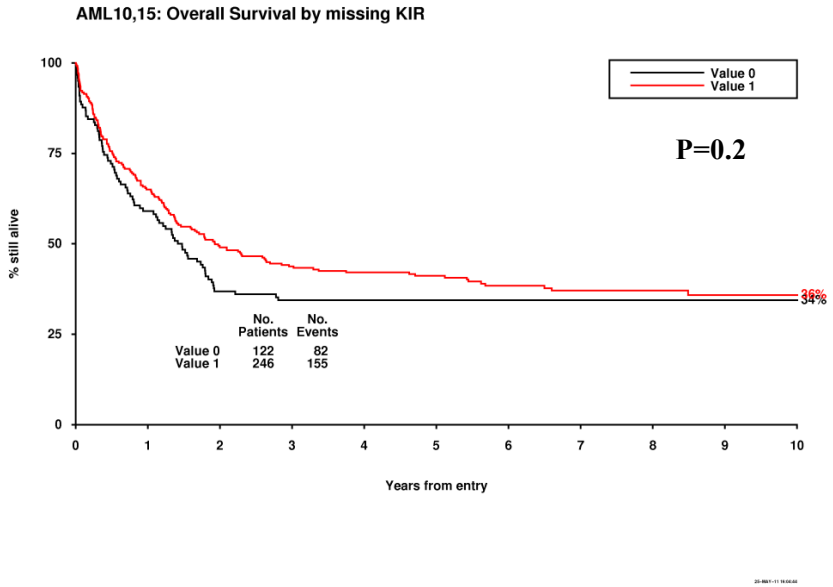


Figure 4.5 The role of KIR haplotype on AML outcome in MRC/NCRI trial patients. Patient KIR haplotype did not affect a) overall survival or b) relapse risk in AML. Value 1=patients with haplotype B.

a.



b.

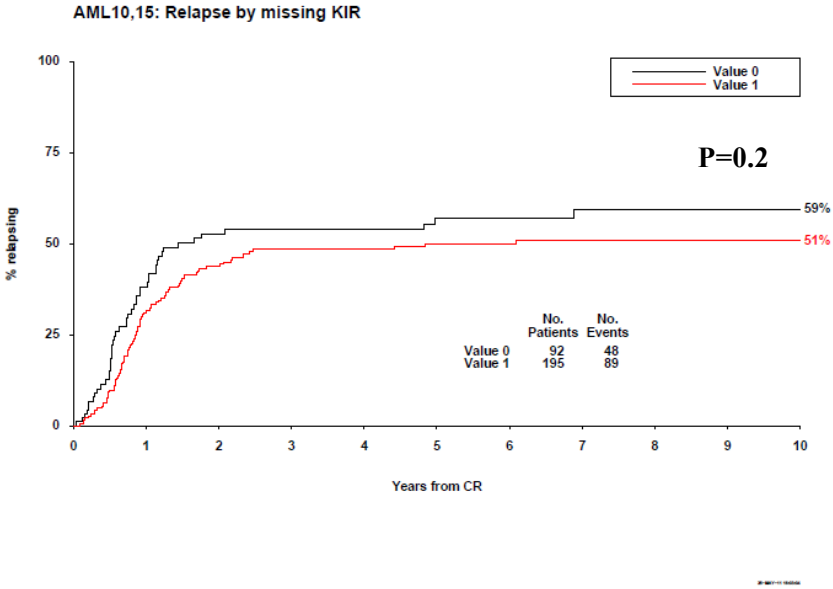
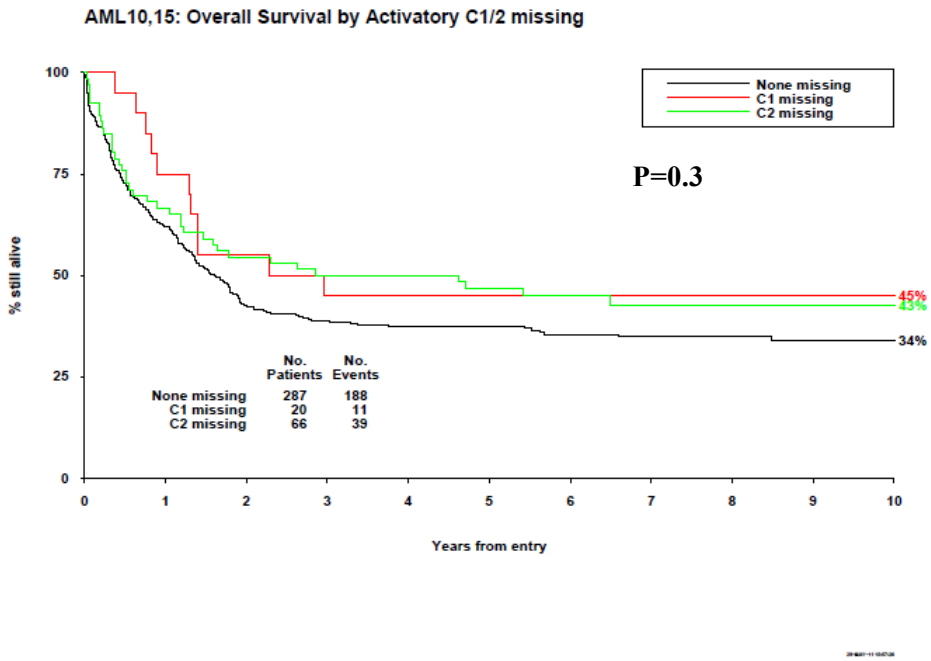


Figure 4.6 The role of inhibitory KIR ligands on AML outcome in MRC/NCRI trial patients. Missing a ligand for inhibitory KIRs did not affect a) overall survival or b) relapse risk in AML. Value 1= patients missing an HLA ligand for their inhibitory KIRs

a.



b.

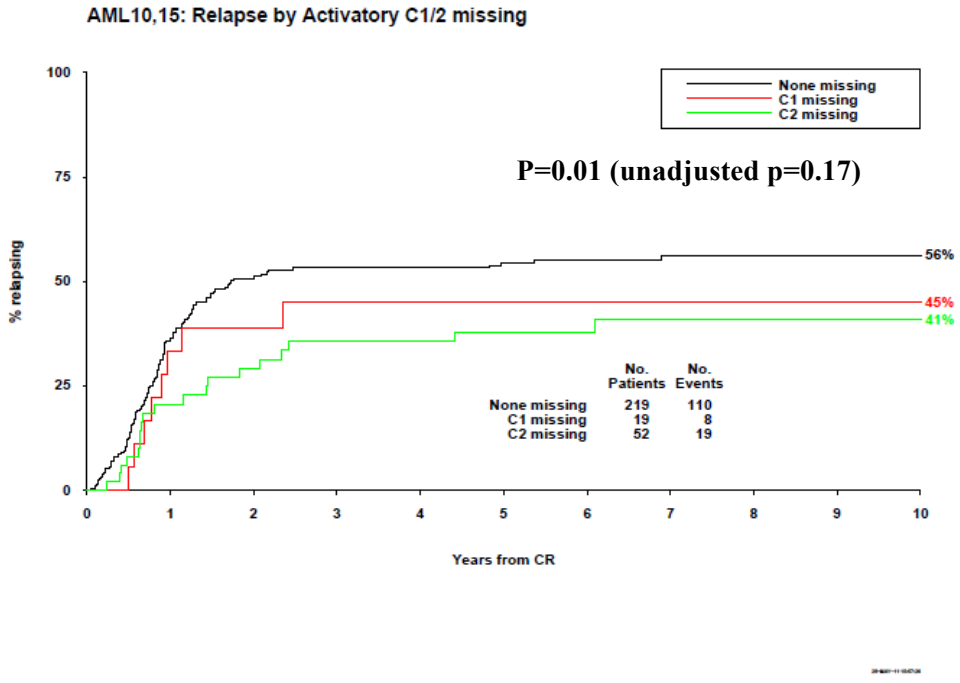


Figure 4.7 The role of activatory KIR ligands on AML outcome in MRC/NCRI trial patients. Missing a ligand for activatory KIRs a) did not significantly affect overall survival but b) may be associated with a reduced relapse risk in AML

4.5 Discussion

I have shown that the frequency of KIR2DS2 was significantly lower in secondary AML compared to *de novo* AML and normal controls suggesting that inheritance of KIR2DS2 may be protective for the development of secondary AML. I have also shown that individuals lacking the activatory haplotype B KIR genes are more prone to develop t-AML. Less convincingly, this data also suggests that individuals missing a ligand for their activatory KIRs may have a reduced relapse risk after AML chemotherapy. These data suggest an important role of activatory KIRs in immunosurveillance of AML.

Does a particular KIR genotype predispose to developing AML?

The fact that we only see a protective relationship from activatory KIRs in secondary AML and not *de novo* AML is perhaps not surprising. Secondary AML often follows a slow time course starting with a dysplastic clone, maybe a few blasts in marrow and proceeding over sometimes many years to develop into a full blown leukaemia. During this time, a multitude of genetic and epigenetic changes are occurring with oncogenes being switched on and tumour suppressor genes being switched off.[212] It is therefore probable that immune editing would have more capacity to adapt to and control this process than a malignant transformation which develops over a matter of weeks, as is often the case with *de novo* AML. Supporting the role of NK-mediated immune editing in protecting against secondary AML, higher frequencies of NK cells are found in low risk MDS as compared to high risk MDS.[212] Also, cytolytic function of NK cells is disrupted in MDS,[213] with reduced NK cytotoxicity in the RAEB forms at high risk of going on to develop AML[214] and increased NK-mediated cytotoxicity in low and intermediate risk MDS.[215]

Linking an activatory KIR with disease is consistent with data from the infectious diseases setting where an activatory KIR genotype limits development of AIDS[216] and HCV.[217] The role of KIR2DS2 as being particularly important has also been identified in the autoimmune setting where individuals with KIR2DS2 have an increased risk of developing the diseases rheumatoid arthritis, type 1 diabetes and psoriatic arthritis.[218-220]

Do individuals with 'favourable' or haplotype B KIR genotypes, or those missing a ligand for their KIRs have better outcome after chemotherapy for AML?

I found that the three 'favourable' KIRs, haplotype B and missing a ligand for inhibitory KIRs are not associated with improved outcome after chemotherapy for AML. However, individuals missing a ligand for their activatory KIRs possibly have reduced relapse risk after chemotherapy. The first three important negative findings indicate that previous results linking these variables with improved outcome in AML after SCT are specific to NK alloresponses and cannot be extrapolated to non-

allograft treatments. The particular environment that NK cells engraft in after stem cell transplantation, the cytokine and chemokine profile and the fact that the regenerating cells belong to a different individual, are all factors that differ from the current population, and which could impact upon the process of NK-mediated immune surveillance. The finding that missing a ligand for activatory KIRs improves outcome is consistent with evidence citing the role of activatory KIRs in NK education, where the responsiveness of NK cells expressing activatory KIRs is down regulated in the presence of their HLA C ligand.[148] My data suggest that in individuals where this educational down regulation does not take place (as would be the case in individuals missing that ligand), NK cells may be more responsive and therefore better able to recognise and kill leukaemic cells emerging during relapse. These data therefore provide important clinical evidence to support for the role of activatory KIRs in NK education. It should be noted that throughout this study, the statistically significant results I have reported have been adjusted to take into account factors known to affect outcome in this cohort (age and performance status as well as trial) and both the adjusted and unadjusted p values have been statistically significant. For the result linking individuals missing a ligand for their activatory KIRs with reduced relapse risk after chemotherapy, only the adjusted p value is significant (0.01) while the unadjusted value is 0.17. This result should be interpreted with caution since there is a possibility that age and performance status may influence the activatory NK response to leukaemia. These factors could account for the discrepancy, since older, frailer patients are more likely to have aggressive disease, and be less susceptible to the effects of immunosurveillance. Further analysis of this variable, stratifying according to age, performance status and cytogenetic risk group in a larger cohort would be necessary to address this limitation.

A general concern of studies testing prediction of disease risk and outcome by presence or absence of a gene is that genetic analysis does not account for variations in expression of the protein coded for by the gene. Overall, KIR gene expression correlates well with RNA transcription,[221] and as previous studies had shown KIR to exhibit 94% sequence homology, [222] genotype-phenotype discrepancy was not initially thought to be a large problem with KIR genes. However, rare alleles of KIR3DL1 have been shown to be expressed at a lower level than others.[223,224] There are also allelic variants of other KIRs which lead to sequestration of the receptor in the cell cytoplasm[225] or secretion of the receptor from the surface.[226] My study is thus limited by the fact that the genotyping method used was not sensitive enough to detect these alleles. These non-expressed alleles are however rare. The commonest non-expressed allele is KIR2DL4 but as my genotyping method enabled identification of the null allele and KIR2DL4 is not one which is incorporated into any of the missing ligand models (as its ligand is, as yet unknown), this should not affect the conclusions of this study. Genotype of activatory KIRs correlates well with phenotype and most activating KIR loci encode a single frequent allele.[227,228]

A further concern with KIR genotyping studies is the effect of multiple testing on the statistical significance of results, that is, the risk of a false positive finding increases with the number of variables incorporated in the model. If enough variables in a data set are explored without a prior hypothesis, a positive answer from one can occur due to chance alone. For this reason, it was decided we could not analyze the data to ask the question: is the effect of KIR genes mediated by cytogenetic subgroups? This is unfortunate as cytogenetic stratification would be interesting, cytogenetics having been shown to impact greatly on AML outcome and it being biologically plausible that immune effects such as KIR profile may be not have a chance to take effect in the aggressive disease typically seen in individuals with poor cytogenetics. Not separating these individuals from the rest of the cohort may have therefore masked a possible effect. Selected analysis of this question may be possible in the future.

To account for multiple testing, in collaboration with the NCRI MRC statistician, I restricted statistical significance at the 0.01 level rather than the usual 0.05. Nevertheless, the findings in secondary AML make a further study with a larger cohort of secondary AML patients worthwhile. An important group to look at are patients with previous haematological malignancies such as Hodgkins and non Hodgkins lymphoma, Myeloma and Waldenstrom's macroglobulinaemia as these patients have a relatively high risk of developing AML following their treatments. If we find that these patients segregate to the poor risk KIR genotypes (low numbers of activatory KIRs/negative for KIR2DS2/negative for KIR haplotype B), those deemed higher risk could be counseled appropriately, screened more regularly, and perhaps have it built it into their initial treatment algorithm to be particularly careful with alkylating agents etc.

It would also be interesting to study MDS patients to determine whether KIR 2DS2 is under represented in those who progress rapidly to AML. Such data could improve the predictive power of the IPSS score. Individuals at high risk of early progression could be selected for earlier therapeutic interventions and SCT while a watch a wait strategy could be safely adopted in lower risk patients.

How might activatory KIRs reduce the risk of secondary AML? As discussed in the introduction, when NK cells activatory KIRs bind a ligand on a target cell, this triggers NK-mediated killing of that target cell provided the activatory signal is not inhibited by inhibitory receptor signaling. It therefore follows that individuals with a greater number of activatory KIRs are perhaps better equipped for this process. One interesting possibility is that KIR 2DS2 has a particular binding affinity for an, as yet unknown, ligand expressed on AML blasts. While 2DS2 can bind HLA C1 molecules on target cells, the binding affinity for the interaction between activatory KIRs is much lower than that for their inhibitory.[229] This raises the possibility that this receptor has evolved to bind to an alternative target. Further studies are required to identify 2DS2-binding ligands on AML blasts.

A further possibility is that the effect of activatory KIRs on immune editing is mediated via T cells, the NK cells acting in a co stimulatory role and making T cells more sensitive to small quantities of antigen.[230]

In terms of practical applications of these data, identifying a genetic basis, which may influence immune editing of AML, is an important step forward in our understanding of the immune response to the disease. Results from genetic studies can provide an invaluable tool for directing future biological studies.

Chapter 5 Phenotypic changes in surface receptors and cytotoxic function of NK cells in AML

The observation that numbers of NK cells are increased in AML at presentation[174,200,231] suggests that NK cells are part of the immune response to leukaemia. However, it is unclear whether these NK cells are functionally competent to recognise and kill their leukaemic targets. Indeed it is possible that NK cells from AML patients are functionally defective because of unfavourable immune editing of the immune system by the leukaemia (a process explained in section 1.9.)

In this chapter I describe my attempts to characterise NK phenotype and function in AML and to answer the question whether NK cells in AML patients are normal or defective. As detailed in chapter 2, I have prospectively collected cell samples and outcome data on a cohort of 32 AML patients sequentially presenting at the Hammersmith Hospital in London during the period of my PhD to provide the samples for this work.

5.1 Background

With the exception of some studies on activatory receptors in AML,[174,231] little has been published on NK cell surface receptors in this context. As discussed in my introduction, it is clear that surface NK receptors are critical for recognition and killing of malignant target cells. It is also known that the percentage expression of receptors vary between individuals[89] and that expression density of certain activatory NK receptors correlates with cytotoxic ability.[91,232] That AML blasts may affect NK surface receptor phenotype has been shown, where the number of certain activatory receptors was reduced in AML patients.[174] However, these studies tend to focus on one or two receptors and/or single aspects of NK cytotoxicity and a comprehensive characterization of NK phenotype and function in AML has yet to be performed.

The main receptors used by NK cells to identify target cells are the C-type lectin receptors, KIRs and NCR. Cytotoxic function occurs via three main mechanisms: perforin/granzyme release, cytokine production and death receptor pathways. In this chapter I measure NK phenotype and function by comparing AML patients with normal controls. The functional ability of CD56 bright NK cells differs to that of CD56 dim with the latter being more efficient at cytotoxic killing. Recent work has shown that the balance of CD56 bright and dim cells can be altered in disease states such as HIV and after stem cell transplantation. [233,234] The CD56 bright/dim ratio is also associated with alteration in NK functional ability in the post transplant period.[235] I therefore include an assessment of CD56 surface expression to assess the role of this factor in AML patients receiving chemotherapy.

After characterising NK cell surface receptor expression, I investigated the cytotoxicity of all NK cells, defined as CD56+, CD3- cells. Then, in view of my results from chapters 3 and 4 indicating an

impact of KIR genes on NK-mediated cytotoxicity towards AML cells, I extended my investigation to see whether KIR expression affects the cytotoxicity of NK cells. It has been shown in the setting of HIV, that NK cells expressing particular KIRs show enhanced NK cytotoxicity.[236,237] Expression of particular KIRs also affects the ability of NK cells to achieve full maturity depending on the KIR-ligand setting in which they develop (detailed in section 1.8).[145-147]

I also assessed the important NK receptor ligands on the blast cells of patients to see whether any changes in NK phenotype were related to disease-specific factors on the AML blasts. This also enabled me to look for evidence of immune escape where blasts could adapt their phenotype to evade immune responses.

5.2 Aims of this chapter

1. Is NK surface receptor phenotype in AML different from that of healthy individuals?
2. Do any surface receptor abnormalities seen in AML normalise in remission?
3. Is NK cell cytotoxicity abnormal in AML patients?

    a. Cytotoxic abnormalities in all NK cells

    b. Are cytotoxic abnormalities in NK cells specific to cells expressing particular KIRs?

    c. Do cytotoxic abnormalities in NK cells affect all triggering mechanisms or are they restricted to cytotoxicity triggered by cell contact?

    d. Do KIR ligands affect the cytotoxicity of NK cells?

4. Do any cytotoxic abnormalities seen in AML normalise in remission?
5. What is the repertoire of NK ligands on AML blasts?
6. Is expression of NK ligands on leukaemic blasts associated with changes in NK receptor phenotype or function?
7. Do individuals with abnormal phenotype have worse cytotoxic function?
8. Is it possible to induce abnormalities by exposing normal NK cells to AML blasts?

5.3 Results

After patient characteristics, results are organised as to address the aims in section 5.2.

Patient characteristics

Patient samples (n=32) were collected prospectively from the Hammersmith Hospital and compared with remission samples on those same patients where available (n=12) and with healthy control individuals who were matched closely for age and sex (n=15). AML patients who were eligible for therapy were treated with the standard UK first line chemotherapy treatment of Daunorubicin (50-60mg/m^2 x3 doses over 5 days) and Cytarabine (100mg/m2 x 20 doses over 10 days). Samples from patients who achieved remission after this were taken when their blood counts recovered and before subsequent chemotherapy was started. In some patients, further remission samples were taken after subsequent treatment courses and at longer time points as detailed. Patients received standard supportive care with antifungal, anti viral and antibiotic therapy as appropriate. Patient characteristics are detailed in table 5.1.

Table 5.1 AML patient characteristics

	No. (%)
No. patients	32
Median age in years (range)	61 (21-87)
Sex ratio (M:F)	1.7:1
Cytogenetics	
Good risk	3 (10%)
Intermediate risk	17 (57%)
Poor risk	10 (33%)
Unknown/insufficient sample	2
Primary AML	16 (50%)
Secondary to MDS/MPD	14 (44%)
Secondary to previous therapy	2 (6%)
Presenting WBC ($x10^9$/L)	
Raised (>11)	14 (44%)(median 50, range 16.1-126)
Normal (4-11)	3 (9%)
Cytopaenic (<4)	15 (47%)
Response to first line treatment (DA 3+10)	
Complete remission	14 (45%)
Induction failure/partial remission/not given	17 (55%)
Unknown (patient refused Rx and left UK)	1

5.3-i- Is the NK surface receptor phenotype in AML different from that of healthy individuals?

Comparing NK surface receptor phenotype in AML with that of healthy controls (fig. 5.1 and 5.2), I found an increase in NK cells expressing the inhibitory receptor NKG2A (p=0.0463, fig. 5.2d) and a reduction in the expression of the activatory receptor NKp46 (p=0.0068, fig. 5.2c). There was no difference in surface expression of CD56, NKG2D, KIRs, NKp30 or NKp44 on NK cells in AML patients compared to normal controls (fig. 5.2a-d).

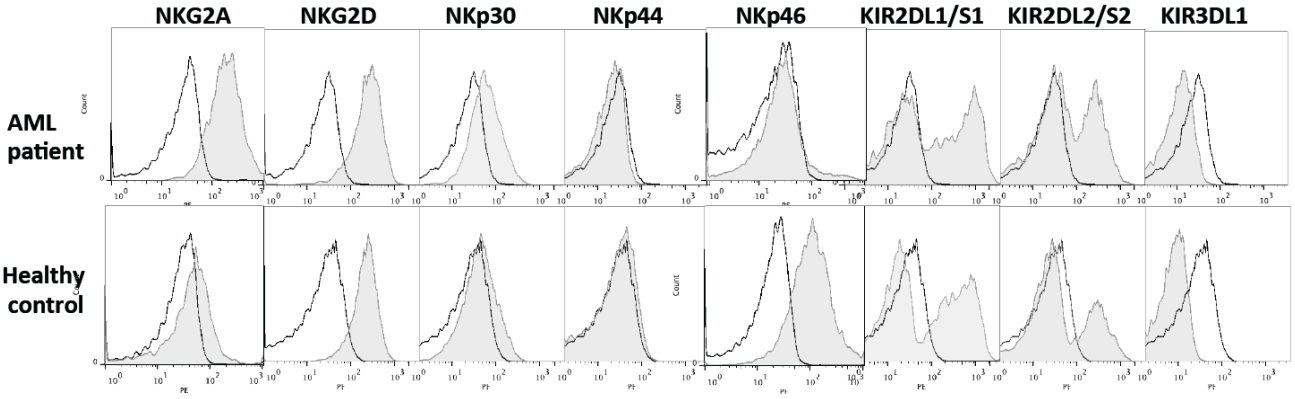


Figure 5.1 Examples of histograms showing NK surface phenotype in a representative AML patient and healthy control. Open histograms represent isotype-matched monoclonal antibody staining. Filled histograms represent staining with specific PE-congugated monoclonal antibodies

5.3-ii-Do any surface receptor abnormalities seen in AML normalise in remission?

Having found an abnormality in the balance between inhibitory and activatory surface receptors, I examined whether these changes normalised when patients were in remission after chemotherapy (fig. 5.3). The increased NKG2A expression seen in AML patients at presentation did not, in fact it continued to increase (p=0.008) in all but 3 patients whereas the reduced NKp46 expression seen at AML presentation, normalised in remission (p=0.01).

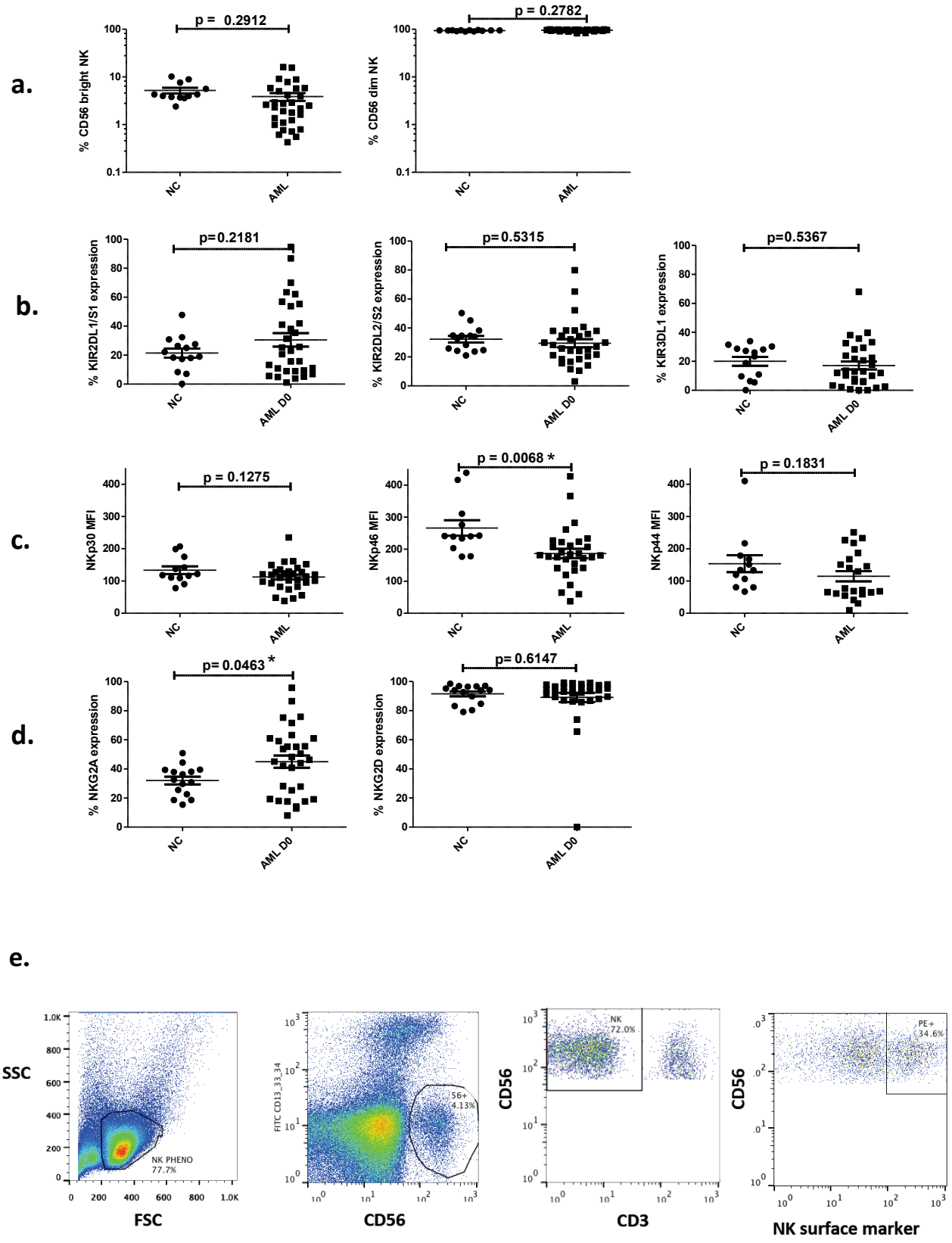


Figure 5.2 NK receptor expression in AML patients compared to normal controls. a) Proportions of CD56 bright and dim NK cells were not different between the groups b) There was no difference in surface expression of KIRs c) NKp46 expression was lower in AML but NKp30 and NKp44 were not different to normal controls d) AML patients showed an increase in NK expressing the inhibitory receptor NKG2A but no difference in NKG2D expression e) Gating strategy to identify NK from PBMC

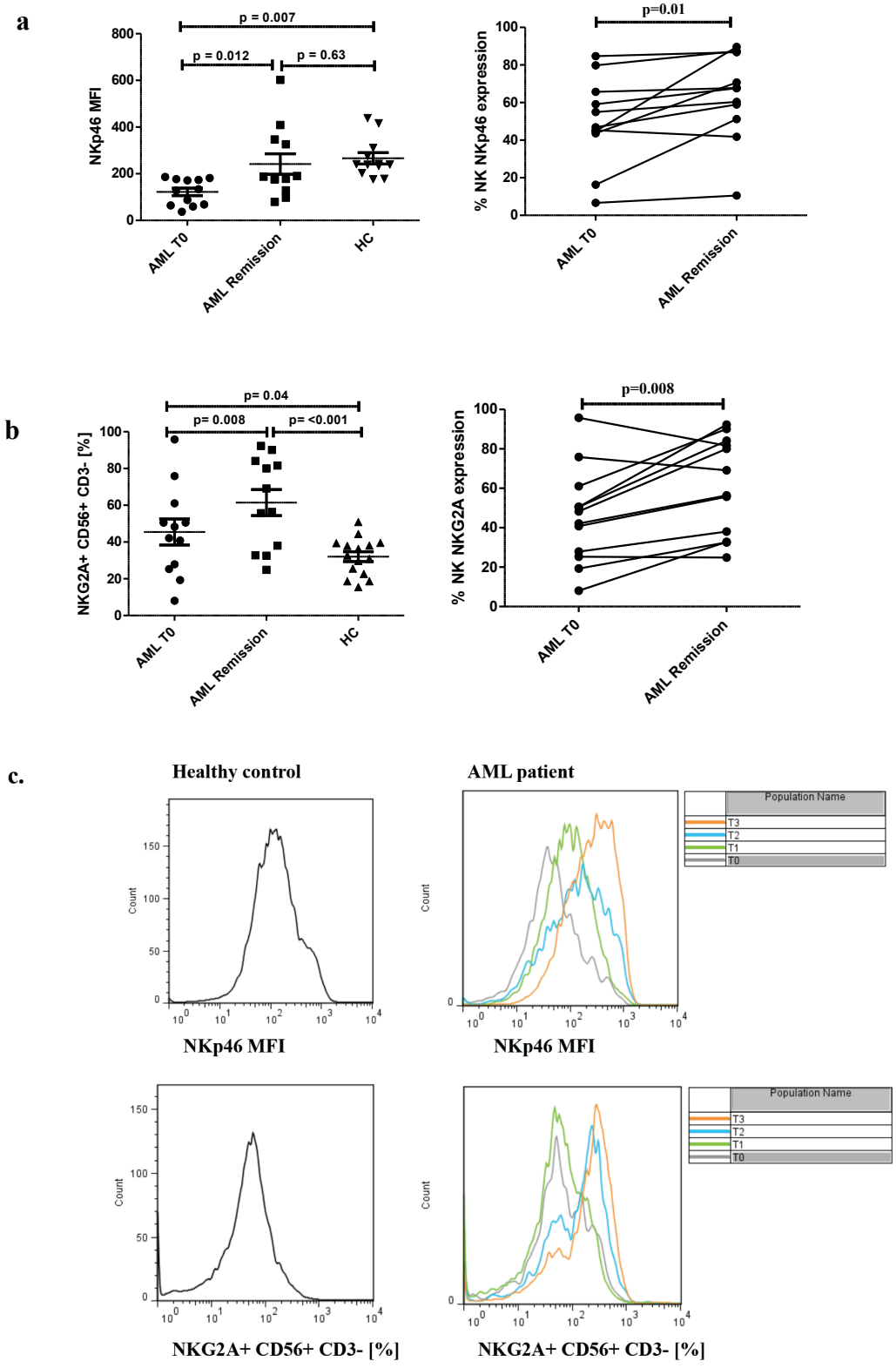


Figure 5.3 NK phenotypic abnormalities in AML were partially restored in remission. (a) NKp46 expression was restored to normal levels (b) NKG2A expression remained elevated. P values denote significance of paired T tests comparing AML T1 with remission and unpaired T tests comparing AML with healthy controls. Horizontal bars denote mean expression. (c) NKp46 and NKG2A expression in a representative healthy control (left column) and AML patient at serial time points (right column): diagnosis (T0), following induction chemotherapy (T1), following consolidation chemotherapy #1 (T2), and following consolidation chemotherapy #2 (T3). Abbreviations: HC: healthy control

I then examined all receptors, even those without a statistically significant abnormality in AML, to see whether any changes occurred in patients after chemotherapy. NKG2D expression was unchanged in remission (fig. 5.4a). Expression of KIR2DS1/L1 and 2DS2/L2 were unchanged in AML patients at remission compared to presentation although KIR3DL1 was down regulated in remission samples (fig. 5.4b). There was a statistically significant up regulation of NKp30 (p=0.02).

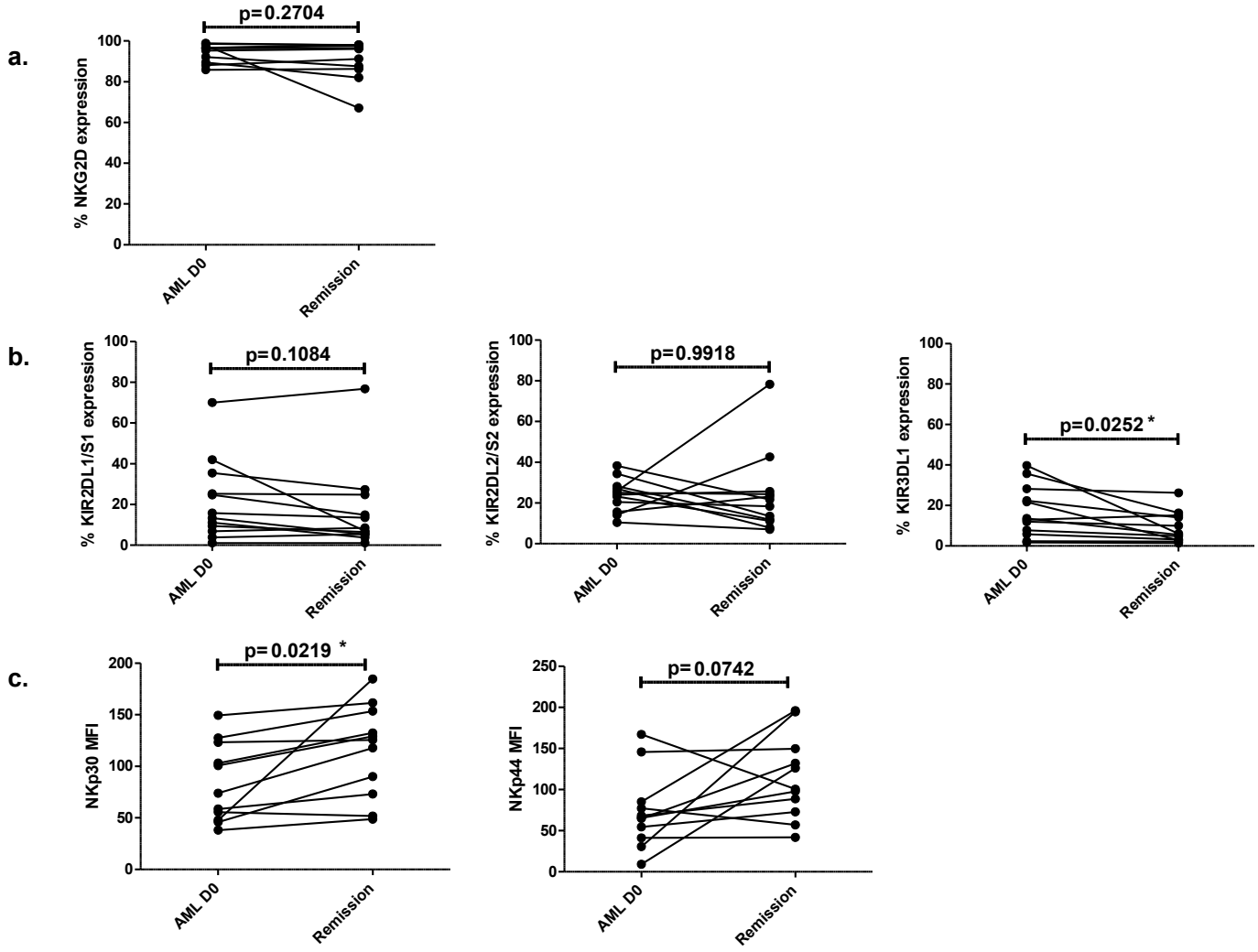


Figure 5.4 Expression at presentation and remission of NK receptors whose expression was not significantly altered at AML presentation a) NKG2D expression was unchanged in remission b) Expression of KIR2DL1/S1 and 2DL2/S2 were unchanged in remission. KIR3DL1/S1 was reduced c) NCRs tended to increase in AML remission compared to presentation (paired t tests).

5.3-iii- Is NK cell cytotoxicity abnormal in AML patients?

a. Cytotoxic abnormalities in all NK cells

Looking at the cytotoxicity of all CD56+, CD3- NK cells, there was strikingly reduced 107a (p=0.0002), TNFα (p=0.008) and IFNγ (p=<0.0001) production in AML patients towards leukaemia target cells (K562) compared to the NK cells of normal controls (fig. 5.5a). Fas ligand cytotoxicity was normal. When NK cell-mediated cytotoxicity in AML patients towards their own AML blasts was measured, cytotoxicity was as low, or lower than towards the K562 leukaemia cell line (fig. 5.5b).

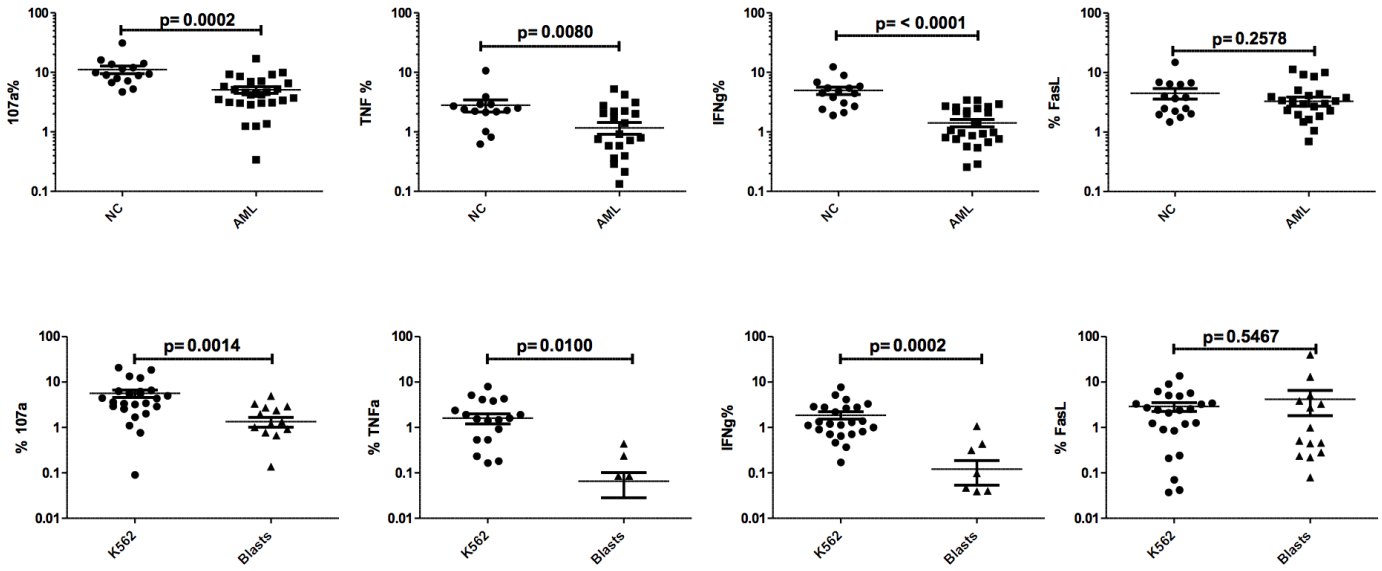


Figure 5.5 NK cytotoxicity and effector function in AML patients at diagnosis compared to healthy controls a) Total NK (CD56+, CD3-) cytotoxicity (CD107a degranulation), TNFα and IFNγ production and Fas ligand expression against K562 leukaemia targets in 32 patients with AML and 15 healthy controls. Cytotoxicity towards K562 was significantly reduced in AML patients compared to normal controls in all measures except Fas ligand b) NK response at AML presentation to autologous blasts was as defective or more defective than towards K562 cells in all measures except Fas ligand. Horizontal bars denote mean expression. Abbreviations: NC: healthy control, AML: acute myeloid leukaemia (unpaired t tests).

b. Are cytotoxic abnormalities restricted to NK cells expressing particular KIRs?

Cytotoxicity of KIR+ NK cells (fig. 5.6a) and KIR- NK cells (fig. 5.6b) towards leukaemic cells was significantly reduced in AML patients compared to normal controls. There was no difference between one particular KIR positive NK clone and another. The defect is seen in NK cells expressing all the different KIR receptors assessed. There was however, more cytotoxicity from KIR positive cells than from KIR negative cells.

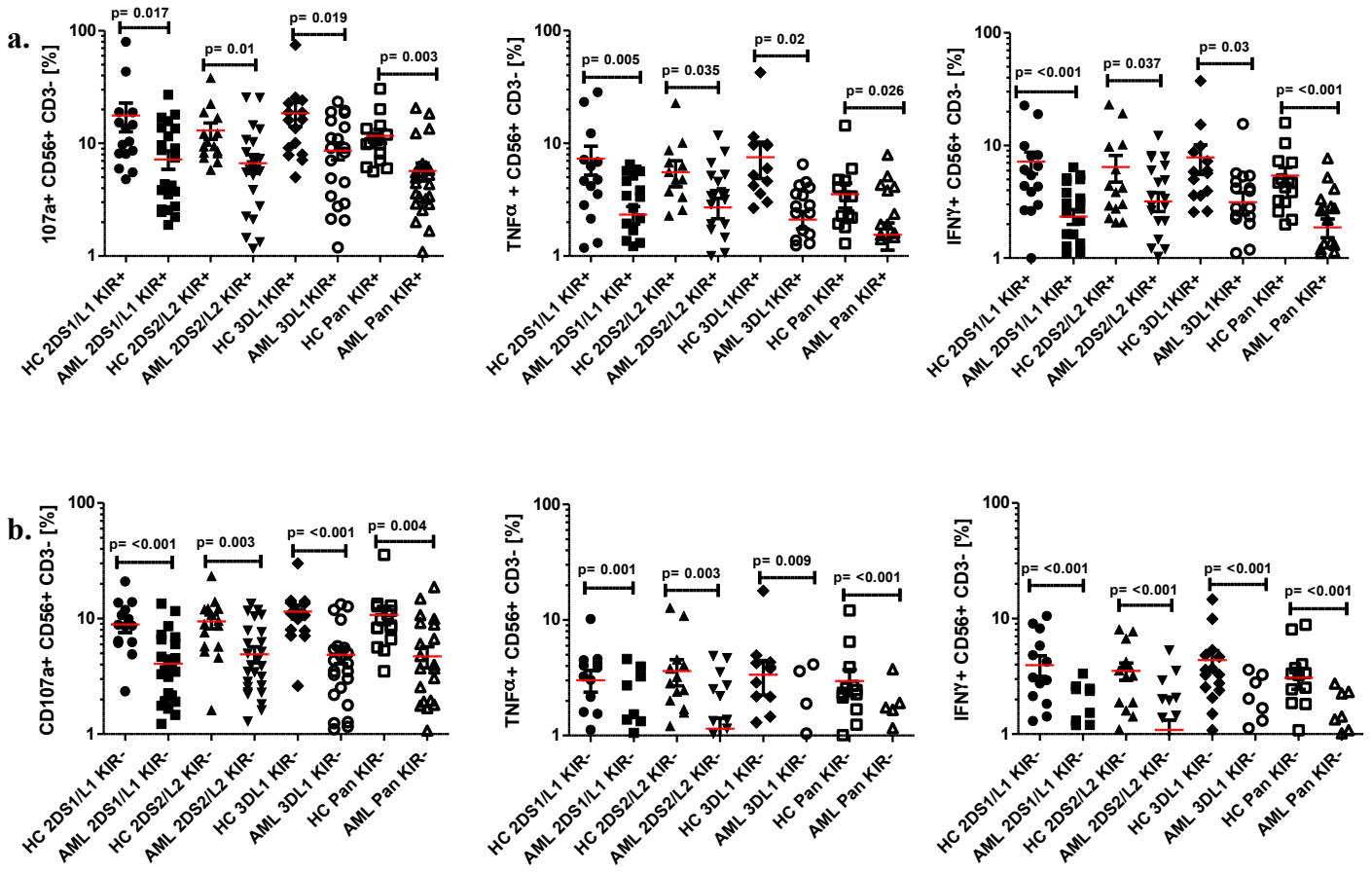


Figure 5.6 KIR specific NK cytotoxicity and effector cytokine production in AML patients at diagnosis compared to healthy controls. a) comparison of NK cytotoxicity and effector cytokine function in KIR+ subsets; b) comparison of NK cytotoxicity and effector cytokine function in KIR- subsets. Figures show greater cytotoxicity and effector cytokine production from healthy control NK than AML patient NK cells and from KIR+ than KIR- NK cells irrespective of which particular KIR is expressed. Horizontal bars denote mean within group. Abbreviations: HC: healthy control, AML: acute myeloid leukaemia (unpaired t tests).

c. Do cytotoxic abnormalities in NK cells affect all triggering mechanisms or are they restricted to cytotoxicity triggered by cell contact?

NK cells from AML patients stimulated with PMA and Ionomycin maintained their ability to degranulate and produce TNFα and IFNγ (fig 5.7). This is in marked contrast to the results in figures 5.5 and 5.6 showing AML patients having reduced NK function after stimulation with leukaemic cells. This indicates that the defect in AML is specific to NK stimulation by cell contact and that the abnormality is not a structural one of the NK cell itself but rather in its ability to be stimulated by leukaemic cells to kill.

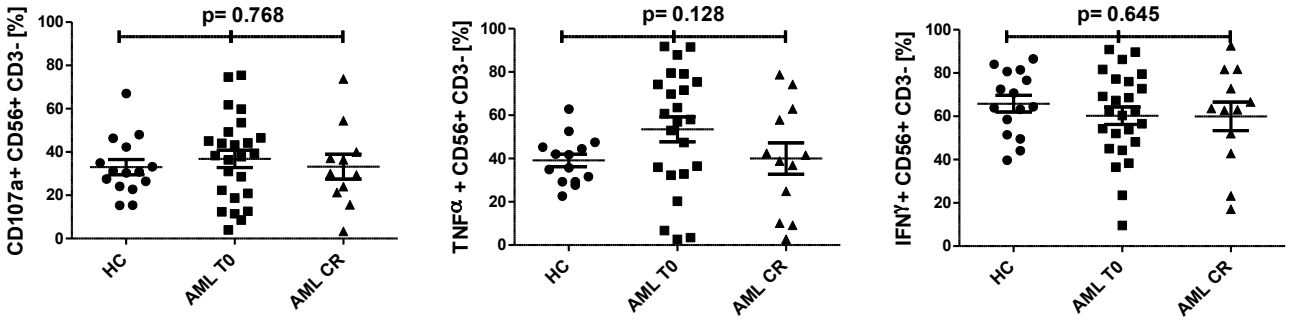


Figure 5.7 NK cells stimulated with PMA/Ionomycin maintain normal function in AML patients. NK cytotoxicity (CD107a degranulation), TNFα and IFNγ production in AML patients at diagnosis (T0), remission (CR) and healthy controls (HC) following stimulation with PMA/Ionomycin. Horizontal bars denote mean expression. Error bars denote standard deviation between individuals within group. Three groups compared using one way ANOVA tests. Abreviations: HC, healthy control; T0, AML presentation; CR, complete remission.

d. Do KIR ligands affect the cytotoxicity of NK cells?

In view of a number of pre-clinical studies indicating that exposure to KIR ligands (in particular HLA A, B and C) is important for NK education both in facilitating cytotoxic function and in maintaining tolerance (see section 1.8), as well as clinical studies in SCT suggesting that differential NK-mediated GVL depends on inherited KIR-KIR ligand variability,[26,28,148] I investigated whether I could find correlates to this work in clinical samples from patients. In case any effect of KIR ligands on cytotoxicity was something specific to NK undergoing immune editing by AML blasts, I also looked in normal control and remission samples. I did not find any differences in cytotoxicity between individuals with different KIR-KIR ligand profiles either for inhibitory (fig. 5.8) or activatory (fig. 5.9) KIRs.

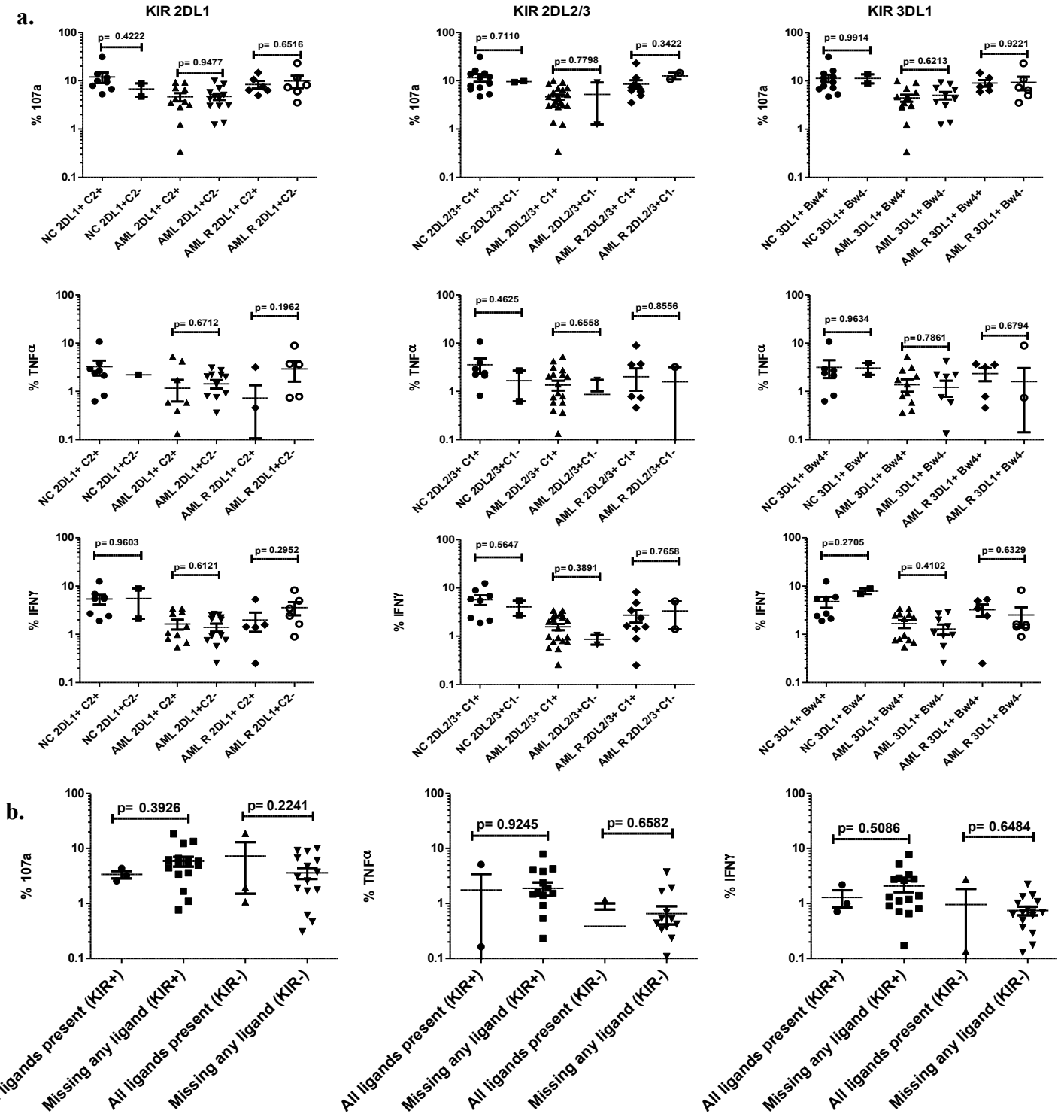


Figure 5.8 The role of inhibitory KIR ligands on NK cell (CD56+, CD3-) cytotoxicity towards K562 leukaemic target cells after 5 hour incubation. In each graph unpaired t tests compared those with a ligand for their inhibitory KIR (KIR2DL1+ C2+, first column; KIR2DL2/3+ C1+, second column, 3DL1+ Bw4+, third column) and those without in three groups: from left, healthy donors (NC), AML at presentation (AML) and AML at remission (AML R). Columns represent from left to right: degranulation, TNFα and IFN γ production. There was no beneficial effect of a) Missing a particular HLA ligand for inhibitory KIR or b) missing any HLA ligand for inhibitory KIRs on NK function.

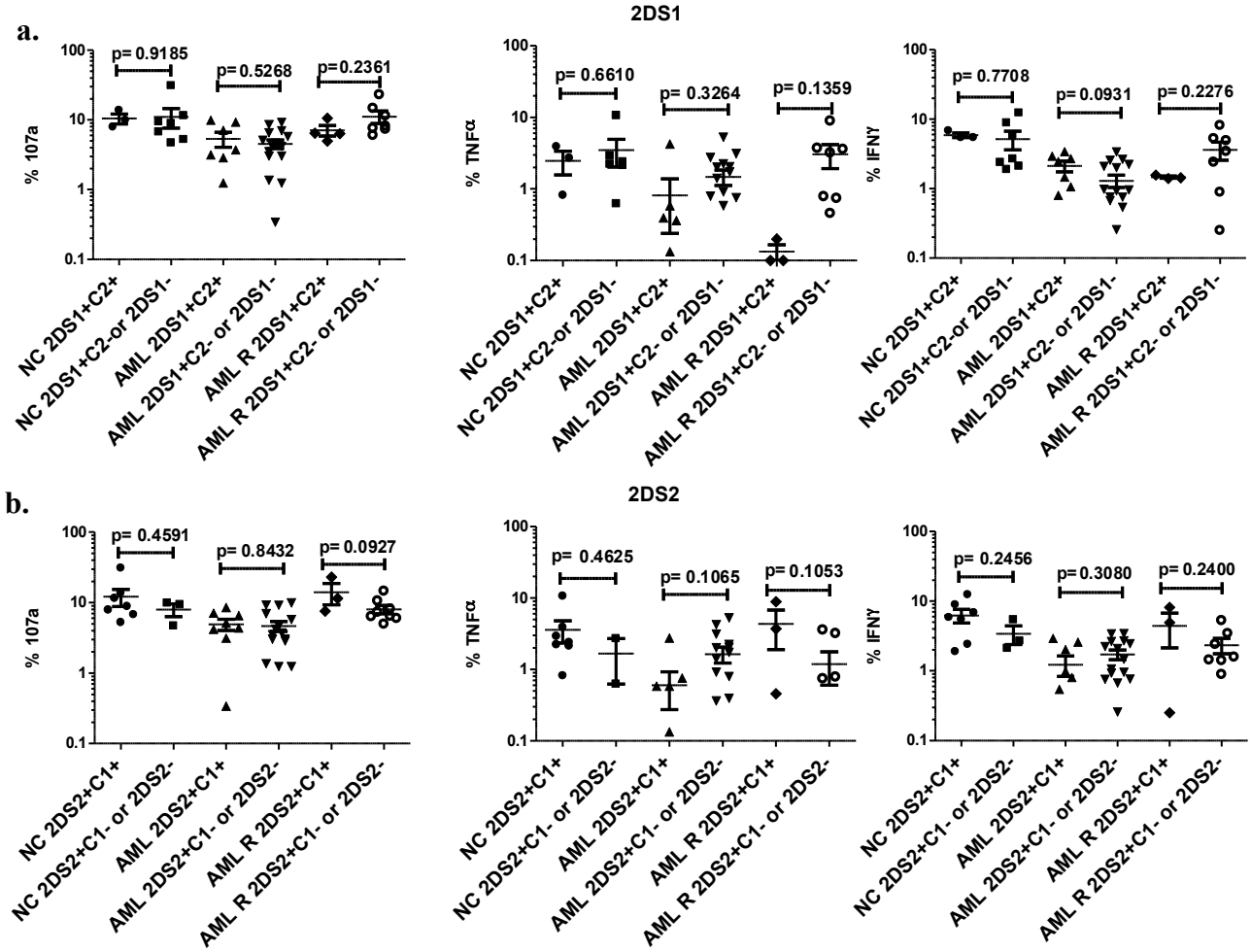
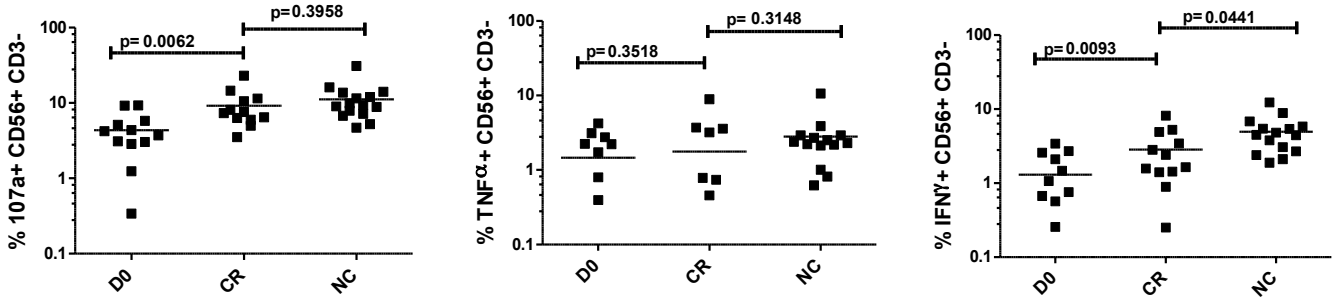


Figure 5.9 The role of activatory KIRs on NK cell (CD56+, CD3-) cytotoxicity towards K562 leukaemic target cells after 5 hour incubation. In each graph unpaired t tests compared those with a ligand for their activatory KIR (KIR2DS1+ C2+ or KIR2DS2+ C1+) and those without in three groups: from left, healthy donors (NC), AML at presentation (AML) and AML at remission (AML R). Possessing a ligand for a) 2DS1 or b) 2DS2 does not affect NK cytotoxicity or effector cytokine production in any group. Columns represent from left to right: degranulation, TNFα and IFN γ production.

5.3-iv-Do any cytotoxic abnormalities seen in AML normalise in remission?

The reduced NK cytotoxicity towards leukaemia cells in AML partially normalised in remission (fig. 5.10a). Degranulation function was restored and IFNγ production was partially restored. Patients who achieved remission were a subset who did not have abnormal TNFα production at diagnosis suggesting that this could be used to predict response to treatment (as further discussed in chapter 6.) Aside from a few individuals, there was no restoration of cytotoxic function towards autologous blasts after the first course of chemotherapy (fig. 5.10b).

a. K562 leukaemia cell line targets:



b. Autologous blast targets:

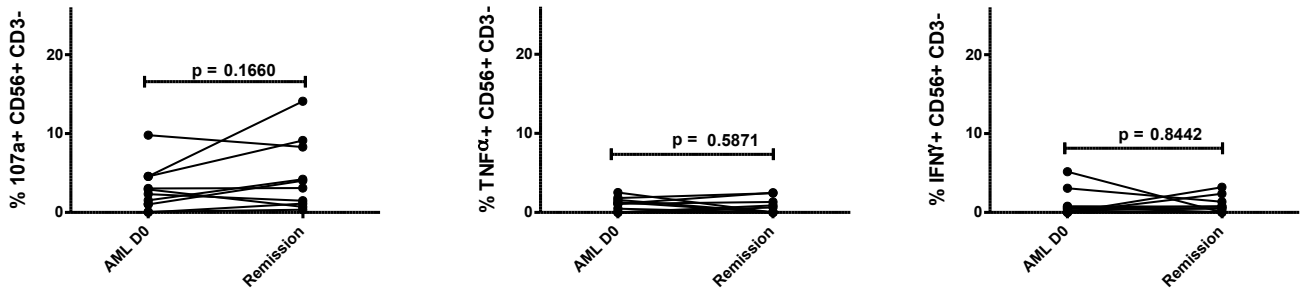


Figure 5.10 Partial normalisation of NK cytotoxic abnormalities in AML remission a) There was normalisation of degranulation and partial normalisation of IFNγ production but not TNFα towards K562 leukaemia cells. Paired t tests comparing D0 with remission, unpaired t tests comparing remission with healthy controls b) Aside from a few individuals, there was generally no improvement in cytotoxicity towards autologous blasts (paired t tests). Abbreviations: D0, AML presentation; CR, complete remission; NC, healthy controls

5.3-v-What is the repertoire of NK ligands on AML blasts?

Figure 5.11 shows that DR4/5 (the ligands for TRAIL), Fas (which binds Fas Ligand) and HLA E (the ligand for NKG2A), had a range of expression in different AML patients, with high levels on the blasts of some individuals and low or no expression on the blasts of others. All blasts expressed class 1 HLA molecules (HLA A, B, C-the ligands for KIR receptors) and these were not down regulated in these AML patients, in fact all blasts analysed had extremely high expression of HLA class 1. MICA/B (the ligands for NKG2D) expression however, was low or absent in most of the patients.

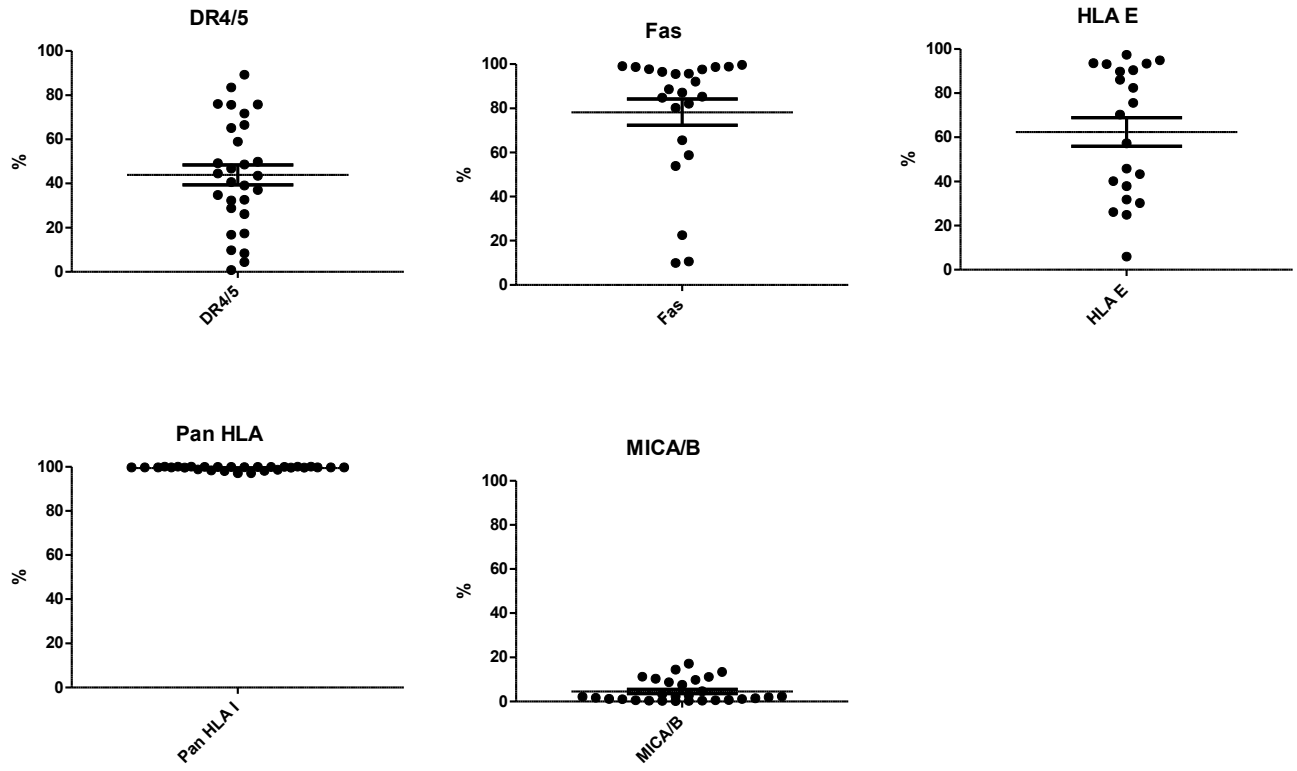


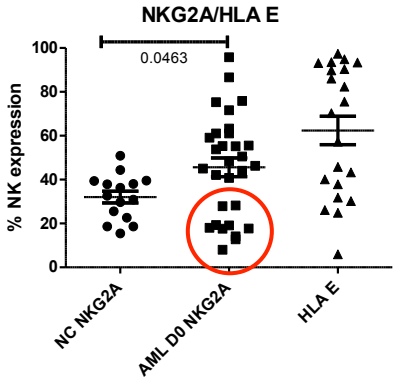
Figure 5.11 Expression of NK receptor ligands on the AML blasts of 32 patients. X axes represent the ligand of interest. Y axis represents percentage of AML blasts expressing that ligand.

5.3-vi-Is expression of NK ligands on leukaemic blasts associated with changes in NK receptor phenotype or function?

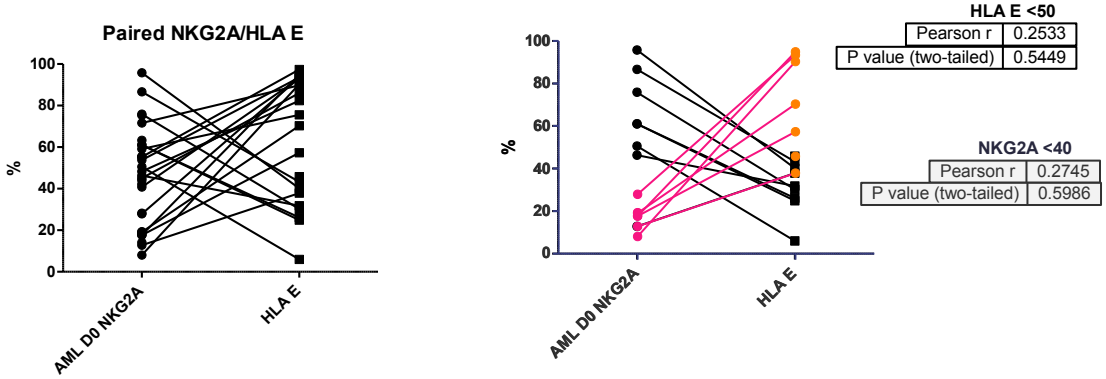
NKG2A and its ligand HLA E

Although I show above that NKG2A is significantly upregulated in AML (fig. 5.2d), it is clear that a subset of AML patients did not have high NKG2A levels (circled in red below, fig. 5.12a): their NKG2A expression appeared lower than normal controls. While this may just be normal variation, I thought it would be interesting to look at whether expression of HLA E, the ligand for NKG2A, influences expression of the receptor. I hypothesised that low HLA E expression on AML blasts may explain a low NKG2A expression, and that those individuals with high HLA expressing blasts may elicit a high NKG2A expression in response. What graphs 5.12b show, is that the opposite may be true. In patients who express low levels of HLA E on their blasts, NKG2A expression is high and in patients with high HLA E expression, NKG2A expression is low. In view of this pattern, I looked to see whether a ratio of HLA E to NKG2A at presentation could provide an informative predictor of NK cytotoxicity in AML patients but found no relationship with cytotoxicity (fig. 5.12c).

a.



b.



c.

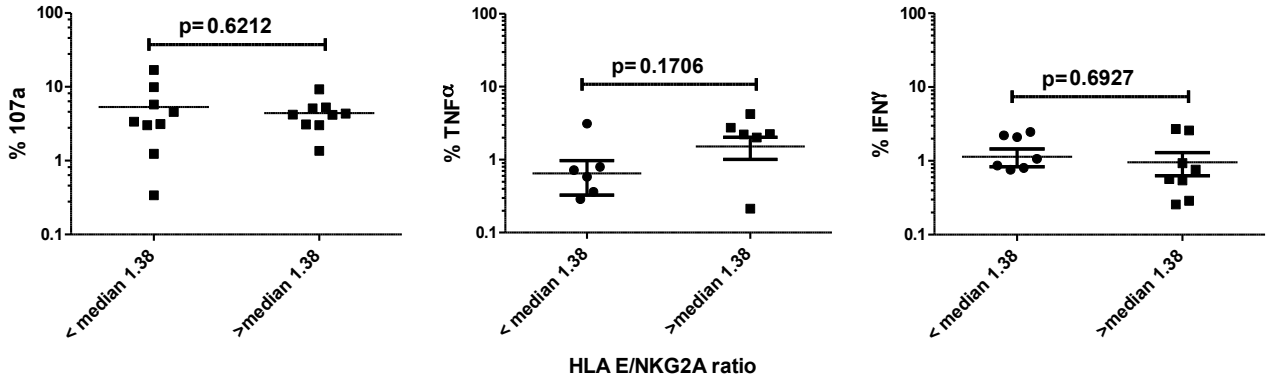


Figure 5.12 Relationship between NKG2A expression and that of its ligand HLA E in AML a) While most had a raised proportion of NKG2A expressing NK cells, a subset of AML patients had low NKG2A (red circle) b) Low NKG2A expression was associated with high HLA E expression on blasts, although this correlation did not quite reach statistical significance c) HLA E/NKG2A ratio did not predict NK cytotoxicity.

5.3-vii-Do individuals with abnormal phenotype have worse cytotoxic function?

Different abnormalities in NK surface receptor phenotype were associated with different abnormalities in cytotoxic function: high NKG2A was associated with reduced TNFα production (fig. 5.13a) and low NKp46 expression with reduced degranulation and IFNγ production (fig. 5.13b).

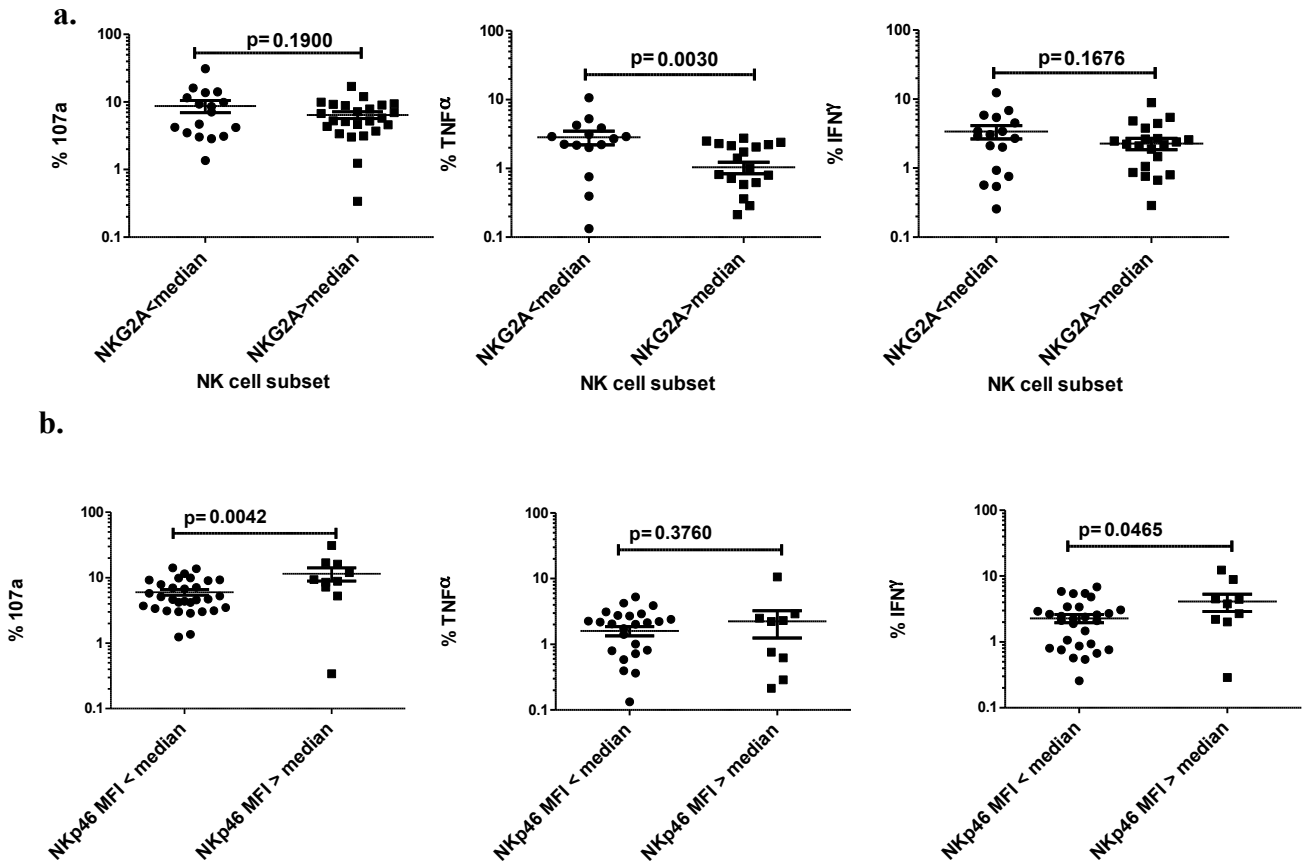


Figure 5.13 The effects of abnormal NK surface phenotype on NK cytotoxicity and effector cytokine production. Individuals were separated into those with receptor levels above and below the median. Unpaired t tests compared the two groups a) High numbers of NK expressing surface NKG2A were associated with reduced TNFα b) Low NKp46 was associated with reduced CD107a and IFNγ production

5.3-viii-Is it possible to induce abnormalities by exposing normal NK cells to AML blasts?

To test the effect of AML on NKp46 and NKG2A expression, ex-vivo selected NK cells from healthy controls were co-incubated with primary AML blasts from patients at a 10:1 ratio for 24 hours and their phenotype assessed in 4 independent experiments. There was no significant difference in the expression of NKG2A and NKp46 in control NK cells after co-culture with leukaemia cells + IL-2 (200 iU/mL) compared to NK cells incubated for 24 hours in the absence of AML blasts + IL-2 (200 iU/mL)(data not shown). The effect of AML blasts on cytotoxicity and effector function of peripheral blood NK cells from 3 healthy controls was then tested. After 24 hours co-culture of NK cells with primary AML blasts (ratio 10:1) + IL-2 (200 iU/mL), the NK effector function was assessed against K562 leukaemia targets (ratio 1:1). Co-incubation resulted in significant impairment of NK cytotoxicity and effector function with marked reduction in TNFα production (p=0.02), IFNγ production (p=0.01) and a trend to reduced CD107a degranulation (p=0.07) against K562 leukaemia targets (fig. 5.14). Experiments performed without Il-2 resulted in poorer NK cell yield after incubation but a similar pattern of NK inhibition to that seen in cells cultured with Il-2. Using NK from the same healthy individual I assessed the effect of cells from two different AML patients on the function of healthy NKs and found that both inhibited cytotoxicity (fig. 5.15).

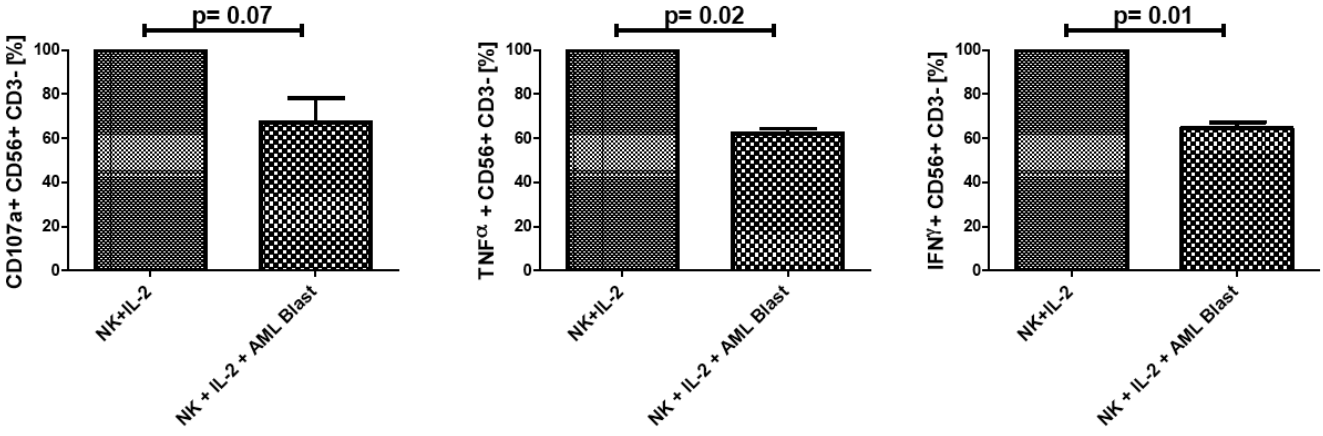


Figure 5.14 AML cells induce impairments in NK effector function in vitro. The reduction in healthy donor NK CD107a degranulation, TNFα and IFNγ production against K562 leukaemia target after 24 hours of co-culture with primary AML blasts (ratio of 10:1) + IL-2 (200 iU/mL), is presented. The results are expressed as a normalised ratio against healthy donor NK.

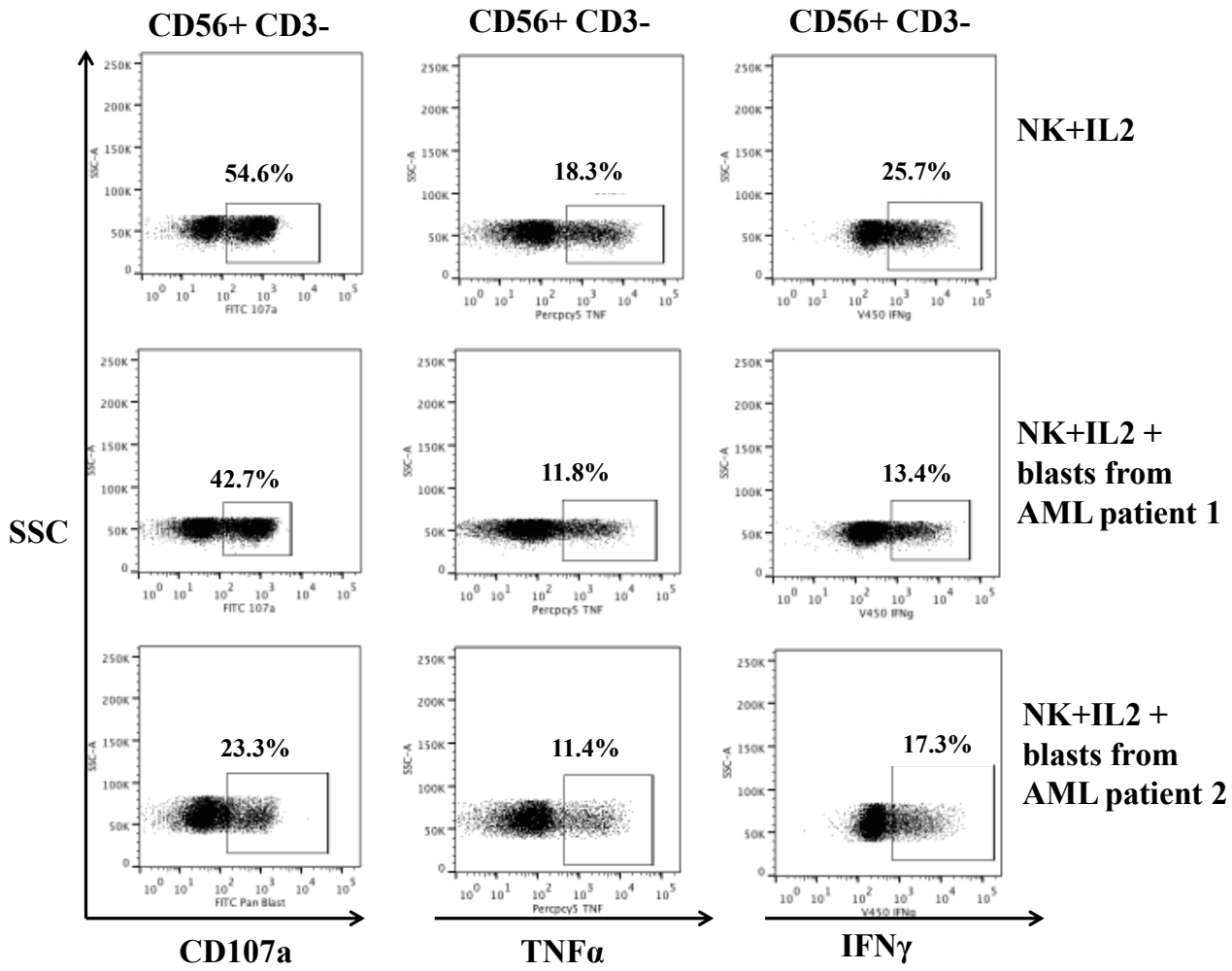


Figure 5.15 Representative FACS plots showing the effect of co-incubating AML blasts from 2 different patients on healthy donor NK cell cytotoxicity and effector function are presented. After 24 hours of healthy donor NK co-culture with primary AML blasts (ratio of 10:1) + IL-2 (200 iU/mL), the effector function of NK cells was assessed against K562 leukaemia targets (ratio 1:1). Plots are gated on CD56+CD3- NK cells.

Summary of results

In section 5.3 I showed that in AML patients, the NK receptor surface phenotype was different to that of normal individuals. There were greater numbers of cells expressing the inhibitory receptor NKG2A and lower expression of the activatory receptor NKp46. This abnormality creates an imbalance in the circulating NK cell population in AML, with cells more predisposed to inhibit cytotoxicity than to activate. I then showed that NK cytotoxicity towards leukaemic cells was reduced in AML patients compared to normal controls and that this reduction affected both degranulation and production of cytokines TNFα and IFNγ but was not specific to KIR-expressing NK cells. The abnormal cytotoxic function in AML only occurred when NK were stimulated by contact with leukaemic target cells, the ability of AML NK to be activated by other stimulants

(PMA/Ionomycin) remained intact. I then show that there is partial restoration of NK phenotype and function in AML patients who achieve remission. Finally, I show that the abnormalities seen in AML patients are inducible in the NK of healthy donors after incubation with AML blasts, suggesting that the AML 'edits' the immune system by impairing the ability of NK cells to recognise and kill enabling it to evade the host immune response.

5.4 Discussion

In this chapter, I showed in a prospectively collected cohort of 32 AML patients that NK cells of AML patients have an abnormal surface phenotype and reduced cytotoxic function. I found evidence for immune editing of NK cells in AML with a shift in the balance of NK surface receptors away from NK activation towards NK inhibition and a reduced ability to degranulate and produce cytokines. My findings suggest a dysfunction in NK-mediated immune surveillance in AML and identify specific areas of NK dysfunction, which could be targeted by future therapeutic interventions. I showed that while some of these changes normalised in remission, some did not, possibly explaining the high risk of relapse post chemotherapy and why adoptive immunotherapy of autologous NK cells in AML has had limited treatment benefits. The discussion of my results is structured around my aims for this chapter.

Is the NK surface receptor phenotype in AML different to that of healthy individuals?

My finding that numbers of NK expressing the inhibitory receptor NKG2A was raised and expression of the activatory receptor NKp46 was reduced in AML patients compared to normal individuals provides evidence in support of the hypothesis that AML blasts can potentially edit the immune system to avoid elimination by NK cells. A raised NKG2A level has also been seen in hepatitis C (HCV) infection[238,239] where it is associated with reduced cytotoxic killing of infected cells[239] suggesting that a similar process may occur in infective disease. Further support of our observation that NKG2A levels are important for regulating immune responses comes from experiments in patients with psoriasis, an autoimmune disease, where low NKG2A levels occur.[240]

My finding of NKp46 down regulation in AML is supported by work from Costello et al, showing a reduction in NKp46 expression in AML patients.[174,231] Our results do however, differ in that they also found a reduction in NKp30 and no change in NKG2A. One possible reason for this discrepancy is that they used different methods for gating NK cells. While we excluded blasts with a separate gate (CD13, 33, 34) and then identified CD56+, CD3- cells to phenotype, they excluded blasts using low expression of CD45 without other steps to remove any blasts which may be CD45 high or considering NK cells which may be CD45 low. It is possible therefore that Costello's NK cell population differed significantly to ours. They also excluded all patients with blast counts over 50 because they found it difficult to separate the NK population from these samples. It is therefore also possible that these excluded patients may explain why our results are different. In our cohort, 16% of AML patients (5/32) had blast counts >50 x 10^9/L.

Do any surface receptor abnormalities seen in AML normalise in remission?

The partial restoration of normal NK phenotype in AML remission where NKp46 normalises but NKG2A continues to climb has also been seen in melanoma patients post chemotherapy[241] and on clearing the HCV virus.[239] The normalization of surface receptors might be expected when NK are no longer exposed to AML blasts; however, it is unclear why NKG2A continues to rise. It is possible that this increase could be secondary to chemotherapy. AML chemotherapy interferes with regenerating bone marrow stromal cells[242] which enable NK cells to develop a mature phenotype.[243] Chemotherapy may also interfere with dendritic cells, which are important for normal NK cytotoxic function.[244] High NKG2A levels are seen after ATG induction treatment for kidney transplant[245] where they normalise after a year. It has also been shown that in cytotoxic T cells, Il-15, TGFβ and Il-12 are associated with up regulation of NKG2A in vitro[246-248] suggesting that chemotherapy-induced changes in the cytokine milieu might explain continuing abnormalities in NK surface receptors. Also, after SCT, high Il-15 levels are associated with higher activatory receptors[249] and Il-15 can be used *in vitro* to induce this in cells from AML patients,[250] suggesting that it may be possible to use Il-15 therapeutically to reverse some of the changes we found in NK cells.

Is NK cell cytotoxicity abnormal in AML patients?

AML NK cells degranulated significantly less ($p=0.0002$), and produced significantly less TNFα ($p=0.008$) and IFNγ ($p=<0.0001$) in response to leukaemia target cells compared to NK cells from healthy volunteers. This further supports the notion that AML blasts increase their chances of survival by altering or 'immunoediting' the NK response. The finding that KIR-expressing cells exhibited more cytotoxicity and cytokine production than KIR-negative NK cells supports my conclusion in chapters 3 and 4 that KIRs play a role in shaping the immune response to leukaemia. However, the fact that this effect was seen with all KIR-expressing cells with no difference found between cells expressing one type of KIR from cells expressing another, means we did not demonstrate a mechanism for why particular KIR genes such as 2DS2 may be beneficial. One limitation of my method, which may explain this, is that KIR antibodies cannot as yet distinguish between activatory and inhibitory KIRs. Therefore an increased cytotoxicity from activatory KIR-expressing NK cells, could have been masked by the fact that cytotoxicity from inhibitory KIR-expressing cells was measured with them. It will be interesting to repeat these experiments when further developments in biotechnology allow us to better differentiate the two. Of course, a further possibility is that, as a range of receptor-ligand interactions as well as chemokines and cytokines play a role in triggering cytotoxicity on target cell recognition[251] it may be that although KIR+ cells are more cytotoxic than KIR- ones, KIRs are not the primary factor in this process.

A possible mechanism by which AML may induce abnormal NK-mediated cytotoxicity, is abnormal expression of the immunosuppressive tyrosine-converting enzyme Indoleamine 2, 3-dioxygenase (IDO). This has been suggested as a mechanism for reduced NK cytotoxicity in melanoma patients.[252] Abnormal IDO expression also influences T cell function in AML patients.[155] In view of these data, I looked to see if this was the case for NK cells in AML. I identified which of my cohort were IDO positive and which negative by RT PCR using cDNA extracted from my samples. However, I did not find a difference in NK cell function between NK pre-incubated with IDO positive and IDO negative blasts (data not shown). Although further experiments would be needed to confirm, it does suggest that IDO is not important for NK function in AML. I also looked to see whether down-regulation of transcription factors such as eomesodermin in NK cells exposed to AML blasts may explain our findings in view of data from a murine AML model suggesting that this mechanism is associated with reduced expression of NK activatory receptors, cytotoxicity and cytokine production.[157] I did not find that eomesodermin was down regulated in human NK cells exposed to AML blasts (data not shown). A further mechanism which may be interesting to explore, is high expression by AML blasts of the immunosuppressive type 1 immunoglobulin CD200 which has been linked to reduced NK cytotoxicity in AML.[253]

Cytotoxicity and cytokine release from AML NK cells in response to PMA/ Ionomycin stimulation was preserved. This suggests that the immune editing process is targeting the immune synapse. Synapse dysfunction occurs in AML patient T cells where AML induces an inability in T cells to form functional immune synapses with AML blasts due to abnormal actin cytoskeletal formation.[11] It is therefore possible that a similar mechanism may explain our data for NK cells.

Do any cytotoxic abnormalities seen in AML normalise in remission?

I showed that the reduced NK cytotoxicity towards leukaemia cells in AML partially normalises in remission. Degranulation function is restored, IFNγ production is partially restored, but TNFα production remains abnormal. A split in abnormal NK degranulation and cytokine-producing function is also seen after URD and cord blood SCT.[254] *In vitro* experiments in mice show that NK cells exposed to AML blasts lose their cytokine-producing and cytotoxic function at different time points after exposure to disease.[157] Different immunosuppressive drugs used post SCT affect NK receptor expression and cytotoxic function differentially: cyclosporin (CSA) is associated with NKG2A+ NCR + KIR- expressing NK cells and reduced IFNγ production but normal cytotoxic function, whereas Rapamycin and Mycophenolic acid (MPA) treatment (MPA is the active metabolite of Mycophenolate) is associated with NKG2A- NCR- KIR+ NK cells and the loss of both

cytotoxic and IFNγ production[255]. While there is less literature about the specific effects of induction chemotherapy on NK function it is possible that a similar mechanism could explain my results.

In the process of NK maturation, NK cells acquire degranulation activity before they start producing IFNγ.[111] As these effector functions are different to start with, it is not entirely surprising that exposure to AML cells might affect one more than the other. There is a wealth of evidence to suggest that production and secretion pathways of granules and cytokines are different: Cytokines are produced *de novo* on target cell stimulation, whereas granules are preformed. Perforin and cytokines are trafficked to the cell surface in separate carriers: vesicles and recycling endosomes respectively.[71] Vesicle secretion is highly polarised via the actin cytoskeleton for release only at the target cell synapse whereas cytokines are released at sites all over the cell surface.[71] Also, signal transduction pathways may be different even downstream of the same receptor: Vav-1 is required for cytotoxicity but not IFNγ production,[256] DAP12 is required for IFNγ secretion but not cytotoxicity,[257] CD45 is required for cytokine production (via DAP12/syk/zap70) but not cytotoxicity (via DAP10),[258] PI3K isoforms p110γ and p110δ are important in cytokine production but not cytotoxicity[259] and Phospholipase C-2 is essential for cytotoxic granule secretion but not cytokine production.[260] AML is heterogeneous and its effects on NK cells may also be heterogeneous. A larger study cohort enabling us to separate AML subtypes is required to understand immune editing in different AML groups.

What is the repertoire of NK ligands on AML blasts?

My results of NK ligand expression on AML blasts show no reduction in HLA class 1 in AML, low levels of MICA/B, and variable expression of death receptors 4 and 5, HLA E and Fas. Previous studies have indicated that HLA class 1 ligands are usually down-regulated in AML patients and that this is a major mechanism by which tumor cells escape NK mediated immune surveillance.[163] We did not replicate this finding. Possibly this is because we used a pan class 1 antibody which included HLA-A, whereas it may be that only HLA B and C alleles class 1 alleles are down regulated in AML.[163] My results showing low or negative MICA/B expression on the blast surface are supported by other studies showing similar results.[164,261] It is also consistent with the mechanism that AML cells shed their surface MICA into the serum and thereby avoid the NKG2D-mediated immune response.[164,166]

Is expression of NK ligands on leukaemic blasts associated with changes in NK receptor phenotype or function?

There appeared to be a pattern of individuals with high NKG2A having lower expression of the NKG2A ligand, HLA E but this correlation was not statistically significant and HLA E expression did not correlate with cytotoxic changes in AML. Upregulated HLA E has been reported in a number of tumours including lymphoma,[262] colon cancer,[263] melanoma[264] and AML.[265] HLA E expression on AML blasts is induced by IFNγ produced by CD56 bright NK cells post SCT. As higher HLA E expression encourages NKG2A-mediated inhibition of NK cells, modification of HLA E is therefore an example of tumour immune evasion.[265] Tumor expression of HLA E may be reduced with selenium treatment leading to increased killing of AML tumour cells via less NKG2A-mediated inhibition[266] indicating that HLA E expression may be exploitable in a clinical setting. We did not find a relationship between expression of HLA E on blasts and NK cytotoxicity or response to treatment in our cohort, however. The exact role HLA E in AML has yet to be clarified. This may be due to the small cohort size, or it could indicate that no relationship between HLA E and AML exists. Certainly data about its role in other cancers has been conflicting with high HLA E expression associated with both better prognosis[267] and worse prognosis in colon cancer.[268] One further possibility is that the role of HLA E in AML has a greater effect on the NKG2A expressed by cytotoxic T cells and further work should be directed towards T cells rather than NK cells.[269]

Do individuals with abnormal phenotype have worse cytotoxic function?

Different abnormalities in NK surface receptor phenotype are associated with different abnormalities in function: high NKG2A being associated with reduced TNFα production and low NKp46 expression being associated with reduced degranulation and IFNγ production. This separation of different functions is similar to that occurring in remission discussed above under aim 4 and may be explained in a similar manner. My findings that raised NKG2A in AML patients is associated with reduced cytotoxicity are consistent with data from stem cell transplantation (SCT), showing that raised NKG2A levels are associated with reduced cytotoxicity and a reduced GVL effect towards AML.[270] This paper also suggests that NKG2A-mediated NK interactions may be more important in inhibiting GVL towards AML post SCT than the absence of a KIR-KIR ligand mismatch, which also corroborates my data finding no clear effect of a mismatch on cytotoxic NK function.

Is it possible to induce abnormalities by exposing normal NK cells to AML blasts?

Since abnormal NK cells were found in AML patients, and remission reversed some of the abnormal phenotypic and functional changes in AML-NK cells, I explored the ability of primary AML blasts to modify NKp46 and NKG2A expression and effector function in healthy control NK cells. Although there was no change in NKp46 and NKG2A expression, co-culture of AML blasts with healthy donor NK cells impaired their effector function and cytotoxicity. These findings imply that while NK cells from healthy donors can exert anti-leukaemia effects, the immunotherapeutic benefit from NK cell immunotherapy and the GVL effect may be limited by an immune-editing function of AML cells. Il-2 is often given as part of adoptive therapy to support NK expansion when NK are infused into AML patients. I therefore investigated whether this cytokine might prevent immune editing by AML. In my experiments, a similar pattern of NK inhibition was seen with and without IL-2, indicating that Il-2 does not overcome the immune editing effects of AML. Future studies to investigate whether the addition of cytokines such as Il-15, IFNγ or TGF-1 inhibitors [250] could circumvent this immune editing process and improve the function of NK immunotherapy will be interesting to pursue.

The main limitation of this work is that my study has limited power. More experiments on greater numbers of patients may strengthen my findings. As AML is a relatively rare disease, I was curtailed in collecting samples from individuals as and when they presented between September 2009 and early 2012. However, my data demonstrate significant abnormalities in the NK response to AML supporting the concept that immune editing by AML facilitates its survival.

Chapter 6 Using NK studies to predict clinical outcome in AML

In the previous chapter, I demonstrated abnormalities in the NK-mediated immune response towards AML and showed results relevant to the concept of immune editing in this disease. My data also provide functional support for the importance of the NK-mediated immune response to AML. What has not yet been shown is whether studying NK cell responses can help predict the outcome of patients with AML. I have collected blood samples, cytogenetic and clinical data from AML patients between 2009 and 2012 and have recorded the treatment regimen and outcomes of these patients. In this chapter I test whether abnormalities in NK cell phenotype and function predict clinical outcome in AML.

6.1 Background

As discussed in section 1.1, there are a number of known factors which impact upon clinical outcome in AML. These can be divided into factors associated with the patient and factors associated with the disease. Patient factors associated with poor clinical outcome include advanced age and performance status (how able the patient is to carry out the activities of daily living).[271] Disease related factors include whether AML has occured secondary to therapy for a previous malignancy or other haematological disorder, [272] as well as cytogenetic abnormalities[2,3] [273] and molecular mutations.[274] It is important to note that cytogenetic and molecular changes can help predict good as well as poor outcome (see table 1.1). Another patient related factor is the strength of NK-mediated immunity towards AML. This has not yet been looked at and I will do so in this chapter, which addresses the following aims:

6.2 Aims of this chapter

i-What are the clinical outcomes of the Hammersmith Hospital cohort of AML patients?

ii-Does having secondary AML impact upon NK immunity and outcome compared with *de novo* AML?

iii-Do NK surface receptor abnormalities in AML affect response to treatment?

iv- Do cytotoxic and effector cytokine abnormalities in AML predict response to treatment?

6.3 Results

6.3-i-Clinical Outcomes

Clinical outcomes of the 32 AML patients studied in chapter 5 are presented in table 6. 1. This cohort had a high proportion of poor-risk patients: half had secondary AML (see table 5.1 in previous chapter) and nearly half went on to have stem cell transplantation (SCT) in first remission. This makes looking at typical outcome measures such as relapse and survival more difficult because any data collected post SCT will correspond to donor NK immunity rather than patient. However, response to the first course of chemotherapy is a valuable outcome measure, which I have for all but one patient (who left the UK before treatment). In this cohort, only half the patients achieved complete remission after the first course of chemotherapy (DA: Daunorubicin and Cytarabine), with a further five patients doing so after a second or occasionally a third course of salvage treatment (typically Flag-Ida chemotherapy: Fludarabine, Cytarabine, GCSF and Idarubicin). A third of patients either did not respond at all or were too frail to undergo chemotherapy treatment.

Table 6.1 Clinical outcomes of AML patients

Clinical Outcomes	**No. (%)**
No. patients	31 (1 outcome unknown: refused Rx, left UK)
Complete remission after #1 (DA 3+10)	15 (48%)
Eventual CR (after 2-3#s Flag Ida)	5 (16%)
No Response to chemo/not fit for Rx	11 (36%)
Outcome at 6 months post chemotherapy for those achieving first remission: n=19	
SCT in CR1	8 (two relapsed < 1 yr, both died, 6 alive in CR)
SCT in CR2	1 (currently in CR 6 months post SCT)
Relapsed, no further Rx	3
Alive without SCT	2
Non-leukaemia death	2 (one sepsis during #2, one cardiac during #3)
Not yet 6 months post chemo but in CR	4

Abbreviations: CR, complete remission; SCT stem cell transplantation; Rx, treatment

6.3-ii-Does having secondary AML impact upon NK immunity and outcome compared with *de novo* AML?

In view of the large numbers of secondary AML patients in this cohort, and my results from chapter 4 suggesting that secondary AML may have distinct kinetics which facilitiate NK-mediated immunity in AML, I investigated whether this predicted response to chemotherapy. Secondary AML is generally thought to be associated with poor outcome.[272] In this cohort, as figure 6.1a shows, there was no difference in response to induction chemotherapy between patients with secondary and *de novo* AML. Surprisingly, patients with secondary AML were as likely to respond as *de novo* AML. This corresponds to my functional data (fig. 6.1b) showing similar NK cytotoxicity in secondary and *de novo* AML.

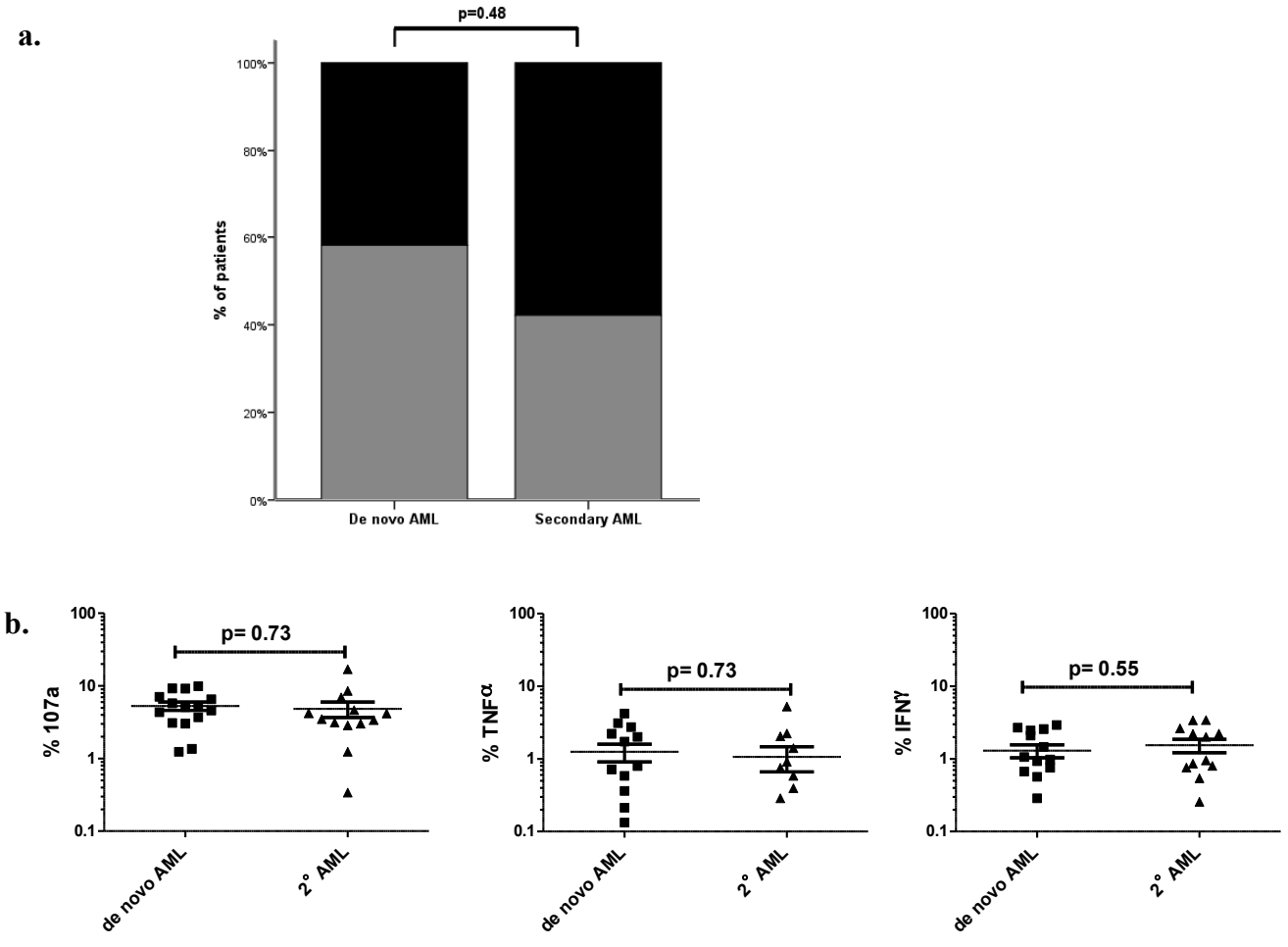


Figure 6.1 Secondary versus *de novo* AML: response to treatment and NK cytotoxicity. There is no significant difference between those with *de novo* AML (n=16) and secondary AML (n=16) with respect to a) response to first line treatment (Chi square test) and b) NK cytotoxicity (unpaired t tests). Grey bars represent proportion of patients achieving complete remission.

6.3-iii-Do NK surface receptor abnormalities affect response to treatment?

Those with an abnormally high frequency of NKG2A expressing NK cells, were less likely to respond to chemotherapy (31% achieved CR compared to 78% of those with a frequency lower than the median, p=0.041). Interestingly, individuals with low NKp46 expression at presentation did not have a higher risk of failing chemotherapy (fig 6.2).

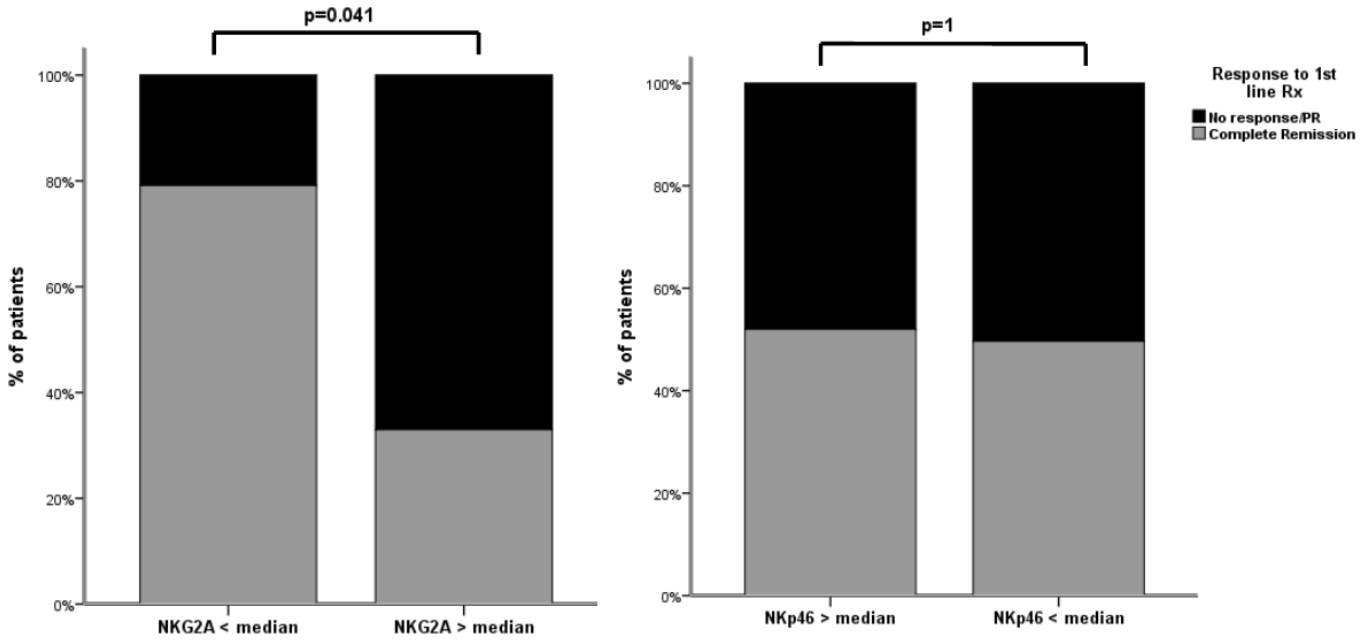


Figure 6.2 NK surface receptor phenotype and response to AML chemotherapy. Receptor expression in 32 AML patients was assessed. Patients were divided into those whose levels of NKG2A and NKp46 were above or below the median. Figure shows that high NKG2A predicted poor response to treatment. Low NKp46 did not.

Having shown that frequency of NKG2A expressing NK cells predicted response to treatment, I looked whether the combination of levels of NKG2A and expression of its ligand HLA E on AML blasts at diagnosis could be an even more useful marker for predicting response. Using my data in chapter 5 (fig. 5.12 showing that patients with high NKG2A often have low blast HLA E expression), I created a ratio of HLA E to NKG2A expression. Figure 6.3 shows that this ratio is a helpful predictor of treatment outcome with higher ratios indicating better response.

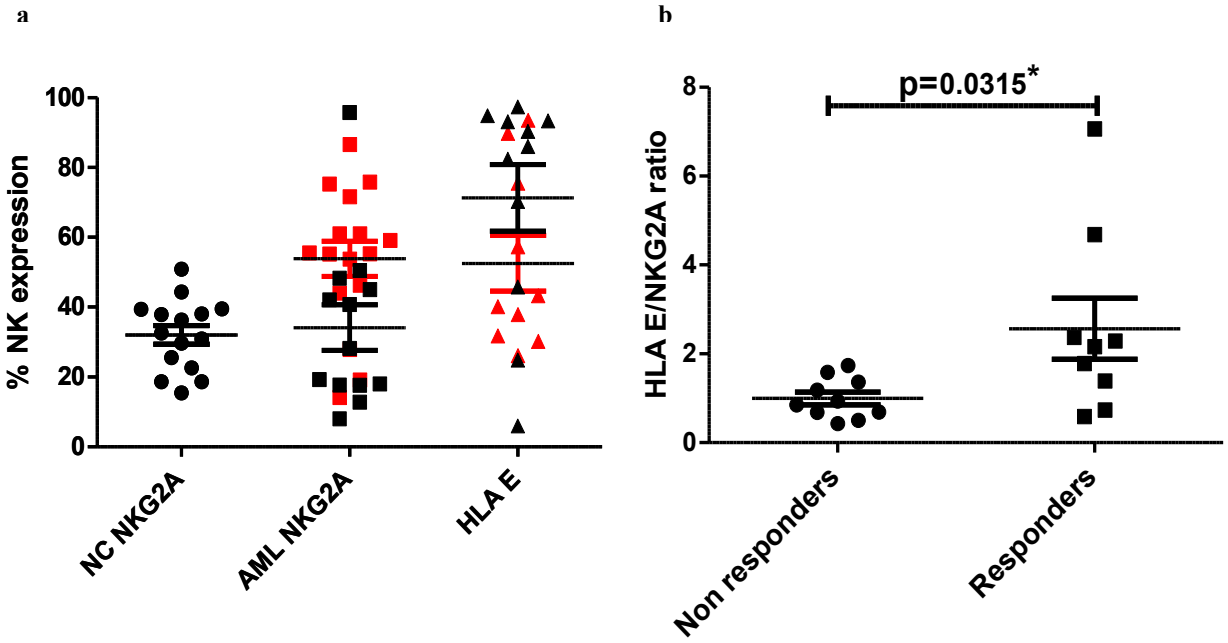
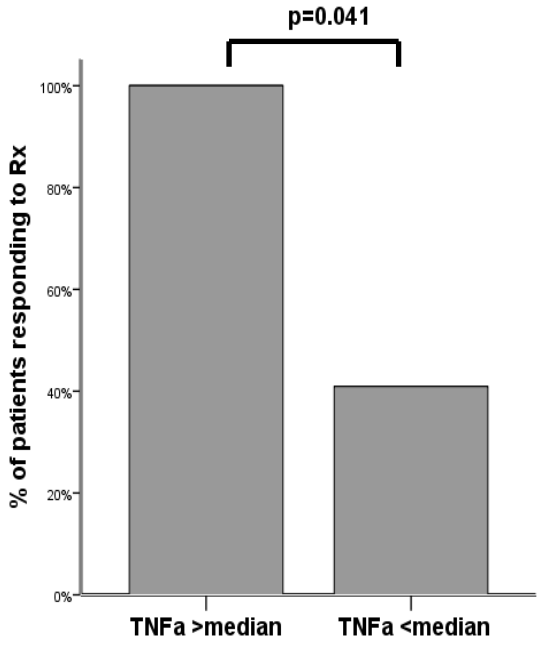


Figure 6.3 NKG2A and HLA E together helped predict response to treatment. a) Non-responders (in red) showed a pattern of higher NK NKG2A levels and lower blast HLA E expression than responders (in black) b) The HLA E/NKG2A ratio is significantly higher in those who respond to AML treatment.

6.3-iv-Do cytotoxic and effector cytokine abnormalities in AML predict response to treatment?

Looking at abnormalities at AML presentation using median cut-offs to separate those with high and low cytotoxicity and effector cytokine producing function, NK production of TNFα at D0 predicted those who will achieve remission with induction chemotherapy. All AML patients presenting with levels of TNFα production greater than the median achieved CR, compared to only 39% (9/23) of patients with TNFα production below the median (p=0.041) (fig. 6.4a). There was no impact of NK degranulation or IFNγ production at diagnosis on the probability of achieving remission post-chemotherapy. Also, those who did not respond to induction chemotherapy had TNFα levels significantly lower than normal controls (p=0.019) (fig. 6.4b), with a possible (but not statistically significant) gradient where higher D0 TNFα indicated more rapid response. These results suggest that TNFα could possibly be used as a marker to predict chemotherapy response.

a.



b.

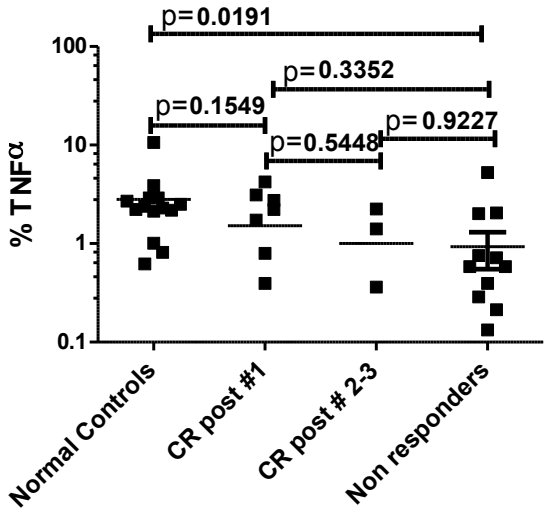


Figure 6.4 NK cytotoxicity and response to treatment a) TNFα production at D0 predicted for achieving remission after initial chemotherapy (Chi^2 test) b) TNFα production from CD56+ CD3- cells against K562 leukaemic targets at AML presentation: Non-responders produced significantly less than normal controls and there appears to be a possible a dose effect with greater production of TNFα at presentation corresponding to earlier treatment response

6.4 Discussion

Clinical outcomes: Clinical outcome data in this cohort of patients are limited by several factors. First, as this study was conceived of, performed and analyzed within the time frame of a PhD, follow up has been relatively short. This matters less for the patients recruited at the start of the study, who have been followed up for two years, the time interval during which AML relapse risk is highest.[275] However, for patients recruited towards the end of the study (and 13% were within 6 months of diagnosis at the time of analysis), the risk of relapse is still high. These later patients would therefore need to be excluded from any outcome measures beyond response to induction. Another subset of patients for whom outcome measures are limited, are those who underwent SCT. This group accounts for a substantial proportion of my patients: nearly a third underwent SCT within 6 months of initial treatment. This reflects the relatively high proportion of individuals not responding to initial induction, as well as a high proportion of secondary AML many of whom were put forward for early SCT by virtue of this regardless of early response. While most patients who underwent SCT have so far done well, with 78% still in remission at time of analysis, this remission does not reflect their own immune surveillance of AML, but rather that of a normal donor and therefore is not of use for the purposes of this study. These factors mean that many of the usual outcome measures in AML patients receiving chemotherapy, that is, relapse-free survival at 3 years, relapse-free survival at 5 years and overall survival, are not applicable to this cohort. Working within these limitations, I measure of outcome in this study as response to induction therapy.

Secondary AML and outcome: Patients with secondary AML were not different to the patients with *de novo* AML, either in response to treatment, or in terms of NK-mediated cytotoxicity and effector cytokine production. This contrasts with other studies where secondary AML is generally associated with poor outcome.[272] Although recruited sequentially, the secondary AML patients in this cohort had a genetic and molecular profile of slightly better risk than is typical in secondary AML: While 50% (8/16) had complex karyotype, monosomies, abnormal 3 or 7, or the MLL mutation, 31% (5/16) had a normal karyotype.

A further possibility is that abnormalities in NK phenotype and function are independent of other risk factors such as having secondary AML. This could be interpreted as a strength, supporting the concept that NK function is important for outcome in all individuals with AML and that the process of immune editing occurs irrespective of the trigger for disease.

NK surface receptor abnormalities and outcome: I show that levels of NKG2A expressing NK cells in AML patients can help predict response to chemotherapy treatment: only 30% of those whose NK cells express abnormally high NKG2A levels achieved complete remission after the first cycle of chemotherapy. This compares to 80% of those whose NKG2A levels are lower than the median

(p=0.041). This clinical evidence supports my data from chapter 5 and further indicates that NKG2A levels are important in AML.

While up regulation of NKG2A is an example of AML-induced immune editing of NK cells to facilitate blast survival, modification of HLA E expression is something that the blast can itself adjust in an attempt to evade immune control. As NKG2A and HLA E levels appear to be linked in AML patients, combining the two factors may provide a superior method of predicting response than either one alone. I found that HLA E expression alone does not predict for treatment outcome (data not shown) as there are some patients who do not down regulate their HLA E and still have a poor response to treatment. However, when we combine HLA E levels as a ratio to NKG2A, we see a clear association between low ratio and poor response (p=0.0315), indicating that this may be a helpful predictor of how the process of immune editing/escape may influence a patient's outcome to chemotherapy. It will be important to validate this finding in prospective studies.

Cytotoxic abnormalities and outcome: I show that individuals not responding to first line chemotherapy are more likely to produce low levels of TNFα at diagnosis than patients who achieve remission. TNFα could therefore potentially be used as a marker to predict those patients to whom either a palliative approach or a more aggressive induction may be more suitable, thus avoiding unnecessary or unhelpful treatment.

My findings are consistent with work showing that reduced NK-mediated cytotoxicity is associated with poor outcome post AML treatment[13] and is consistent with the concept of immune editing of NK cells by AML. Why this process targets NKG2A and TNFα particularly is as yet unknown. Certainly, as mentioned in the discussion for chapter 5, there are a myriad of reasons why immune editing might target one cytotoxic mechanism and not another owing to the very different cellular pathways involved.

The interpretation of these findings needs to be cautious. The selection of the sample (see discussion above) and the short follow up limit the inferences that can be drawn. Further studies should attempt to replicate this finding and evaluate the potential role of TNFα and NKG2A as predictors of clinical outcome.

Chapter 7 Discussion

This thesis has investigated the role of NK cell immunity in AML. Because this is a very extensive topic, three main questions have been considered, questions which seemed central to identifying a potential role for NK cells in the immune control of AML:

1. Do individual killer immunoglobulin receptor (KIR) genes (coding for specific NK surface receptors) affect the risk of developing leukaemia and the outcome of treatment aimed at cure, i.e. chemotherapy and stem cell transplantation?

2. Are NK cells abnormal in surface phenotype or cytotoxic function in AML patients and does the individual's NK cell receptor profile or cytotoxicity affect outcome of leukaemia remission induction treatment?

3. Are abnormalities found in NK cells induced by the AML or do they occur because of genetic predisposition?

These questions have been addressed in the previous chapters. Below I summarise the findings.

7.1 Summarised findings addressing aims of PhD

7.1-i-Do individual killer immunoglobulin receptor (KIR) genes (coding for specific NK surface receptors) affect the risk of developing leukaemia and the outcome of treatment aimed at cure, ie. chemotherapy and stem cell transplantation?

Finding: KIR2DS2 and greater numbers of activatory KIRs may be protective against the development of secondary AML and the activatory KIR haplotype B may specifically protect against therapy-related AML

Chapter four assessed the role of KIRs in AML patients receiving chemotherapy. This large cohort of 499 AML patients enabled me to investigate firstly whether KIR genotype was different in individuals developing leukaemia compared to normal individuals and, secondly, whether KIR genotype predicted outcome. I found that individuals with secondary AML had lower rates of the activatory KIR2DS2, as well as lower total numbers of activatory KIRs compared to the normal donor population and those with *de novo* AML, suggesting that activatory KIRs and in particular KIR2DS2 may be protective against development of secondary AML. The subset of secondary AML who developed their disease following therapy for a previous malignancy (t-AML) were also significantly less likely to possess the activatory KIR haplotype B suggesting that this KIR haplotype may protect individuals from developing t-AML.

Finding: The largely activatory B haplotype KIR genes 2DS1, 3DS1, 2DL5a were associated with reduced relapse after SCT for AML but not for other haematological malignancies

Chapter 3 assessed the role of KIRs in stem cell transplantation. Results are presented showing that AML patients receiving matched sibling T deplete transplants from donors with 3 specific 'favourable' KIRs (encoding mostly activatory receptors), were four times less likely to relapse than patients whose donors did not possess these genes (p=0.02). As the study cohort included those with lymphoid malignancies as well as chronic myeloid leukaemias, it was possible to assess whether the effect of KIRs was disease specific. It was found that donor 'favourable' KIRs only conferred benefit to patients with acute myeloid leukaemia indicating a protective process specific to this disease.

Finding: Neither KIR genotype, nor KIR ligands predicted outcome in AML patients receiving chemotherapy

Following the finding presented in chapter 3 that donor KIRs predicted relapse risk in AML patients receiving SCT as well as several studies showing the importance of missing KIR ligands in predicting outcome of SCT, the next step was to explore KIR groups in AML patients receiving standard initial remission induction chemotherapy (chapter 4). The lack of any impact of KIR genotype or missing KIR ligands on outcome after chemotherapy suggests that KIR group genotype and expression only impact the outcome of treatment in the context of allogeneic stem cell transplantation. This could either be because alloreacting NK cells also use non-KIR related genetic differences to exert an antileukemic effect, or because the immunoablation of SCT provides a unique milieu where newly generated donor NK cells can exert a GVL effect.

Relevance of KIR results: KIRs are an important group of NK surface receptors which when bound to their ligands, mediate NK education, self-tolerance and recognition of target cells via inhibition or activation of NK cell activity. The fact that within the human population there is distinct variability in the inheritance of KIR genes leading to a range of genotypes, led me to investigate whether this variability might account for some of the variability in predisposition for, and outcome from leukaemia. It appears that this is indeed the case in AML.

Implications of KIR results for biological understanding/basic research: As KIR are largely confined to NK cells (and a very small subset of T cells), the first conclusion which can be drawn from these results, is that NK cells do play an important role in the immune response to AML. However, the fact that KIR2DS2 only conferred benefit against the development of secondary AML indicates that NK cells only affect the course of disease when leukaemia develops over a longer time scale – more likely to occur in tAML in contrast to *de novo* AML where rapid accumulation of leukaemic blasts overwhelms the NK response. This has implications for understanding the kinetics of the NK

response to AML. That KIR genotype can influence patient outcome, supports investment in further functional studies to delineate NK-mediated immune responses to leukaemia. Both in AML patients treated with SCT and those receiving chemotherapy, the KIRs which conferred most benefit encoded activatory receptors. This suggests that individuals with an NK cell KIR gene profile balanced in favour of NK activation are better equipped to kill AML tumour cells. Further identification of the ligands for activatory receptors and the expression (or modification of expression) of these ligands on the AML target in the evasion of NK mediated immune response would therefore be worthwhile.

Implications of KIR results for risk stratification: Many factors improve the likelihood of a successful outcome after SCT as discussed in previous chapters. Most donor-related factors identified relate to the T cell response. These include modifying the degree of HLA match to influence the GVHD/GVL balance and using donor sex mismatch to provide female donor T cells specific for recipient male minor histocompatiblity antigens encoded by Y-chromosome genes. My results support the increasing evidence that the NK cell-mediated immune response is important in the SCT setting and that selecting donors with an activatory KIR genotype may improve patient outcome.

The findings presented in this thesis that KIR2DS2 may be protective against secondary AML could enhance risk stratification of patients with myelodysplastic syndrome. Currently the IPSS score takes into account factors known to impact risk of development of AML. It would be of interest to explore whether KIR genotype might be an additional risk factor for disease progression. It will also be of interest to validate whether individuals with poor risk KIR genotypes (low numbers of activatory KIRs/negative for KIR2DS2/negative for B haplotype), are more likely to develop AML secondary to treatment for haematological diseases such as Hodgkin's and non Hodgkin's lymphomas, Myeloma and Waldenstrom's. If so, those deemed higher risk could be counselled appropriately, screened more regularly, and perhaps have it built it into their initial treatment algorithm to be particularly careful with alkylating agents and other particularly leukaemogenic drugs.

Implications of KIR results for future therapeutic interventions: The fact that KIR2DS2 only conferred benefit against the development of secondary and not *de novo* AML suggests that optimal NK responses do not occur in the presence of very rapid accumulation of leukaemic blasts. This supports the practice of reducing the blast count as much as possible before attempting NK-based immunological therapies. Strategies to improve the NK-mediated immune response to AML could incorporate KIR genotype. For example, adoptive transfer of NK cells expressing high numbers of activatory KIRs either by choosing donors according to genotype, or by developing ways to manipulate grafts to increase KIR expression.

7.1-ii- Are NK cells abnormal in surface phenotype or cytotoxic function in AML patients and does the individual's NK cell receptor profile or cytotoxicity affect outcome of leukaemia remission induction treatment?

Finding: AML patients exhibited abnormal NK surface receptor phenotype with higher numbers of NK cells expressing inhibitory NKG2A receptors and lower NK cell expression of activatory NKp46 receptors

Chapter 5 assessed the surface phenotype and cytotoxicity of NK cells in AML patients at presentation and remission compared with normal controls. The results demonstrate a distinct change in the balance of NK activatory and inhibitory receptor expression in AML patients biased towards inhibition of NK cell immunity.

Finding: AML patients exhibit reduced NK cytotoxicity both in terms of the ability to degranulate and to produce cytotoxic cytokines IFNγ and TNFα

This striking finding of a marked reduction in the ability of NK cells to kill leukaemic target cells in AML patients supports the hypotheses that the NK response is important in AML, and that successful immune editing of NK cells by AML cells occurs, favouring evasion of NK control and survival of AML blasts. Interestingly, this effect was not specific to KIR expressing cells but was found in all NK cells.

Finding: Surface receptor abnormalities and cytotoxic abnormalities only partially normalise in complete remission

While abnormal NKp46 expression, degranulation and IFNγ production all normalised when AML patients achieved remission, both NKG2A expression and TNFα production remained abnormal. This may be a mechanism contributing to the high relapse rates in AML indicating that the immune editing of NK cells persists, either because only small levels of residual leukaemia are needed to maintain the defective phenotype or because the effect involves haematopoietic stem cells which continue to produce abnormal NK cells. NKG2A expression and TNFα production were also the factors that best predicted response to treatment, and future studies focusing on these molecules are indicated.

Finding: Expression of NKG2A together with its ligand HLA E on AML blasts at presentation, and TNFα production predicts response to remission induction treatment in AML

Patients failing to achieve remission had higher NKG2A levels and lower HLA E to NKG2A ratios than non-responders. Lower TNFα production at presentation was also a risk factor for treatment failure.

Implications of functional results for biological understanding/basic research: The work presented in this thesis adds potentially useful data to emerging evidence that NK immune responses in AML are defective and that patients with more functional NK cells do better. However, links between these findings and clinical outcomes still remain largely circumstantial. Therefore, my findings serve best as hypothesis-generating starting points to better define NK cell / AML interactions.

Implications of functional results for risk stratification: My results indicate that NKG2A on NK cells and HLA-E on AML blasts as well as the ability of NK cells to produce TNFα, could be incorporated into a prognostic model for predicting remission induction. This could aid current algorithms used to stratify AML patients into those suitable for standard chemotherapy, and those for whom a more intensive regimen, or a palliative approach would be more appropriate.

Implications of functional results for future therapeutic interventions: These findings suggest that treatment outcome in AML could be improved by the adoptive transfer of NK cells from healthy donors in remission at a time when the leukaemia burden is low enough to avoid immune editing of infused NK cells. Alternatively it would be worthwhile exploring whether cytokines such as IL-15 could normalise the NK cell population and improve immune control of residual leukaemia in remission.[250] Encouraging tumour cell expression of NK receptor ligands such as HLA E[266] is also a possibility in AML. The induction of higher TNFα production by NK cells may help patients respond to chemotherapy and would also be a worthwhile research avenue to pursue.

7.1-iii- Are abnormalities found in NK cells induced by the AML or do they occur because of genetic predisposition?

Finding: Abnormal phenotype and function can be induced in normal NK cells by exposure to AML blasts

This indicates that the abnormalities in surface phenotype and cytotoxic function I found in the NK-mediated immune response in AML patients occur through immune editing and not because of an innate defect in the patients' NK cells. This provides experimental support for a dynamic interaction between AML and NK cells. That AML cells "need" to regulate NK function strongly suggests that healthy unmodified NK cells could have the potential to control leukaemia and supports attempts to improve outcome for AML by NK cell therapy. However the rapid and efficient disarming of normal NK cells by AML blasts raises the problem of how to deliver NK cell therapy without the cells being rendered ineffective.

7.2 Limitations of this thesis

7.2-i- Biological constraints:

Incomplete correspondence between genotype and phenotype: There will be individuals who possess the KIR gene of interest but do not express the receptor on the surface of their NK cells. As discussed in chapter 4, this problem is less pernicious in the study of KIR genes than for many other genes as there is relatively good correlation (about 94%) between genotype and phenotype.[222] This is however, something that needs to be taken into consideration.

KIRs are also expressed on a small fraction of T cells: Focus on NK function may have been too narrow. Mechanistic explanations for functional differences in KIR groups that explain the anti-leukaemic potential are lacking. More extended functional studies looking at factors such as licensing of NK by AML, SCID mouse AML models, exploring differential disease control by NK according to leukaemia subtypes as well as the role of cytokine production will all help to expand our understanding of this area.

7.2-ii- Clinical constraints:

Limited follow up: Longer follow-up of the Hammersmith Hospital series to enable analysis of relapse and overall survival would have been useful. This would allow me to assess other factors such as the role of KIRs and leukaemia-induced NK functional abnormalities in relapse outcome, exploring age effects and time to remission, all of which would enrich this study.

Limited scope for analysing different types of AML: A larger cohort of patients for my functional study with longer follow up would have facilitated investigation of different subgroups of AML. For example, it would have been interesting to explore AML M3 – maybe this specific AML subtype with its favourable outcome is affected differently. As my functional cohort is weighted towards poor risk patients, a larger cohort of good and intermediate risk patients may show bigger effects of immune surveillance which are masked by the preponderance of poor risk patients who may do poorly whatever treatment we give and whatever NK-related genetic and functional equipment they possess. My results from chapter 4 indicate that further investigation of MDS/AML and therapy related-AML is also warranted.

7.2-iii Statistical constraints:

Limited power: As with many scientific studies, greater power could improve the accuracy of my results and reduce the risk of committing Type 1 errors. Larger cohorts for my studies would also

have enabled me to investigate different AML subsets and better take into account known risk factors such as cytogenetics, which could strengthen my findings.

Selection bias: All three studies assume the sample investigated represents the general AML population. This is of particular concern with the third prospective study detailed in chapters 5 and 6 where I extrapolate conclusions to the whole AML patient population from findings generated from a study of only 32 patients. To minimise selection bias, I included consecutive new AML patients missing only those who arrived and started chemotherapy on a weekend. All patients I approached consented to the study. In the SCT study the fact that matched sibling SCT was the only mode of treatment meant that the AML population I investigated was higher than average risk and that conclusions from this study cannot therefore be extrapolated to other transplant situations.

The MRC cohort, as discussed in chapter 4, draws from the MRC AML 10 and 15 trials. While the original trials were designed to minimise selection bias as far as possible, the present study cohort was necessarily constrained by the DNA samples available. There was therefore, a higher proportion of patients from more recent time points (where samples had not been used up in previous studies). However, within the population for whom samples were available, we attempted to ensure that the cohort was representative of the general AML population in terms of age, morphological subtypes and cytogenetic risk groups to minimise this problem.

Multiple testing: In all three studies, multiple testing is a potential source of random error (the increased probability of statistical Type I error; that is, mistaking a random finding for a statistically significant one). I tried to minimise this by making my research as hypothesis-driven as possible, attempting to answer only specific pre-determined questions. The MRC observational and AML prospective study were however, by nature exploratory, and therefore some non-hypothesis-based findings with significant differences emerged during the analysis, which we retrospectively sought to validate. To reduce error due to multiple testing I decreased the p value indicative of significance in the MRC study to <0.01.

Limited outcome measures due to early death or SCT: In spite of attempts to minimise selection bias, the prospectively collected AML cohort included a preponderance of patients with poor risk and/or secondary AML which meant that a greater proportion of patients either did not respond to chemotherapy, or had an early SCT to prevent relapse. While this strategy of early SCT worked, in that the majority of those undergoing SCT in first remission were still in remission at the time of writing, it limited my analysis as I could not use the typical measures of disease relapse or overall survival to assess outcome in this cohort.

7.3 Future directions

7.3-i- Clinical/therapeutic future directions

a) Studying KIR genotype in patients with myelodysplastic syndrome (MDS) and in patients after therapy for other haematological malignancies: My finding that 2DS2 appears to protect against secondary AML encourages me to study MDS patients to see whether KIR2DS2 is under-represented in those who go on to develop AML. Similarly, it will be important to study a cohort of patients after treatment for haematological malignancies such as Hodgkin's, NHL and myeloma (who have a significant increased risk of secondary AML) to see whether poor risk KIR genes segregate to these individuals. Such data would have important implications in helping to predict secondary AML patients and could augment current predictive algorithms. In particular it could help select individuals for earlier therapeutic interventions and SCT and identify patients in whom a watch and wait strategy and/or less intensive treatment could be adopted. This study would be relatively straightforward to carry out in a unit with a large cohort of MDS and lymphoma patients. The time lag for development of AML from MDS would be suited to a retrospective observational study.

b) Blocking immune editing with new therapeutic agents: After SCT, high IL-15 levels have been demonstrated to be associated with higher numbers of activatory receptors[249] and this cytokine has been used *in vitro* to induce expression of activatory receptors in cells from AML patients.[250] This work suggests that it may be possible to use IL-15 to reverse some of the changes we found in NK cells. Selenium can reduce expression of HLA-E and might thereby enhance AML killing by NK cells.[266] A trial using selenium to block HLA-E and improve the HLA E/NKG2A ratio in AML patients should be considered.

c) Clinical trials using donors with activatory/2DS2/Favourable KIRs for SCT and as donors for adoptive NK immunotherapy: My results in chapters 3 and 4 suggest that KIR genotype may improve NK mediated immune surveillance. Incorporating this into donor selection procedures for SCT and adoptive NK immunotherapy could improve outcome for patients receiving SCT from HLA matched donors and a prospective study using selected donors would be worthwhile. The concept of "favourable KIR" donors could also be extended beyond the SCT setting to treatment of AML in remission with alloreactive NK cells from selected donors. Especially where the patients' KIR groups are unfavourable, the use of allogeneic NK cells could have greater antileukemic potential than autologous NK cells.

d) Investigating Bortezomib to up-regulate TRAIL ligands: Bortezomib can be used to up-regulate the DR4/5 ligands for TRAIL. This has been shown in chronic myeloid leukaemia to enhance

susceptibility of quiescent CD34+ CML cells to cytotoxicity by *in vitro* expanded natural killer cells.[276] Further investigation of the role of the TRAIL-mediated death receptor pathway of NK cytotoxicity and the potential to enhance NK cytotoxicity to AML stem cells would therefore be important to explore.

e) Using antibodies to block negative KIRs: Preliminary studies have shown that antibodies can be used clinically to block inhibitory KIRs in AML patients.[277] In view of my results highlighting the role of activatory KIRs, further exploration of this antibody to alter the balance between activatory and inhibitory KIRs in AML patients using these agents will be interesting.

7.3-ii- Biological aspects - future directions

a) Further functional work to elucidate mechanisms of AML-initiated immune editing of NK cells:

1) Co-culture studies should be repeated with a larger group of normal individuals using different AML subtypes to delineate whether certain healthy individuals are more susceptible to immune editing than others and whether certain AML subtypes are more immune-modulatory than others. I would also want to explore whether the suppression is mediated by all AML blasts or CD38 negative CD34 positive AML stem cell like cells.

2) Although my data so far suggest that cell contact between blasts and NK cells is necessary for immune editing to occur, it should be confirmed using trans-well experiments and supernatants to assess the role of secreted factors more carefully.

3) Further studies using confocal microscopy and assessment of the cytoskeleton could be used to explore the immune synapse between NK cells and AML to determine whether there are similar abnormalities to the findings in T cells.[11]

4) Investigations of molecular mechanisms underlying immune editing by AML: in my initial studies, I could not identify any candidate enzymes or transcription factors (IDO or eomesodermin), to explain abnormalities seen in NK cells in AML patients. The role of CD200 expression on blasts would be of relevance here.[253]

b) Identifying the ligands for activatory KIRs on blasts: The results in this thesis support the growing body of evidence that NK cells expressing activatory KIRs are particularly important in mediating the NK response towards AML. However, identification of the exact ligands for many activatory KIRs (as well as other NK receptors such as the NCRs) remains elusive. Candidates include heat shock proteins and as yet unknown leukaemia antigens. Work to identify these ligands could inform

functional studies. For example, ligand expression on blasts could be manipulated to minimise immune evasion with therapeutic implications.

7.4. Immunotherapy for AML – concluding remarks

While the results in this thesis provide strong evidence for the importance of NK cells in the immune response against AML, there is evidence for similar immune editing of T cells in AML raising the questions: how much do the innate and adaptive immune responses to AML interact? And do T cells or NK cells dominate in AML immune regulation? A further complexity that remains to be addressed is the role of the bone marrow milieu and in particular the role of mesenchymal stromal cells (MSC) in modulating immune responses to AML. In a larger context, despite accumulating evidence for the interaction of the immune system with AML, it is as yet unclear how powerful the immune system is in shaping the outcome of AML in comparison with better-defined prognostic indicators. These questions seem important because they underpin the level of enthusiasm we should have for developing immunotherapy strategies to augment NK or T cell (or possibly B cell) responses in AML to achieve sustained remissions.

Clear evidence for T cell responses against AML comes from the SCT setting, where AML-specific T cells in the graft as well as donor lymphocyte infusions correlate with improved GVL. Further evidence comes from work with tumour vaccinations against leukaemia antigens such as Wilms tumor protein 1 (WT1) and PR1 (derived from proteinase 3) showing anti-leukaemic responses from antigen specific T cells which correlate with clinical response.[22] There has been less work focusing on the B cell response to AML. Humoral responses have however, been very useful in the context of serum screening of tissue banks and cell lines which can direct us to new leukaemia associated antigens[278,279]. The immune cells in the bone marrow provide a special milieu for AML. MSC inhibit normal tumour apoptosis and provide a tolerant environment for AML to grow[280,281]. Induced abnormalilites in the MSC microenvironment can induce leukaemia[282] and blocking chemokines such as CXCR4 can impair the bone marrow's role as a tumor sanctuary from chemotherapy[283] . The NK response is therefore by no means the only component of the immune reaction against AML. It may be that some subsets of AML are more T-cell than NK-cell controlled. Mutations in the NPM1 gene are a relatively common finding in AML[284]. Recent work confirms that in the majority of AML patients with this mutation, NPM1 induces specific CD4 and CD8 T cell responses which may explain why mutated NPM1 is associated with a good prognosis[285]. A patient who maintained a long-term response after a rare spontaneous remission to AML 11 years before, shows that while T cell responses are no different to normal, NK tumour responses remain increased suggesting that NKs may have a particular role in maintenance of long term remission.[286]

In considering the role of immunity in AML we have to be mindful that the immune milieu may be drastically different at presentation, at remission and again at relapse. At presentation, in addition to the abnormalities I describe in NK cells, a similar process may occur with the T cell response as indicated by the impairment of T cell synapsing and T cell cytotoxicity. Myeloid-derived suppressor cells (MDSC) derived from the leukaemic clone may also interfere with the immune response. MDSC are myeloid cells which proliferate in solid tumors and suppress the T cell response via a range of mechanisms including altering L arginine availability, inhibiting reactive oxygen species and inducing regulatory T cells[287,288]. Whether AML blasts have MDSC-like activity is as yet unclear, and further studies to explore this are indicated since MDSC are a potential target for immunotherapy.[287] The presence of MDSC at AML presentation may explain the defective function of both T cells and NK cells[11,155]. A critical question pertaining to immune regulation is whether the immune abnormalities normalise after remission when the leukaemic burden is minimal. This is important because first remission of AML would be the most promising moment to introduce immunotherapy. Data presented in this thesis shows only partial normalisation of the NK response. Cytokines such as Il-15, an important growth factor for NK and T cells, could be used to improve their number and function to sustain remission.[250] Antibody therapies targeting antigens common to leukaemia cells such as CD33[289] are also proving useful for prolonging remission in some AML patients.[290] At relapse, there is evidence that the immune system has again been disabled. This raises the question whether the treatment itself may play a role in disabling immunity.

Chemotherapy may affect the immune system positively or negatively. While cytarabine and daunorubicin directly kill tumour cells and stop cell division, the effects on the immune system are poorly studied. Reducing leukaemic bulk may help normalise immune function but chemotherapy can also induce lymphopenia. For example, after AML chemotherapy in children, B cell depletion reduces influenza vaccine responses.[291] NK cells are produced in the marrow and unlike T cells, do not have a significant peripheral stem cell memory pool. Indeed some T cells capable of clonogenic proliferation remain after induction.[292] In contrast, NK cells may be more severely depleted by chemotherapy. Chemotherapy may, nevertheless, have favourable effects on the immune response - T cells are more responsive after chemotherapy and have greater proliferative potential,[293] because lymphodepletion provides a cytokine rich environment conducive to the proliferation of tumour specific T cells. This lymphopenic drive has been exploited for adoptive cell transfer in both mice and men.[294-296] After AML chemotherapy, improved cytokine release by T cells, particularly of antileukaemic IFNγ has been reported.[297] The removal of immune cells which compete with tumour specific T cells for cytokines (a phenomenon known as 'the cytokine sink') is another benefit of chemotherapy.[294] Finally, although it inhibits regeneration of bone marrow stromal cells,[242] chemotherapy may improve the function of antigen presenting cells, killing tumour cells by

apoptosis so increasing APC uptake and presentation of tumour antigens to T cells,[298,299] and by activating APC either by release of LPS into the bloodstream through damaged mucosal barriers[300] or via release of APC-stimulating pro-inflammatory cytokines such as TNFα.[301]

Much needs to be learned about the specific immune modulating effects of conventional chemotherapy and newer agents: certain drugs are more helpful in augmenting immune effects against leukaemia than others. Fludarabine, an effective antileukaemic drug which augments the effect of cytarabine[302] induces lymphopenia and the subsequent increase in IL-15, causing a burst of T and NK cell regeneration.[197,292] Cyclophosphamide successfully reduces regulatory T cells which suppress tumour immunity[303] and 5 Azacytidine increases tumour antigen presentation.[304] Even steroids, well known for their immunosuppression, can be beneficial by inhibiting the transcription of genes involved in immune cell effector functions, a function useful in the treatment of inflammatory and autoimmune disease. In keeping with this, steroids reduce NK cell cytotoxicity.[305] Recently, however, endogenous steroids have been shown to promote the appropriate selection of a desirable thymic T cell repertoire.[306] The anti-proliferative agents such as mycophenolate mofetil, cyclosporin, azathiprine, tacrolimus and rapamycin are also likely to have complex beneficial and detrimental immune effects. Lenalidomide, the immune-modulatory drug developed as a TNFα blocker, is of particular interest. Lenalidomide can render the marrow microenvironment less hospitable to myeloma, (reviewed in[307]) and directly kill mutated cells in MDS (reviewed in[308]). It also stimulates T cells, and improves NK function via increased T cell IL-2 production and a direct effect on NK cell-mediated cytotoxicity.[309-311] Therapy with cytokines such as IL-15 can also improve NK proliferation and function in the bone marrow.[312,313]

In conclusion, for many reasons, AML has proven to be relatively resistant to treatment. However the disease may yield to a combined attack with chemotherapy, new "biological" agents and immunotherapy. Within the next decade, I anticipate considerable progress in improving the cure rate of this major leukaemia of older individuals by coordinated multi-modal treatment. Immunotherapy seems likely to play an important role in this.

Appendix: Supplementary material

8.1 Consent form: functional study of NK cells in AML

Imperial College
London

Directorate of Cancer Services – Division of Clinical Haematology

Consent Form: Natural Killer (NK) Cells & Acute Myeloid Leukaemia study

The participant should complete the whole of this sheet him or herself.

(please tick each statement as it applies to you)

I have read the Information Sheet ☐

I have been given the opportunity to ask questions and discuss the proposed studies ☐

I have received sufficient information about these studies. ☐

I have received satisfactory answers to all my questions ☐

The consent process has been explained to me by: Prof/Dr/Mr/Mrs/Ms…….

I understand that I am free to change my consent at any time, without having to give a reason and without affecting my future medical care.

I understand that relevant sections of my medical notes and data collected during the study may be accessed by responsible individuals from the study Sponsor (Imperial College), NHS trust or regulatory authorities. I give permission for these individuals to access my records.

I consent to give samples for research into blood diseases for this research project ☐

and for other ethically approved projects ☐

Signed.....…… Date:.....

(NAME IN CAPITALS).....……..

Hospital Number:……

Physician seeking consent: signature..... Date:.....

(NAME IN CAPITALS).....……

8.2 Patient information sheet: functional study of NK cells in AML

Imperial College
London

Directorate of Cancer Services – Division of Clinical Haematology

Natural Killer (NK) Cells & Acute Myeloid Leukaemia study

Information Sheet: Consent for Research Samples
Laboratory Studies in Blood Diseases
We would like to ask your permission to use samples of your blood and bone marrow for the purposes of medical research. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you decide not to take part, your treatment will not be affected by your decision. If you choose to participate, your donated samples will be sent to the laboratory of Dr. Katy Rezvani at the Hammersmith Hospital, Imperial College London. Dr Kate Stringaris is a young haematologist who is studying for her PhD in Dr Rezvani's laboratory and the results of this study will be submitted as part of her degree.

What is the purpose of this study?
The purpose of this study is to look at the role of the immune system in fighting malignancies involving blood cells. We have known for many years that the immune system is very important in fighting cancer. We wish to investigate this in greater detail by taking samples from patients with blood malignancies to look at the ability of the immune cells to specifically recognise and kill blood cancer cells. Natural killer cells are part of the innate immune system and appear to be particularly important in the response towards leukaemia. This project investigates the role of natural killer cells, their receptors and their function in patients with haematological malignancies before, during and after treatment with a view to identifying factors which may influence the natural killer cell response. This work may enable us to find ways to boost the immune response to leukaemia, or provide targetted natural killer cell infusions to improve patient outcome after standard chemotherapeutic approaches with the aim of reducing relapse. If you would like additional information regarding the exact nature of the research, we would be happy to discuss this in more detail at your request.

Why have I been chosen?
You have been diagnosed with a haematological malignancy and therefore could help with this study by participating.

Do I have to take part?
Your participation in this study is entirely voluntary. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide not to take part then this is fine and it will not affect the standard of care that you will receive.

What will happen to me if I take part?
As part of your treatment your doctors will be taking samples of blood and bone marrow at regular intervals. At first this will be to give a precise diagnosis so as to decide on your treatment. Later on samples will be taken to study your response to treatment. The blood samples will be removed from a vein in your arm and the marrow samples will be drawn from your hip.

We would like to ask you to donate an additional 3 tablespoons of blood once a week during your first month of treatment. In addition you will have a bone marrow aspirate before your treatment starts and another, usually after the first course of chemotherapy. We would also like to take an additional tablespoon of bone marrow at these times.

What are the possible disadvantages of taking part?
At no time will these additional specimens be collected if it is felt that this would jeopardise your health in any way or the collection of the samples required for the management of your disease. The research samples will be taken at the same time as samples are taken for your routine care and participating in this study will not require any extra tests or needles for you.

What are the possible benefits of taking part?
It is highly unlikely that our results will change any part of your treatment. However, they might provide us with information that improves our understanding of your disease and helps other patients in the future.

What if something goes wrong?
Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation. If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Dr Kate Stringaris, contact details below). The normal National Health Service complaint complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Office.

Will my taking part in this study be kept confidential?
It may be necessary to compare the results from the research laboratory with the progress of your disease. For this reason it will not be possible to completely anonymise your samples and in addition we may wish to study your medical records. However we keep the information collected about you during the course of the research strictly confidential.

What will happen to my samples?
When your sample arrives in the laboratory, it will be given a number (known as a unique identification number). It will be stored under this number and not under your name. Dr. Stringaris will hold the information necessary to link you with the unique identification number and hence the sample. Any information about you which leaves the hospital will also have your name and address removed so that you cannot be recognised from it. Samples will be frozen and stored in liquid nitrogen in the department of Haematology, Hammersmith Hospital, until they are used in experiments. They are likely to be used within 3 years although storage time may be longer. If there are any remaining samples not used in this particular study, rather than disposing of them, we will use them in future, ethically approved studies into leukaemia.

Who is organising and funding the research?
This research project has been submitted and accepted for PhD registration at Imperial College London and to organisations that sponsor medical research e.g. the charity LEUKA.

Who has reviewed the study?
Before the research could be registered for a higher degree and the funding agency agreed to fund the research, the project was sent to other scientists working in the same area to confirm that the research is worthwhile and justified. The project has also been approved by the local Ethical Review Board.

What will happen to the results of the research studies?
We hope that the results of our research may be published in medical and scientific journals. You may have copies of these publications if you wish but your name will not appear as it will be anonymous. If you would like an update on the progress of the research please ask Dr. Kate Stringaris or Dr Katy Rezvani (contact details at the end of the page).

It is possible that some of the information that we obtain from our research will be of value to other researchers or even to commercial biotechnology companies. We may wish to enter into an agreement with a company such that we obtain some financial sponsorship for our work. If this were to happen, any information obtained from your blood samples would be completely anonymised so that you could not be identified. Your samples will not be used for any tests in which the information obtained could be used to any disadvantage by another organisation, e.g. insurance companies. If at any time you decide that you do not wish for your samples to be used for research purposes, then we will arrange for them to be discarded.

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8.3 Supplementary table 1. Optimising DNA extraction

Experiment to assess optimum cell number to use for DNA extraction method described in 2.3 showing that above 10 x 10^6 cells the purity began to drop as the column became overloaded.

No. cells from same patient sample applied to column	**DNA concentration acheived**	**Purity of DNA (260/280)**
5 x 10^6	110.24 ng/µl	1.89
5 x 10^6	143.03 ng/µl	1.87
10 x 10^6	272.48 ng/µl	1.89
20 x 10^6	456.8 ng/µl	1.4

8.4 Supplementary table 2. Univariate analyses in Cox models for factors affecting transplant outcome (n=261)

Variable	**No pts (%)**	**TRM**	**OS**	**Relapse**	**GVHD (ii-iv)** data on n=192
Risk Group					
Standard	105	16 (15%)	75 (71%)	15 (14%)	26 (30%)
High risk	156	39 (25%)	59 (38%)	63 (40%)	59 (57%)
		P= 0.014*	**P= <0.001***	**P= <0.001***	**P= <0.001***
CD34+ dose					
<median 5	130	38 (29%)	52 (40%)	44 (34%)	40 (47%)
>median 5	131	17 (13%)	82 (63%)	34 (26%)	45 (43%)
		P= 0.001*	**P= <0.001***	**P=0.033***	P=0.573
Graft type					
Bone Marrow	41	15 (37%)	18 (44%)	10 (24%)	
PBSC	220	40 (18%)	116 (53%)	68 (40%)	
		P= 0.002*	P=0.123	P=0.711	P= 0.203
Patient age					
<median 36 yrs	130	25 (19%)	70 (54%)	40 (31%)	49 (47%)
>median 36 yrs	131	30 (23%)	64 (50%)	38 (2%)	36 (41%)
		P= 0.466	P=0.47	P=0.9	P=0.43
LC30					
<median 600	131	40 (31%)	61 (47%)	35 (27%)	41 (43%)
>median 600	130	15 (12%)	73 (56%)	43 (33%)	44 (45%)
		P=<0.001*	P= 0.13	P=0.388	P=0.759
Transplant era					
<median July 01	130	35 (26.9%)	59 (45%)	38 (29%)	45 (46.9%)
>median July 01	131	20 (15.3%)	75 (57%)	40 (30%)	40 (42%)
		P= 0.021*	P=0.144	P=0.934	P=0.468
Donor KIRs					

Favourable KIRs	87	15 (17%)	51 (59%)	21 (24%)	22 (35%)
Not favourable KIRs	159	33 (21%)	77 (48%)	53 (33%)	58 (49%)
		P=0.350	**P=0.05**	P=**0.043***	P= 0.074
Donor KIR Haplotype					
B	156	25 (16)	86 (55%)	46 (30%)	49 (45%)
A	90	23 (26%)	42 (47%)	28 (31%)	31 (45%)
		P=0.072	P=0.20	P=0.79	P=0.837
No. Inhib. Donor KIRs					
< median 8	99	22 (22%)	48 (49%)	35 (35%)	31 (43%)
>median 8	145	26 (18%)	79 (52%)	38 (26%)	49 (45%)
		P=0.374	P=0.36	P=0.13	P=0.801
No. activ. Donor KIRs					
<median 3	107	25 (23.4%)	56 (52%)	32 (30%)	38 (47%)
>/=median3	138	23 (17%)	72 (52%)	41 (30%)	42 (42%)
		P=0.218	P=0.98	P=0.97	0.472
Missing KIR ligand					
No	76	20 (26%)	38 (50%)	20 (26%)	25 (43%)
Yes	126	22 (18%)	65 (52%)	43 (34%)	43 (47%)
		P=0.107	P=0.83	P=0.25	P=0.620
No. missing ligands					
No missing ligand	76	20 (26%)	38 (50%)	20 (26.3%)	25 (43%)
One missing	68	12 (18%)	36 (53%)	21 (31%)	24 (50%)
Two missing	49	6 (12%)	25 (51%)	19 (39%)	16 (46%)
		P=0.135	P=0.94	0.34	P=0.777

8.5 Supplementary table 3. Competing risk analysis

Multiple event stratified Cox model (time to first event analysis)

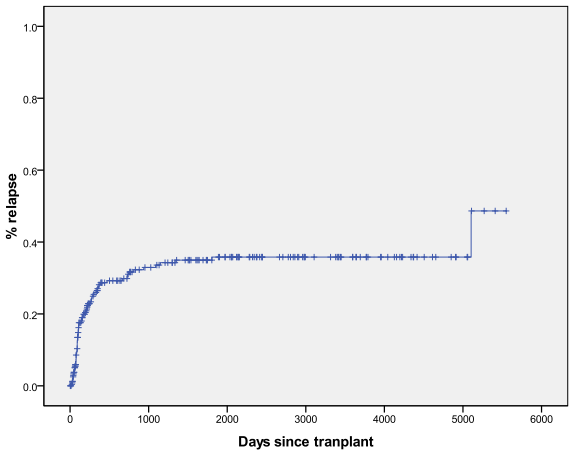
	Coefficient	Hazard Ratio	Robust SE	P value
Risk Group	1.52664	4.603	0.341	**<0.001**
CD34 dose	-0.61422	0.541	0.295	**0.037**
PBSC graft	0.00447	1.004	0.464	0.99
Patient age	-0.05726	0.944	0.285	0.84
LC30 count	0.75153	2.120	0.381	**0.049**
SCT era	-0.50735	0.602	0.340	0.14
3 Favourable KIRs	-1.00939	0.364	0.364	**0.0056**
KIR haplotype	1.08354	2.955	0.687	0.12
Inhib KIRs>8	-1.62430	0.197	0.483	**<0.001**
Activ KIRs>3	0.78797	2.199	0.575	0.17
Missing KIR ligand	0.19691	1.218	0.292	0.5

Liklihood ratio test p value = <0.001.

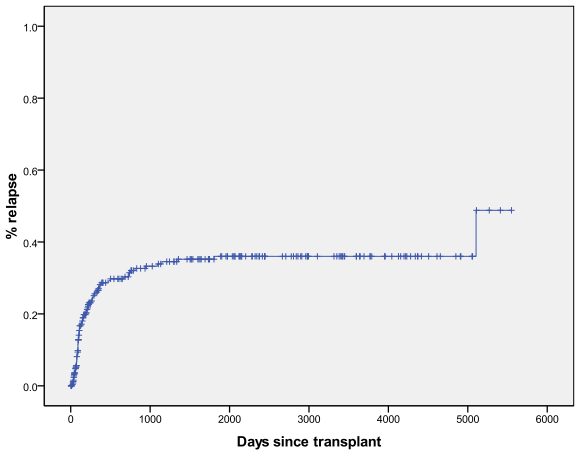
8.6 Supplementary figure 1. Patient representation for NIH cohort

Showing that the sample of patients for whom donor samples were available is representative of the whole patient population transplanted at the NHLBI since 1993 in terms of outcome:

A. Relapse rates in 246 patients with donor samples available



B. Relapse rates in 261 patients transplanted since 1993



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