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Functional consequences of genetic polymorphism of the KIR3DL1/S1 receptor

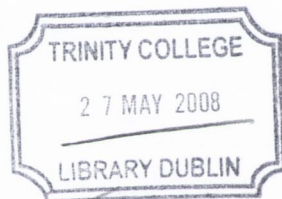
**A thesis submitted to the University of Dublin
For the degree of Doctor of Philosophy**

by

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Nov 2007

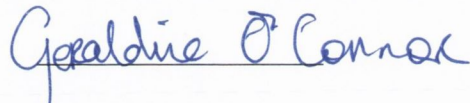


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DECLARATION

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Summary

Natural Killer (NK) cells are crucial effector cells of the innate immune system that function to eliminate virally infected and transformed cells. The activation of these cells is controlled in part by the expression of cell surface receptors including inhibitory receptors specific for HLA antigens. Expression of these inhibitory receptors allows NK cells to become activated in the event of down-regulation or loss of HLA expression, as occurs in some viral infections and transformation events. One important family of HLA-specific receptors are killer immunoglobulin like receptors (KIR). The 3DL1 member of the family is a highly polymorphic receptor that recognises a range of HLA-B allotypes that contain the Bw4 public epitope.

One of the aims of this study was to assess the role of genetic polymorphism of the 3DL1 gene on the interaction of 3DL1 with Bw4. In parallel, the effect of sequence variability within the Bw4 grouping on this interaction was also investigated. To this end, we used the common 3DL1 allotypes *001, *002 and *005 and measured their interaction with a panel of HLA-B molecules (including B*1513, B*2705, B*3801, B*5101 and B*5801). This interaction was measured in a number of different ways. Jurkat T cells expressing a chimeric construct consisting of the extracellular domain of 3DL1 allotypes fused to the intracellular CD3 ζ -chain were stimulated with a panel of B cell lines cells expressing a single defined HLA allele. Interaction between 3DL1 and HLA antigens resulted in the production of an NFAT signal, which could be measured by means of transient transfection with a reporter plasmid. Secondly, a panel of donors were characterised for the expression of 3DL1 allotypes and methodologies for the generation of NK cell clones with specific receptor expression profiles were optimised. These NK cell clones, along with 3DL1⁺ polyclonal NK cell populations were used as effector cells against the same panel of HLA-expressing 721.221 cells in a cytotoxicity assay. A third approach was to measure the level of activation of resting NK cells by exposure to the class I negative 721.221 parental cell line and the reduction of this activation when cells were stimulated with the panel of HLA-transfected 721.221 cells. These complementary approaches demonstrate that sequence variability within both the 3DL1 receptor and HLA-Bw4 allotypes has functional consequences in terms of NK cell recognition of target cells. Thus, inhibitory allotypes of KIR3DL1 differ in their ability to recognise HLA-Bw4 ligands, and an individual 3DL1 allotype differs in its interaction with different Bw4 allotypes.

3DS1, which segregates as an allele of 3DL1, has a short cytoplasmic tail characteristic of activatory receptors. As it is very similar to 3DL1 in the extracellular domains, it has been assumed that it will recognise a HLA-Bw4 ligand. As expression of this receptor had not been demonstrated, a FLAG-tagged 3DS1 construct was generated and transfected into the Jurkat T and THP-1 monocytic cell lines. These cells were then stained with an anti-FLAG antibody to check for expression of the 3DS1 construct at the cell surface. Cell surface expression of 3DS1 was found in both these transfected cell lines. In addition, we describe the first serological reagent (the Z27 antibody) capable of recognising 3DS1 and distinguishing it from other 3DL1 allotypes. Using this antibody, we identified the population of 3DS1⁺ cells and demonstrated that 3DS1 is expressed on a variable proportion of NK cells and CD8⁺ T cells. Crosslinking of 3DS1⁺ NK cells with this

antibody provided evidence that ligation of this receptor results in the activation of NK cells, as measured by the expression of the activation marker CD69. Similar to assays performed with the inhibitory 3DL1 allotypes, Jurkats cells expressing a chimeric construct consisting of the extracellular domain of 3DS1 and the CD3 ζ chain were stimulated with the panel of 721.221 HLA transfectants. No interaction between 3DS1 and Bw4 was observed in this assay. Polyclonal 3DS1⁺ NK cells were used as effector cells against the panel of 721.221 HLA transfectants in the presence or absence of the Z27 antibody. There was no 3DS1-dependent difference in the level of killing of these cell lines suggesting that there is no interaction between 3DS1 and the HLA-Bw4 allotypes expressed on these cells. In addition Jurkat cells expressing the extracellular domain of 3DS1 were stained with B*2705 tetramers folded in the presence of both self and virus-derived peptides, but no interaction was detected.

The role of NK cells in HIV infection and in particular in HIV long term non progression (LTNP) was analysed by measuring NK cell function and phenotype in a cohort of HIV⁺, HIV viremic and HIV LTNP patients. NK cell activity, as measured by cytotoxicity against the K562 cell line, was decreased in HIV viremic patients relative to controls. HIV LTNP patients showed an increase in cytotoxicity relative to HIV viremic, but did not reach control levels. The expression of a number of cell surface receptors was altered across the three groups but did not correlate to the functional differences seen.

ACKNOWLEDGEMENTS

Thank you to my supervisor, Dr. Clair Gardiner, for all her help and advice throughout my PhD. In particular, I would like to thank her for being so understanding and supportive over the last two years.

A big thanks to all the members, present and past, of the Natural Killer Cell group. To Orla, who started out with me in the empty lab and provided loads of help, support encouragement and entertainment. To Verónica, for lots of helpful discussions about immunology, science and life in general! To all the current members in the lab; Kieran, Megan, Aisling, Michelle and Kate for all their help and advice.

Thanks to the other members of the department; the Bowie, Mantle, Mills, O'Neill, Scott and Tipton labs and the technical and admin staff for their seemingly endless supply of equipment, reagents, support, help and advice. A big thanks in particular to Ed, Gavin, Seamus, Coilin, Jerrard, Aoife, Jeff, Joe, Sarah, Susan, Peter, Mary-Pat, Liam and Kyran. A special mention for Laura for her incredible attention to detail and for providing so many excuses for (much needed) tea breaks.

A very special thanks to everybody who so kindly and generously donated blood for this project.

I would also like to thank my family- my Mom, for everything she did to support and encourage me throughout my student years. Also my brother, sisters, in-laws, nieces and nephews for all their encouragement. In particular to Lisa and Noreen for providing lots of support and distractions when needed, and ignoring me when I was cranky!

Table of Contents	i
List of Figures	vii
List of Tables	xi
Abbreviations	xii

Chapter 1 Introduction

1.1	Natural Killer cells	2
1.1.1	NK cell cytotoxicity	3
1.1.2	NK cell and cytokines	5
1.1.3	NK cell subsets	6
1.2	NK cell development	7
1.3	The function of NK cells	10
1.3.1	NK cells and cancer	10
1.3.2	NK cells in transplantation	11
1.3.3	Shaping of the adaptive immune response	12
1.3.4	NK cells and viral infection	13
1.3.4.1	NK cell deficiencies	14
1.3.4.2	Viral Immune Evasion	15
1.3.4.3	NK cells and HIV	17
1.4	NK Cell Receptors	19
1.4.1	Activatory Receptors	21
1.4.1.1	CD16	21
1.4.1.2	NCRs	22
1.4.1.3	NKG2D	22
1.4.2	HLA	23
1.4.3	Non-HLA –specific inhibitory receptors	25
1.4.4	Non-KIR HLA-specific receptors	26
1.4.4.1	CD94/NKG2A/C/E	26

1.4.4.2	LILR	26
1.4.5	MHC Receptors in Mice	27
1.5	KIR	28
1.5.1	KIR Gene Complex	29
1.5.2	Variation of KIR gene	31
1.5.2.1	Haplotypic Variation	31
1.5.2.2	Allelic polymorphism	32
1.5.3	Recognition of HLA by KIR	33
1.5.4	KIR Signalling	34
1.5.4.1	Activating Signals	35
1.5.4.2	Inhibitory Signals	35
1.5.5	KIR expression on NK cells	36
1.5.6	KIR expression on T cells	37
1.5.7	KIR Structure	38
1.5.8	KIR and disease	40
1.6	KIR3DL1	42
1.6.1	The role of presented peptide	42
1.7	Aims	45

Chapter 2 Materials and Methods

2.1	Materials	47
2.2	Methods	49
2.3	Growth and Maintenance of cell lines	49
2.4	Cryopreservation of cells	49
2.5	PBMC Isolation	49
2.6	NK cell isolation	50
2.7	NK cell cloning	51
2.7.1	Yssel's Medium	51

2.7.2	RPMI 8866	52
2.7.3	Stem Cell Growth Medium	52
2.8	Polyclonal NK cell expansion	53
2.9	Transfection	53
2.9.1	GeneJuice® transfection	53
2.9.2	Lipofectamine 2000® transfection	54
2.9.3	Electroporation	54
2.10	Cell stimulation	55
2.11	Measurement of luciferase activity	55
2.12	Measurement of SEAP activity	56
2.13	FACS analysis	56
2.14	Tetramer formation and staining	56
2.15	IFN- γ intracellular staining	57
2.16	CD107a staining	57
2.17	⁵¹ Cr release assay	58
2.18	Plasmid transformation and Purification	59
2.19	DNA isolation	59
2.20	RNA isolation	60
2.21	cDNA synthesis	60
2.22	PCR	61
2.22.1	KIR3DL1 allelic gDNA typing	61
2.22.2	KIR3DL1 cDNA typing	61
2.22.3	High Fidelity PCR	61
2.23	Generation of FLAG-tagged KIR3DS1	69
2.24	PCR CleanUp	69
2.25	Gel extraction	70
2.26	3' A overhang addition	70
2.27	Restriction digest	70
2.28	Ligation	70
2.29	TOPO TA cloning	71
2.30	Statistical Analysis	72

Chapter 3 Characterisation and cloning of 3DL1+ NK cells

3.1	Introduction	74
3.1.1	KIR3DL1 genetic variability	74
3.1.1.1	Methods of KIR genotyping	77
3.1.2	KIR3DL1 expression	78
3.1.3	Non-KIR HLA-specific receptors	79
3.1.4	NK cell proliferation	79
3.1.5	Choice of experimental model system	80
3.2	Aims	83
3.3	Results	84
3.3.1	Donor Profiling and Selection	84
3.3.1.1	Validation of KIR3DL1 allele typing system	84
3.3.1.2	KIR3DL1 allele expression in a panel of donors	84
3.3.1.3	KIR3DL1 phenotyping	90
3.3.2	NK cell expansion using RPMI-8866 cell line	90
3.3.3	NK cell isolation	94
3.3.3.1	Magnetic bead isolation	94
3.3.3.2	NK cell sorting	96
3.3.4	NK cell cloning	96
3.3.4.1	RPMI-8866-based cloning protocol	99
3.3.4.2	Yssel's medium based protocol	99
3.3.4.3	Stem Cell Growth Medium (SCGM) clones	107
3.4	Discussion	115

Chapter 4 3DL1 polymorphism is functional

4.1	Introduction	123
4.1.1	Variation within HLA-Bw4	123

4.1.2	The role of peptide	126
4.1.3	3DL1 polymorphism	127
4.2	Aims	129
4.3	Results	130
4.3.1	Transfection of the Jurkat T cell line	130
4.3.2	Monitoring of stably-transfected cell lines	135
4.3.3	Optimisation of stimulation conditions	135
4.3.4	3DL1*001 interacts with HLA-B alleles	138
4.3.5	3DL1*002 interacts with HLA-B alleles	142
4.3.6	The role of CD94 in the recognition of HLA panel	142
4.3.7	3DL1*001 specificity in a clonal cytotoxicity assay	145
4.3.8	3DL1*002 specificity in a clonal cytotoxicity assay	145
4.3.9	3DL1*005 specificity in a clonal cytotoxicity assay	148
4.3.10	3DL1*005 specificity in a polyclonal cytotoxicity assay	148
4.3.11	Activation of NK cells by 721.221 cell lines is inhibited by HLA-B*5801	154
4.3.12	3DL1*001 specificity is maintained in primary NK cells	157
4.4	Discussion	162

Chapter 5 3DS1 expression and function

5.1	Introduction	168
5.1.1	2D Activatory KIR	168
5.1.2	3DS1	172
5.1.3	NK cells in HIV Infection	172
5.2	Aims	176
5.3	Results	177
5.3.1	Generation of FLAG-tagged 3DS1 construct	177
5.3.2	3DS1 cell surface expression	183
5.3.3	3DS1 recognition by Z27 antibody	183
5.3.4	3DS1 expression profile	187

5.3.6	3DS1 ligand specificity	192
5.3.7	Tetramer binding studies	192
5.3.8	NK cell function and phenotype in HIV and HIV LTNP	202
5.3.8.1	NK cell function in HIV and HIV LTNP	202
5.3.8.2	Percentage NK cells	205
5.3.8.2	3DS1 in HIV and HIV LTNP	205
5.3.8.3	Expression of NK cell associated receptors in HIV and HIV LTNP	205
5.4	Discussion	218
5.4.1	3DS1	218
5.4.2	NK cells in HIV infection	223

Chapter 6 General Discussion

6.1	Introduction	227
6.2	Diversity in the NK cell response – 3DL1	227
6.3	Activatory KIR	232
6.4	Control of KIR expression	236
6.5	Rapid evolution of KIR genes	237
6.6	KIR in human health and disease	240
6.7	Concluding Remarks	241
6.8	Future Perspectives	241

Bibliography	244
---------------------	-----

Appendix: Publications	274
-------------------------------	-----

LIST OF FIGURES

CHAPTER 1

Introduction

Figure 1.1	“Missing self” hypothesis of NK cell recognition	4
Figure 1.2	NK CD56 ^{dim} and CD56 ^{bright} cell subsets	7
Figure 1.3	The development of NK cells in humans	9
Figure 1.4	NK cells are activated during viral infection	15
Figure 1.5	The domain structure of the KIR	29
Figure 1.6	The Leukocyte receptor complex	30
Figure 1.7	Intron-exon structure of a typical KIR gene (3DL1)	30
Figure 1.8	KIR haplotypes can be divided into group A and B haplotypes	32
Figure 1.9	Signalling of inhibitory KIR	36
Figure 1.10	2DL2 in complex with HLA-Cw3/GAV	39

CHAPTER 3

Characterisation and cloning of KIR3DL1⁺ NK cells

Figure 3.1.1	Generation of NK cell clones for analysis of KIR3DL1 functional polymorphism	82
Figure 3. 1	3DL1 genomic DNA allele typing	85
Figure 3.2	3DL1 allele frequencies in normal healthy donors	89
Figure 3.3	Allelic variants of 3DL1 are characterised by different DX9 staining patterns	91
Figure 3.4	3DL1 alleles influence the frequency of expression on NK cells	92
Figure 3.5	Expansion of NK cells from PBMC culture using the RPMI-8866 cell line	93
Figure 3.6	Flow cytometric analysis of isolated NK cells	95
Figure 3.7	Purity of sorted NK cells	97
Figure 3.8	Cell sorting resulted in pure populations of 3DL1+ NK cells	98
Figure 3.9	Clone growth is influenced by tissue culture well shape	100
Figure 3.10	Influence of IL-15 in NK cell clone growth	102

Figure 3.11	Clone growth was identified by the presence of a lymphocyte population	103
Figure 3.12	Clones exhibited an NK cell phenotype	104
Figure 3.13	NK cell clones show cytotoxicity against the K562 cell line	106
Figure 3.14	3DL1 expression by NK cell clones	108
Figure 3.15	NK cell clones express additional inhibitory receptors for HLA	109
Figure 3.16	Identification of non-clonal NK cell populations	110
Figure 3.17	A comparison of NK cell cloning efficiencies	111
Figure 3.18	StemCell Medium generated clones contain less feeder cells	113
Figure 3.19	A high proportion of StemCell medium generated clones display cytotoxic potential	114

CHAPTER 4 KIR3DL1 polymorphism is functional

Figure 4.1	3DL1/HLA-Bw4 interaction was measured using a system of chimeric receptors	131
Figure 4.2	The polyamine based GeneJuice® transfection reagent does not achieve transfection of the Jurkat cell line.	133
Figure 4.3	Transfection of the Jurkat cell line by electroporation	134
Figure 4.4	3DL1*001 and 3DL1*002 are expressed at comparable levels	136
Figure 4.5	721.221 cell line transfectants express HLA at comparable levels	137
Figure 4.6	Optimisation of stimulation conditions	139
Figure 4.7	Evaluation of SEAP activity as reporter readout	140
Figure 4.8	3DL1*001 interacts with HLA-B allotypes	141
Figure 4.9	3DL1*002 interacts with HLA-B allotypes	143
Figure 4.10	The role of CD94 in the recognition of HLA panel	144
Figure 4.11	3DL1*001 specificity in clonal cytotoxicity assay	146
Figure 4.12	3DL1*002 specificity in clonal cytotoxicity assay	149
Figure 4.13	3DL1*005 specificity in clonal cytotoxicity assay	151
Figure 4.14	3DL1*005 specificity in polyclonal cytotoxicity assay	152
Figure 4.15	Activation of NK cells by 721.221 cell lines is inhibited by HLA	155
Figure 4.16	3DL1*001 specificity is maintained in primary NK cells	159
Figure 4.17	3DL1*001 specificity in short term IL-2 stimulated cultures	161

CHAPTER 5 3DS1 expression and function

Figure 5.1.1	Signalling by Short tailed Activatory KIR	171
Figure 5.1	Cloning Strategy for the generation of a FLAG-tagged 3DS1	178
Figure 5.2	Full length 3DS1 construct was generated by PCR	179
Figure 5.3	Leader-FLAG and FLAG-D0 were generated by PCR	180
Figure 5.4	Leader-FLAG-D0 construct was generated by recombinant PCR	181
Figure 5.5	Presence of FLAG insert was verified by sequencing	182
Figure 5.6	3DS1 is expressed at the cell surface	184
Figure 5.7	Z27 recognises 3DS1 on the surface of NK cells	185
Figure 5.8	Z27 recognises 3DS1 on the surface of 3DS1ECD Jurkat cells	186
Figure 5.9	Sorted Z27dim cells express 3DS1	188
Figure 5.10	Expression of 3DS1 on NK and T cells	189
Figure 5.11	3DS1 is expressed on CD8 ⁺ but not CD4 ⁺ T cells	190
Figure 5.12	Crosslinking of 3DS1 leads to NK cell activation	191
Figure 5.13	3DS1ECD Jurkat cells do not recognise HLA-B on EBV transformed B cells	193
Figure 5.14	3DS1 positive NK cells are not activated by HLA-B	194
Figure 5.15	LILRB1 expression on Baf3 transfectants	196
Figure 5.16	HLA tetramers bind LILRB1	197
Figure 5.17	3DL1*002 and 3DS1 show different affinity for HLA-B27 tetramers	199
Figure 5.18	Binding of HLA-B27 tetramers to 3DS1 was not inhibited by Z27 antibody	200
Figure 5.19	3DS1 cell line shows significant staining with HLA-A2 tetramer	201
Figure 5.20	NK cell cytotoxicity is maintained in LTNPs	204
Figure 5.21	Percentage of NK cells is reduced in HIV and HIV LTNP	206
Figure 5.22	3DS1 expression in HIV	207
Figure 5.23	NK cell receptor expression in HIV	208
Figure 5.24	KIR expression on NK cells	210
Figure 5.25	KIR expression on T cells	212
Figure 5.26	Decreased NCR expression on NK cells in HIV infection	214

Figure 5.27	CD94 expression on NK and T cells	215
Figure 5.28	NKG2A expression on NK and T cells	216
Figure 5.29	Increased LILRB1 expression on NK and T cells in HIV infection	217
Figure 5.4.1	A comparsion of the HLA binding regions of 2D and 3D KIR	221

CHAPTER 6 General Discussion

Figure 6.1	3DL1 genetic polymorphism contributes to NK cell functional heterogeneity	231
Figure 6.2	The role of 3DS1 in NK cell activation	235

LIST OF TABLES
CHAPTER 1 Introduction

1.1	NK cell receptors	20
1.2	KIR recognise broad grouping of HLA Class I molecules	25
1.3	Allelic polymorphism of KIR genes	33
1.4	Association of KIR and disease	41

CHAPTER 2 Materials and Methods

2.1	A comparison of the NK cell cloning protocols used	53
2.2	B*27 Tetramers Peptide Sequence	57
2.3	gDNA KIR3DL1 allele typing primers	62
2.4	PCR conditions for KIR3DL1 gDNA allele amplification	63
2.5	cDNA KIR3DL1 allele typing primers	64
2.6	PCR conditions for KIR3DL1 cDNA allele amplification	65
2.7	Primers for the generation of FLAG-tagged KIR3DS1	66
2.8	PCR conditions for KIR3DS1 amplification	67
2.9	PCR conditions for leader, D0 and leaderD0 amplification	68

CHAPTER 3 Characterisation and cloning of 3DL1⁺ NK cells from normal donors

3.1.1	Amino acid difference in KIR3DL1 alleles	76
3.1	Verification of KIR3DL1 genomic DNA allele typing	86
3.2	Summary of donor KIR3DL1 allele typing and phenotyping	87

CHAPTER 4 KIR3DL1 polymorphism is functional

4.1.1	Amino acid sequence at the Bw4/Bw6 motif	125
4.1	Conditions for GeneJuice® Transfection	132

CHAPTER 5 KIR3DL1 expression and function

5.1	Characteristics of viremic HIV, HIV LTNP and control patients	203
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ABBREVIATIONS

ADCC	Antibody-dependent cell cytotoxicity
AICD	Activation-induced cell death
AICL	Activation-induced C-type lectin
AIDS	Acquired immunodeficiency syndrome
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
APC	Antigen presenting cell
BAT3	HLA-B -associated transcript 3
bp	Base pair
BM	Bone marrow
BSA	Bovine serum albumin
cDNA	Complementary DNA
cFLIP-L	Cellular FLICE-inhibitory protein – long form
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CMVR	CMV retinitis
CTL	Cytotoxic T lymphocyte
DAMP	Danger associated molecular pattern
DAP12	DNAX-activating protein of 12 kDa
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule 1
DNA-PK	DNA protein kinase
dNTP	Deoxyribonucleotide triphosphate
dsRNA	double-stranded RNA
EBV	Epstein-Barr virus
ECD	Extracellular domain
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular regulated kinase
EU	Exposed uninfected
FACS	Fluorescence associated cell sorting
FasL	Fas ligand
FCS	Foetal calf serum
FcR	Fc receptor
FITC	Fluorescein isothiocyanate
GFP	Green fluorescence protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GvH	Graft versus host
GvL	Graft versus leukaemia
GvT	Graft versus tumour
HA	Haemagglutinin

HAART	Highly active anti-retroviral therapy
HCMV	Human CMV
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
HSV	Herpes simplex virus
ICAM	Intercellular adhesion molecule
iDC	Immature DC
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Ile	Isoleucine
IMDM	Iscoe's modified Dulbecco's medium
iNOS	Inducible nitric oxide synthase
IP-10	Interferon-inducible protein 10
I-TAC	Interferon-inducible T cell alpha chemoattractant
ITAM	Immunotyrosine-based activatory motif
ITIM	Immunotyrosine-based inhibitory motif
ITSM	Immunotyrosine-based switch motif
KIR	Killer cell immunoglobulin-like receptor
kb	Kilobase
KLRG1	Killer cell lectin-like receptor subfamily G, member 1
LAT	Linker for T-cell activation
LAK	Lymphokine-activated killing
LAIR	Leukocyte-associated immunoglobulin-like receptor
LFA	leukocyte function associated antigen
LILR	leukocyte immunoglobulin-like receptor
LRC	Leukocyte receptor complex
LTNP	Long term non progressor
LU	Lytic units
MAC	<i>Mycobacterium avium</i> complex
MHC	Major histocompatibility complex
MICA/B	MHC class I chain related A/B
MIP	Macrophage inflammatory protein
MLR	Mixed lymphocyte reaction
mRNA	Messenger RNA
NCR	Natural cytotoxicity receptor
NK	Natural Killer
NKP	NK precursor
PAMP	Pathogen associated molecular pattern
PARP	POLY (ADP-ribose) polymerase
PCP	Polymerase chain reaction
PCP	<i>Pneumocystis Carinii</i> pneumonia

PBS	Phosphate buffered saline
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein
PHA	Phytohaemagglutinin
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 12-acetate
PMN	Polymorphonuclear cells
PVR	Polio virus receptor
RAG	Recombination activating gene
rh	Recombinant human
RT	Reverse transcription
RANTES	Regulated upon activation, normal T-cell expressed and
secreted	
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT	Reverse transcription
SBT	Sequence based typing
SCGM	Stem cell growth medium
SCID	Severe combined immunodeficiency
SD	Standard deviation
SEAP	Secreted alkaline phosphatase
SEM	Standard error of the mean
SHP-1	SH2-containing protein tyrosine phosphatase-1
SSCP	Sequence-specific conformational polymorphism
SSOP	Sequence-specific oligonucleotide primer
SSP	Sequence-specific primer
TAP	Transporters associated with antigen processing
TGF- β	Tumour growth factor- β
Thr	Threonine
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
ULBP	UL16 binding protein
uNK	uterine NK
VZV	varicella-zoster virus
ZAP-70	Zeta-chain (TCR) associated protein kinase-70

Chapter One

Introduction

1.1 Natural Killer cells

Natural Killer (NK) cells are large granular lymphocytes that account for approximately 10% of peripheral blood lymphocytes. Natural Killer cells were so called because of their ability to lyse certain tumour cells without the need for prior exposure and without fine restriction by major histocompatibility (MHC) antigens. Initially described in the 1970s (Baldwin et al, 1973; Kiessling et al, 1975), NK cells are identifiable by their large granular morphology and expression on the cell surface of CD56 but not CD3 antigen. Expression of the natural cytotoxicity receptor (NCR) NKp46 has recently been suggested to be a more definitive marker for the selective identification of NK cells in humans and mice (Walzer et al, 2007). NK cells are potent producers of several cytokines including IFN- γ , granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor α (TNF- α). NK cells are important mediators of the innate immune system. In addition to their role in tumour surveillance, NK cells play an important role in defence against viral and bacterial infection, in pregnancy and in the regulation of the immune response.

In contrast to T cells, NK cells do not require antigen-specific recognition to kill target cells. For many years the receptors involved in recognition of target cells were unknown. In the last 15 years there has been an explosion of knowledge in this area with the identification of several families of receptors that control recognition by NK cells. These receptors include both activatory and inhibitory cell surface receptors and it is the balance of signals received by an NK cell that controls activation. The primary activatory receptors involved in target cell recognition include natural cytotoxicity receptor (NCR) family members and NKG2D. Members of the NCR family (NKp30, NKp46 and NKp44) recognise (as yet unknown) constitutively expressed cellular ligands. The constant presence of activatory signals for NK cells requires that healthy cells also express inhibitory ligands to prevent inappropriate NK cell activation. An important source of inhibitory ligands is the expression of the MHC antigens. These antigens are expressed by virtually all healthy cells, but are frequently down-regulated during infection or transformation. NK cells have evolved two different and complementary receptor systems to allow the detection of these highly polymorphic MHC antigens – CD94/NKG2A (in mice and humans) and KIR

(humans) or Ly49 (mice). The C-type lectin-like CD94/NKG2A heterodimer recognises non-classical MHC antigen (HLA-E in humans, Qa-1 in mice). This relatively non-polymorphic molecule binds to and presents the leader sequence from other MHC antigens (in humans HLA-A, -B, -C and -G). Inhibition though the ligation of CD94/NKG2A is lost in the event of a total loss of MHC expression. The KIR family of receptors in humans are a diverse family of receptors that recognise polymorphic determinants on HLA antigens e.g. the KIR2DL1 receptor recognises a subset of HLA-C molecules HLA-C2, characterised by serine at position 77 and asparagine at position 80, but not HLA-C1 allotypes. Expression of these receptors allows NK cell to be sensitive to the loss of individual HLA allotypes. The Ly49 receptor family functions in a similar manner in mice but is genetically unrelated to the KIR family. The conservation of the CD94/NKG2A system between mice and humans points to this receptor system being evolutionarily older than the KIR/Ly49 families. The convergent evolution of the KIR family in humans and the Ly49 family in mice suggests that there is significant advantage to the ability to discriminate the loss of expression of individual MHC locus in addition to monitoring global MHC expression.

1.1.1 NK cell cytotoxicity

One of the key effector mechanisms of NK cells is their ability to cause the death of infected or transformed cells. The array of triggering and inhibitory NK cell receptors allows for the induction of NK cell cytotoxicity from either an increase in positive signals or a decrease in inhibitory signals. The “missing self” hypothesis of NK cell activation proposes that NK cells are activated by the absence of MHC due the loss of an inhibitory signal to counteract a ubiquitous positive signal ((Ljunggren and Karre, 1990), Figure 1.1).

Lymphokine-activate killing (LAK) activity is a term used to describe killing by activated NK cells. Traditionally, LAK activity refers to NK cell cytotoxicity induced by the direct action of IL-2, or other cytokines, on NK cells. It involves the up-regulation of one or more activatory receptors on the surface of the NK cells and results in a greater potency of NK cell killing as well as an increase in the range of target cells that are susceptible to NK cell lysis.

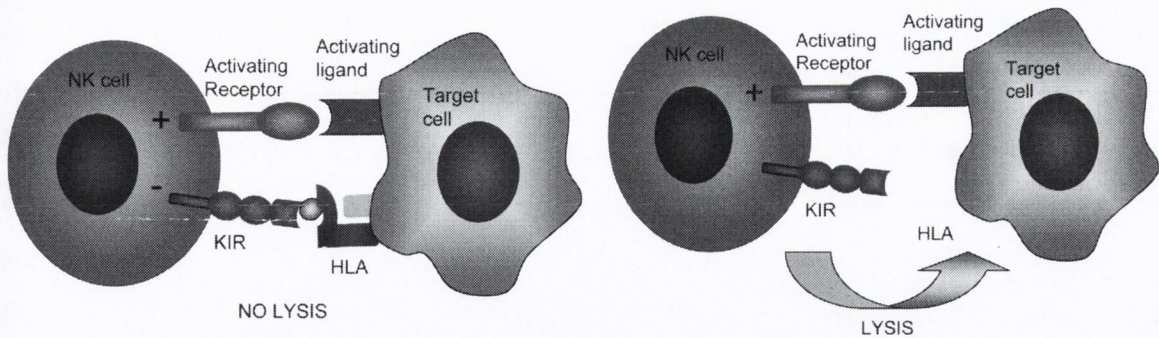


Figure 1.1 “Missing self” hypothesis of NK cell recognition.

Engagement of an inhibitory receptor (such as KIR) by HLA prevents activation of NK cells. In the absence of this signal (“missing self”), NK cells are activated by ubiquitous activatory signals. Activation of the NK cell then leads to lysis of the altered target cell.

NK cells kill target cells by a number of different mechanisms including the exocytosis of cytotoxic granules and the engagement of death receptors. Resting NK cells possess preformed cytoplasmic granules that contain perforin and granzymes. Perforin disrupts the membrane of the target cells (Masson and Tschopp, 1985) and allows entry of granzyme, although the exact mechanisms by which they do this is unclear (Keefe et al, 2005; Metkar et al, 2002). Granzymes are structurally related serine proteases that differ in substrate specificity. Granzyme A and B are believed to be important in NK cell cytotoxicity. Granzyme B is responsible for the rapid induction of caspase-dependent apoptosis in target cells (Trapani and Sutton, 2003). Cleavage of the pro-apoptotic protein Bid results in the release of cytochrome c from the mitochondria, the formation of the apoptosome and the activation of a caspase cascade (Alimonti et al, 2001). Granzyme B has also been reported to directly activate caspases and to directly cleave some caspase substrates including poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK) (Andrade et al, 1998; Froelich et al, 1996). Granzyme A induced cell death does not involve the activation of caspases but is believed to result in the release of reactive oxygen species (ROS) from the mitochondria (Martinvalet et al, 2005). NK cells also express members of the death receptor family, FasL and TNF-related apoptosis inducing ligand (TRAIL) (Mirandola et al, 2004; Montel et al, 1995). Ligation of these receptors by their

ligand (Fas and TRAILR respectively) on target cells induces apoptosis of target cells. TRAIL is constitutively expressed on a small subset of NK cells but expression is induced by IL-2, IFN (type I and II) and IL-15 (Smyth et al, 2003). In mice, cancer cells that do not express FasL can be induced to do so by the production of IFN- γ , rendering them susceptible to NK cell attack (Screpanti et al, 2001).

1.1.2 NK cell and cytokines

A second major effector mechanism of NK cells is the production of cytokines and chemokines. NK cell cytokine production in response to cytokine stimulation is predominately due to the CD56^{bright} subset of NK cells (see 1.1.3), which can produce large amounts of IFN- γ , TNF- α , TGF- β and GM-CSF (Cooper et al, 2001b). CD56^{bright} NK cells appear to have an intrinsic ability to produce more cytokine than CD56^{dim} NK cells, as they consistently produce more cytokine in response to monokine stimulation, co-culture with LPS-stimulated macrophages and PMA and ionomycin stimulation (Cooper et al, 2001a). Production of IFN- γ by NK cell required the presence of two or more signals (Fehniger et al, 1999). IL-1 and TNF- α both synergise with IL-12 for IFN- γ production. IL-18 also stimulates IFN- γ production and induces cytotoxic activity and can synergise with IL-12 to augment these responses. The optimal stimulus of production of cytokine by NK cells differs between cytokine; IL-18 in combination with IL-12 is optimal for induction of production of IFN- γ , while IL-18 with IL-15 results in optimal GM-CSF production (Cooper et al, 2001b).

A number of cytokines affect NK cell function, including type I interferon (IFN), IL-12, IL-18 and IL-15. IFN- α and - β increase NK cell cytolytic activity within 4-6 hours of stimulation and cause NK cell proliferation in vivo. IL-12, initially identified as NK cell stimulatory factor (NKSF), stimulates cytotoxicity and IFN- γ production by NK cells (Chan et al, 1992). This cytokine is believed to be critical in macrophage-induced NK cell activation during certain viral and bacterial infections (Chace et al, 1997; Orange and Biron, 1996). IL-15 is an important cytokine for the development of mature NK cells and the maintenance of NK cells in the periphery (Liu et al, 2000). It can also synergise with IL-12 for IFN- γ and MIP-1 α production by NK cells. TGF- β inhibits IFN- γ production by

NK cells (Bellone et al, 1995) and indirectly inhibits NK cells by inhibiting IL-12 production (Tada et al, 2004).

MIP-1 α , MIP-1 β , MCP-1, MCP-2, MCP-3 and RANTES have been shown to cause chemotaxis of NK cells in vitro (Taub et al, 1995). CD56^{dim} NK cells express high levels CXCR1 and CX₃C-chemokine receptor 1 and show functional responses to IL-8 and fractalkine, the respective ligands for these receptors (Inngjerdingen et al, 2001). CD56^{bright} NK cells express CXCR3 and show strong chemotactic responses to the ligands for this receptor, interferon-inducible T-cell chemoattractant (I-TAC) and interferon-inducible protein 10 (IP-10).

1.1.3 NK cell subsets

The major fraction (approximately 90%) of peripheral blood NK cells is composed of CD56^{dim} CD16⁺ NK cells, and the remainder is made up of CD56^{bright} CD16⁻ NK cells. There are a number of differences between these two NK cell subsets, including the level of natural cytotoxicity and LAK activity, cytokine production and receptor expression (Figure 1.2). CD56^{dim} NK cells are traditionally considered to be poor producers of cytokine in response to monokine stimulation but display high resting cytotoxic activity, while the inverse is true for CD56^{bright} NK cells (Cooper et al, 2001b; Nagler et al, 1989). Constitutive expression of a high affinity IL-2 receptor on CD56^{bright} NK cells allows them to be more sensitive to IL-2 stimulation and increases LAK activity (Caligiuri et al, 1990). CD56^{bright} NK cells lack CD16 expression and as such are not capable of antibody-dependent cellular cytotoxicity (ADCC) (Nagler et al, 1989). KIR expression is mainly confined to the CD56^{dim} population, while CD56^{bright} NK cells express greater amounts of CD94/NKG2A (Jacobs et al, 2001; Lima et al, 2001). CD56^{bright} NK cells are found in higher proportions in tissues, possible due to their distinct chemokine and adhesion receptor expression profile (Campbell et al, 2001).

The relationship between these functionally distinct subsets is unclear. While it has been reported that CD56^{bright} cells represent an NK cell fully differentiated under the influence of

IL-12 (Loza and Perussia, 2004), there is a growing consensus that CD56^{dim} cells are the terminally differentiated NK cell (Mrozek et al, 1996; Ouyang et al, 2007). CD56^{dim} NK cells express a full array of cell surface receptors allowing them to function as sensitive detectors of alterations in HLA expression, while the CD56^{bright} NK cell subset, with large capacity for cytokine production, may play an immunomodulatory role in tissues.

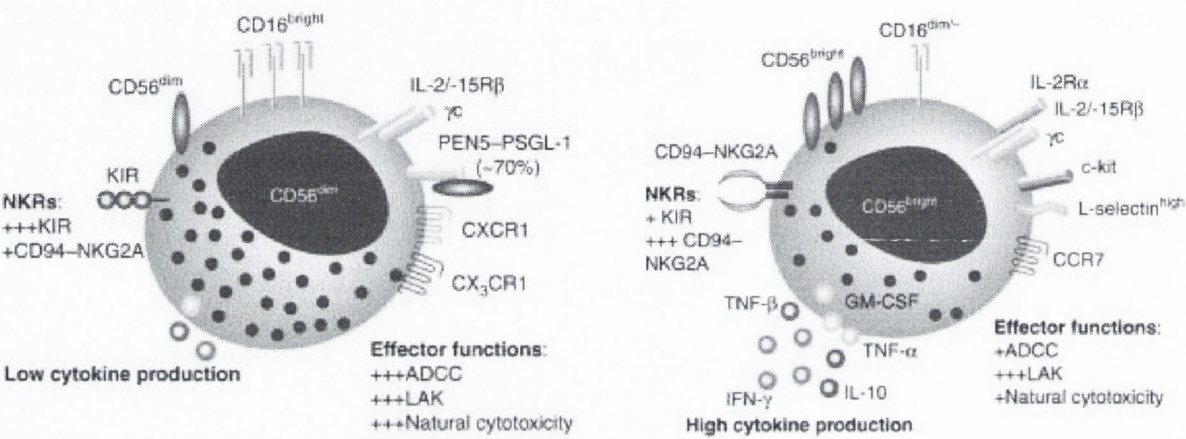


Figure 1.2 NK CD56^{dim} and CD56^{bright} cell subsets

CD56^{dim} and CD56^{bright} NK cells differ in receptor expression, cytotoxic activity and cytokine production. CD56^{dim} NK cells, which represent the bulk of the peripheral blood NK cell population, typically express CD16, KIR, CD94/NKG2A and the medium affinity IL-2 receptor. CD56^{bright} NK cells do not typically express CD16 or KIR, but express high levels of CD94/NKG2A and the high affinity IL-2 receptor. Adapted from Cooper et al, 2001[a].

1.2 NK cell development

NK cells are derived from CD34⁺ haematopoietic stem cells (HSC) in the bone marrow (BM) (Colucci et al, 2003). The bone marrow provides both the mix of cytokines needed for NK cell development and the stromal cells that are required for full maturation of NK cells. There is also recent evidence in support of a role for the secondary lymphoid organs as significant sites of NK cell development (Freud et al, 2005). Unlike T and B

lymphocytes, NK cells do not require antigen receptor rearrangement and are present in SCID, RAG-1 and RAG-2 deficient mice.

The first stage in the development of a NK cells is the commitment of a HSC to the lymphoid lineage and the creation of a common lymphoid progenitor (CLP). The next step involves the generation of a common T and NK cell progenitor (T/NKP), followed by the transition to a NK cell progenitor (NKP). Generation of NKP from HSC is dependent on bone marrow derived factors, including ligands for the class III receptor tyrosine kinases c-kit (ligand KL) and FLT3 (ligand FL), and is marked by the acquisition of the IL-2/15R β . The acquisition of this receptor chain confers sensitivity to IL-15, whose receptor signals through a unique α -chain (IL-15R α), a β chain that is shared with IL-2 (IL-2/15R β) and the common γ c chain. IL-15 is an important cytokine in the development of NK cells, and a defect in IL-15 function leads to impaired generation of NK cells in mice and humans. The transition from NKP to mature NK cell involves the acquisition of NK cell markers and capacity to elicit effector functions. The first receptor expressed by immature NK cells is a member of the NK cell receptor protein 1 (NKR-P1) family, CD161. CD56 is subsequently acquired, along with cytotoxic activity. CD94/NKG2 complexes are expressed next, followed by KIR (Figure 1.3).

It was believed that each mature NK cell would have to express at least one receptor that recognised self-HLA to maintain self-tolerance. This theory (“at least one”) was supported by a study that profiled the receptor expression in a large panel of NK cell clones generated from two donors, which showed that each clone expressed at least one inhibitory receptor for that individual’s HLA alleles (Valiante et al, 1997). In the past two years however a number of studies have called this model into question. In both mice and humans, there is a subset of NK cells that do not express any known inhibitory receptor (Anfossi et al, 2006; Fernandez et al, 2005; Kim et al, 2005). In healthy human subjects, this subset accounts for approximately $13\% \pm 6\%$ of peripheral blood NK cells, and importantly it is hyporesponsive to various stimuli, including HLA-negative target cells. These NK cells exhibit a mature NK cell phenotype, express the full spectrum of activatory receptors and respond normally to the pharmacological activators PMA and ionomycin (Anfossi et al, 2006).

There is therefore no global deficit in NK cell function, but rather a specific dampening of certain NK cell responses in such a way as to prevent unwanted NK cell auto-immunity. There are a number of models that could explain the mechanisms of NK cell self-tolerance (Raulet and Vance, 2006). Hyporesponsiveness may be induced in NK cells lacking inhibitory receptor expression by chronic stimulation upon interaction with autologous cells. This model (the “disarming model”) is similar to the induction of anergy in T and B lymphocytes. In contrast, the “arming model” proposes that ligation of inhibitory receptors on NK cells by their HLA ligands is necessary for the functional maturation of NK cells and in the absence of appropriate receptor expression NK cell maturation is blocking leading to hyporesponsive NK cells. The development of hyporesponsive NK cells in mosaic mice expressing a mixture of MHC class I positive and negative cells suggest that encounters with class I negative host cells dominantly induces tolerance (Johansson et al, 1997). This favours actively induced hyporesponsiveness, as predicted by the disarming model, rather than a failure of ‘arming’ (where interactions with cells that lack ligands for inhibitory receptors are irrelevant).

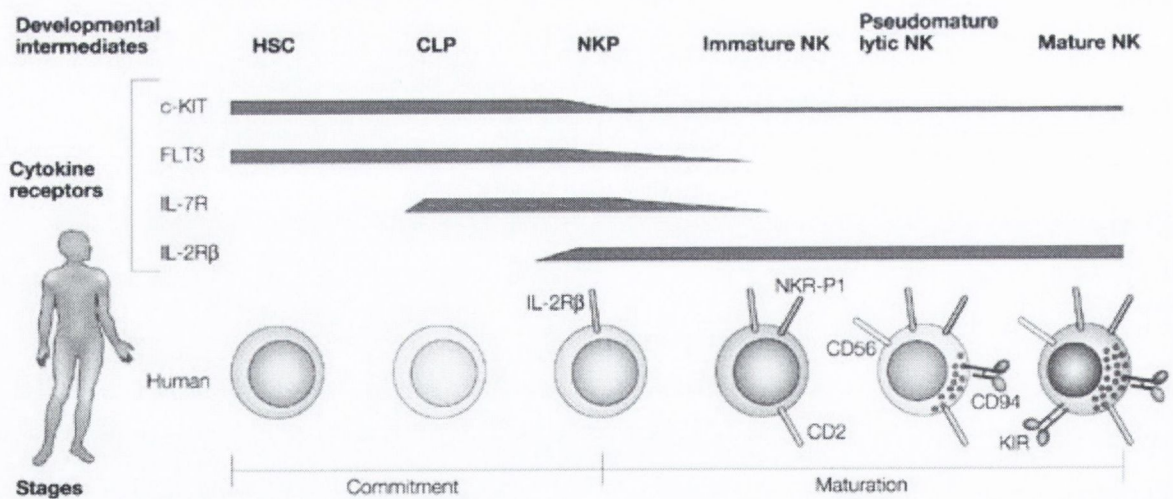


Figure 1.3 The development of NK cells in humans.

NK cell differentiation occurs in the bone marrow under the direction of BM-derived cytokines and stromal cells. Acquisition of IL-2/15Rβ marks the generation of a NK progenitor from the common lymphoid progenitor. NKP then acquire a number of cell surface markers that mark different stages in their development. Adapted from Colucci 2003.

1.3 The function of NK cells

1.3.1 NK cells and cancer

NK cells were initially described due to their ability to lyse certain tumour cells without the need for prior sensitisation, suggesting a possible role for NK cells in immune surveillance against spontaneously arising tumours and in anti-tumour activity. A number of animal models of cancer and metastases have implicated NK cells in anti-tumour immunity. There is a direct correlation between the levels of NK cell activity and resistance to transplanted syngenic tumours (Kuppen et al, 2001). Immunosuppressive doses of cyclophosphamide render mice more susceptible to injected malignant cells; an effect reversed by the adoptive transfer of NK cell clones or NK cell enriched lymphocytes (Riccardi et al, 1981). In humans, patients with Chediak-Higashi or X-linked lymphoproliferative syndromes, who have very low NK cell activity, are more susceptible to malignant lymphoproliferative disorders (Roder et al, 1982).

NK cells express a number of activating receptors that may be important in tumour killing, including the natural cytotoxicity receptors and NKG2D, both of which play a role in the lysis of tumour cells (Pende et al, 2001). NKG2D is of particular interest, as it is known to recognise ligands that are induced on abnormal cells that are undergoing transformation or stress. NKG2D recognised stress proteins including UL-16 binding protein (ULBP) and MHC class-I chain related A and B antigens (MICA and MICB). NK cells can target tumour cells in a number of ways including the down-regulation of HLA molecules on the surface of tumour cells and the expression of Fas and TRAILR on tumour cells. NK cells are also potent producers of IFN- γ , which can block angiogenesis and stimulate antigen presentation by up-regulating MHC class I and class II molecules. The anti-tumour effect of IL-12 is attributed to its ability to induce IFN- γ production by NK cells (Bigda et al, 1993; Soiffer et al, 1993).

Two main strategies for the therapeutic use of NK cells have been considered, activation of endogenous NK cells and adoptive transfer of *in vitro* activated autologous NK cells (Ljunggren and Malmberg, 2007). Activation of endogenous NK cells generally involved

the use of IL-2, frequently in combination with biological response modifiers such as IFN. Trials using this approach have noted a marked accumulation of NK cells at tumour sites, but not to the degree considered necessary for elimination of the tumour. Adoptive transfer of IL-2 stimulated NK cells gave results that are more promising during trials, and were used in a clinical setting, but again results were disappointing (Albertsson et al, 2003). In light of recent developments in the understanding of the control of NK cell killing and NK cell homing to tumour sites, it is hoped that new approaches to the use of NK cells in this setting, that will allow optimal infiltration of the tumour may be developed.

1.3.2 NK cells in transplantation

Haematopoietic stem cell (HSC) transplantation is used as a treatment for leukaemia and lymphoma, and involves the transfer of HSC derived from aspirate of bone marrow, from peripheral blood into which stem cells have been mobilised, or from placental cord blood. The recipient is first treated with chemotherapy and irradiation to kill tumour cells and ablate the bone marrow. After transplantation, the progeny of the transplanted stem cells repopulate the haematopoietic system of the patient. NK cells are the first lymphocyte to appear in peripheral blood after transplantation and account for approximately 80% of circulating lymphocytes for up to three months after transplantation.

Transplant conditions are optimised to reduce the incidence of graft versus host disease (GVHD), caused by mature T and NK cells from the donor attacking host cells. However, it was found that some degree of GVH actually favours the process of engraftment and tumour cell eradication. This improvement can be attributed to donor cells that further deplete recipient bone marrow cells and attack host cells that would otherwise cause an alloreaction against donor stem cells. Similarly, transplanted NK and T cells may attack residual tumour cells in an effect known as graft-versus-leukaemia (GVL) or graft-versus tumour (GVT). A report by Ruggeri *et al* suggests that a mismatch in appropriate HLA groups - KIR ligands - may be more beneficial in stem cell transplants than a complete HLA match (Ruggeri et al, 2002). This will give rise to a subset of donor NK cells in the recipient that is not inhibited by recipient HLA and helps mediate a GVL effect. NK cells

are found to predominantly attack cells of the haematopoietic lineage while sparing epithelial cells, a target of GvH disease. NK cells also attack bone marrow derived antigen presenting cells, preventing them from presenting to T cells, which may lead to GvH disease. NK cells that are activated in the recipient allow for many of the benefits of GvL (including targeting of residual disease and the prevention of GvH), while causing minimal GvH, suggesting that in place of the perfect HLA match, a “perfect mismatch” may be more beneficial. Despite the very promising results from Ruggeri *et al*, further analysis of the role of alloreactive NK cells have provided conflicting evidence. A review of these studies suggests that the transplantation protocol and in particular the NK: T cell ratio in the stem cell infusion may be a critical variable (Witt and Christiansen, 2006). The protocol of Ruggeri *et al* involved intensive depletion of donor T cells and very high stem cell doses together with the lack of prophylaxis for GvH (such as cyclosporin) to promote rapid immune reconstitution. A greater understanding of the conditions suitable for maximising the beneficial role of alloreactive NK cells in bone marrow transplantation is necessary to fully exploit their potential.

1.3.3 Shaping of the adaptive immune response

Initially regarded as ‘crude’ effectors of the innate immune system, there is now mounting evidence that NK cells are important players in setting the stage for an appropriate adaptive immune response through their interaction with dendritic cells (DCs) (Gerosa et al, 2002; Piccioli et al, 2002). DCs are crucial regulators of the adaptive T cell response to pathogen, acting as the main antigen-presenting cell to prime naïve T cells to mount a suitable adaptive response to pathogen. Immature DCs (iDCs), which patrol the periphery, are specialised for antigen uptake and processing. Upon antigen capture these cells switch to an antigen-presenting mode and migrate to the lymph node for interaction with T cells. This switch is characterised by the up-regulation of co-stimulation markers including HLA molecules (Ferlazzo et al, 2001). Evidence of interactions between these two cell types suggests that bidirectional crosstalk regulates the response of both cell types.

Activated NK cells are capable of killing immature DC, despite normal levels of HLA expression by these cells. Upon maturation, these cells up-regulate HLA and become

resistant to NK cell lysis. Killing of iDC by NK cells is mediated almost exclusively by the activatory receptor NKp30, with little if any role for NKp44 or NKp46 (Ferlazzo et al, 2002). NKp30 (and in part NKG2D) are down-regulated on the surface of NK cells in response to TGF- β 1, which may provide resistance to TGF- β 1 producing DCs (Castriconi et al, 2003). This killing of DCs that have failed to become fully activated has been proposed to form part of a 'quality control' system where the quantity and quality of antigen presentation during an infection is controlled in part by NK cells.

Interaction between NK cells and DCs can also induce DC maturation, mediated by NK cell-derived cytokines. Ligation of NKp30 on NK cells by DC promotes the secretion of TNF- α and IFN- γ , which leads to DC maturation (Vitale et al, 2005). Indeed the supernatant from NKp30 triggered NK cells is sufficient to cause DC maturation and production of IL-12. Mature DCs have recently been shown to induce the activation (including cytotoxicity and IFN- γ production) and proliferation of resting NK cells. Proliferation of NK cells and production of IFN- γ appears to be confined to the CD56^{bright} subset of NK cells (Vitale et al, 2004). This subset of NK cells is characterised by a distinct chemokine receptor profile (including CD62L, CCR7 and CXCR3), perhaps allowing these cell to traffic to secondary lymphoid organs for interaction with DCs during an immune response. Different DC derived cytokines are responsible for NK cell activation with IL-12 promoting NK cell IFN- γ production and IL-15 inducing proliferation (Ferlazzo et al, 2004).

During an immune response, NK cells are likely to encounter DCs in inflamed tissue where DC-mediated increases in NK cell cytolytic activity and IFN- γ production promotes both killing of infected cells and generation of a Th1 response. In turn, NK cells can optimise the DC response by editing unsuitable iDC or promoting their maturation. Thus these two cell types can work in concert to generate a maximal adaptive response.

1.3.4 NK cells and viral infection

There is very strong evidence, from both murine studies and human NK cell deficiencies, that NK cells are important mediators of anti-viral immunity. NK cells combat viral

infection through the killing of virally infected targets and the production of cytokines (Figure 1.4). Virally infected cells become a target for NK cell cytotoxicity due to decreased expression of inhibitory ligands (HLA class I) and the expression of stress proteins such as MICA and MICB, which act as ligands for the activatory receptor NKG2D. Virally infected cells produce IFN α and IFN β , which are potent inducers of NK cell cytotoxicity. The NCRs have also been implicated in anti-viral immunity. Although their cellular ligands remain unknown, soluble Ig fusion proteins of NKp46 and NKp44, but not NKp30, specifically bind to haemagglutinin (HA) of influenza virus (IV) and haemagglutinin neuroaminidase of Sendai virus (Arnon et al, 2001; Mandelboim et al, 2001).

IL-12, which is produced during the course of some but not all viral infections, induces IFN- γ production by NK cells (Orange and Biron, 1996). IFN- γ is capable of activating iNOS-dependent and independent pathways of antiviral defence mediated by macrophages. The production of NO by iNOS involves a number of reactive intermediates and products, which can inhibit viral propagation by modifying molecules essential for replication. NK cell derived IFN- γ has been shown to play a crucial role in the induction of a Th1 response in mice (Martin-Fontecha et al, 2004).

1.3.4.1 NK cell deficiencies

Chronic viral infections, particularly by members of the Herpesvirus family, are seen in cases of NK cell deficiency in humans (Orange, 2002). Herpesviruses are a family of double-stranded DNA viruses with large genomes that include cytomegalovirus (CMV), varicella-zoster virus (VZV), Herpes simplex virus (HSV) type I and II, human herpesvirus 8, and Epstein-Barr virus (EBV). The best-known NK cell deficiency is that described by Biron *et al* in 1989, of an adolescent patient with a complete absence of CD56⁺ or CD16⁺ lymphocytes. This patient presented with a disseminated, life-threatening varicella infection, and subsequently developed CMV pneumonitis and cutaneous HSV (Biron et al, 1989). This patient's deficiency, while unknown in origin, was limited to an absence of NK cells, as she had evidence of an intact adaptive immune system with normal varicella-induced lymphocyte proliferation and varicella-specific antibodies. Other NK cell

deficiencies, such as those seen due to familial erythrophagocytic lymphohistiocytosis causing a defect in perforin also increase susceptibility to particular viruses including EBV, CMV, VZV and HSV (Orange, 2002).

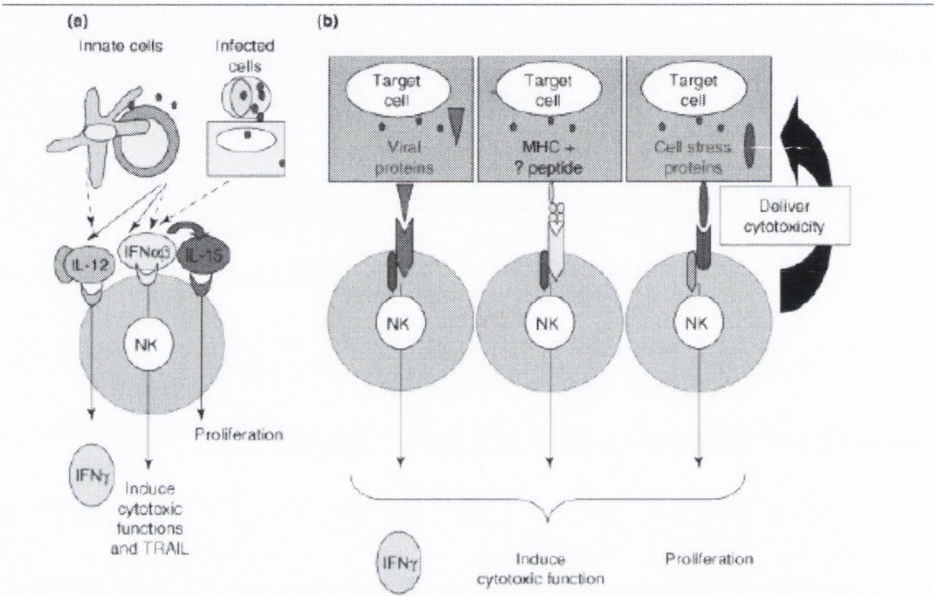


Figure 1.4 NK cells are activated during viral infection

A number of pathways can lead to the activation of NK cells during a viral infection. A, Production of cytokines such as type I interferon, IL-12 or IL-15 by infected cells or accessory cells can lead to NK cell activation. B, Infected cells may also be targeted for NK cell-mediated lysis due to the down-regulation or altered expression of HLA or the expression of stress-induced activatory ligands. Figure adapted from (Lee et al, 2007)

1.3.4.2 Viral Immune Evasion

In further support of the importance of the role played by NK cells in viral infection, are the evasion strategies that viruses have evolved to evade NK cells (reviewed in Orange et al, 2002)). Human cytomegalovirus (HCMV), a DNA virus that causes disease in immuno-compromised individuals, is an example of a virus that targets the immune response at a number of levels. Both murine and human CMV down-regulate host MHC I using several viral proteins (e.g. HCMV proteins US2, US3, US6 and US11) that interfere with distinct

stages of the MHC I biosynthetic pathway (Tortorella et al, 2000). The down-regulation of host MHC I impairs CD8⁺ T cell effector function against HCMV-infected target cells. This down-regulation of an important source of inhibitory signals for NK cells would make infected cells a target for NK cell attack, however the HCMV genome encodes a number of proteins that target the NK cell response. The MHC homologue UL18 binds to the inhibitory receptor LILRB1 expressed on NK cells and other cell types (Cosman et al, 1997). Expression of this protein by infected cells is believed to inhibit cell lysis by LILRB1⁺ NK cells, although it may actually activate LILRB1⁻ NK cells (Leong et al, 1998; Prod'homme et al, 2007). The leader sequence of UL40 contains a nonamer sequence similar to HLA class I proteins that supports the expression of HLA-E. Expression of UL40 leads to the up-regulation of HLA-E on the surface of infected cells, independent of transport-associated with antigen processing (TAP, which is inhibited by US6) and protects the cell from lysis by CD94/NKG2A⁺ NK cells (Tomasec et al, 2000). IFN- γ has been shown to increase the expression of HLA-E and the potency of inhibition of NK cells due to expression of UL40 from clinical isolates (Cerboni et al, 2001).

The HCMV protein UL16 has been found to bind to host cell ligands for the activating NK cell receptor NKG2D (Cosman et al, 2001), and sequester them in the endoplasmic reticulum within the cell, to prevent NKG2D-mediated immune responses. UL16 is an early CMV gene, which is dispensable for viral replication in tissue culture (Kaye et al, 1992). It encodes a type I membrane glycoprotein and is expressed by CMV-infected cells, but is undetectable in virions. Soluble UL16 was found to bind the NKG2D ligands, ULBP-1, -2 and MICB, but curiously not to MICA or ULBP-3. More recently, an additional viral protein UL142 has been shown to down-regulate the cell surface expression of MICA (Chalupny et al, 2006). UL141 targets the ligand of another activating receptor on NK cells. This protein blocks cell surface expression of CD155, which serves as a ligand for the activatory receptors DNAM-1 and CD96 on NK cells (Tomasec et al, 2005). A HCMV structural protein pp65 inhibits NK cells by targeting the triggering receptor Nkp30 (Arnon et al, 2005). pp65 binds to Nkp30 but rather than trigger the receptor, it causes dissociation from Nkp30 of the signalling CD3 ζ chain, leading to a reduction in NK cell activation. Thus the HCMV virus encodes several immunomodulatory proteins that

influence NK cell activation through both inhibitory and activatory receptors, highlighting the importance of the NK cell response.

1.3.4.3 NK cells and HIV

HIV is a retrovirus that infects almost 40 million people worldwide and is estimated to have caused more than 25 million deaths since 1981 (UNAIDS/WHO AIDS Epidemic Update: December 2006). Despite nearly two decades of research aimed at inducing an appropriate adaptive immune response, no successful immunological therapy or vaccine has been developed. This virus targets and compromises an important component of the adaptive immune system – CD4⁺ T cells. HIV further escapes the adaptive immune response through rapid mutation to avoid recognition by CD8⁺ CTL. The innate system is increasingly believed to play an important role in the immune response to HIV (Levy, 2001). Common entry points of HIV are mucosal surfaces that are rich in innate immune cells such as DCs, $\gamma\delta$ T cells and macrophages (Lehner, 2003). These cells are capable of secreting a number of chemokines and cytokines that block HIV replication and transmission in the mucosal surface. While NK cells are known to be important antiviral effector cells in some viral infections, a direct role for NK cells in HIV infection has not been demonstrated.

NK cells may influence the course of HIV infection at a number of levels including the entry of HIV into target cells, the killing of infected cells, production of cytokines that influence the adaptive immune response as well as in the control of opportunistic infections and malignancies. R5 strains of the HIV virus require the chemokine receptor CCR5 as a co-receptor for entry into CD4⁺ T cells. The importance of this receptor in disease progression is evident in patients who are homozygous for the 32-bp deleted CCR5 allele, where virus transmission is rare; patients who are heterozygous for this allele show a slower rate of progression to AIDS (O'Brien et al, 1997). High levels of CC chemokines (including MIP-1 α , MIP-1 β and RANTES), such as those produced by activated NK cells, would be expected to block HIV access to CCR5 and thus limit replication and spread of the virus (Oliva et al, 1998). HIV-infected cells may become targets for NK cell cytotoxicity due to a modulation in HLA expression or an increase in the expression of ligands for triggering NK cell receptors. The viral nef protein has been shown to cause a

selective down-regulation in HLA-A and -B alleles but not HLA-C or -E (Cohen et al, 1999). Many known CTLs are restricted by HLA-A or -B allotypes but few are restricted by HLA-C and as such selective down-regulation of HLA-A and -B may protect HIV-infected cells from CTL attack. This strategy however leaves the target cell vulnerable to lysis by NK cells which express inhibitory receptors for these HLA ligands. HIV leads to the up-regulation of the NKG2D ligands ULBP1, ULBP2 and ULBP3 on the surface of the infected cells and blocking of the NKG2D receptor leads to a reduction in the NK cell mediated lysis of HIV-infected autologous T cell blasts (Ward et al, 2007). However, the nef protein modulates this increase in NKG2D ligands, down-regulating ULBP1, ULBP2 and MICA and causing a reduction in the NK cell lysis of infected cells (Cerboni et al, 2007). Stimulation of NK cells with single stranded RNA from the HIV-1 long term repeat (LTR) leads to the activation of NK cell cytotoxicity and the production of IFN- γ , dependent on cell contact with plasmacytoid DCs or monocytes (Alter et al, 2007a). There is a decreased response to stimulation with this TLR agonist from NK cells isolated from HIV⁺ individuals, in particular viremic individuals, suggesting that activation of NK cells in this manner may only be important in early infection. Destruction of the adaptive immune system leaves HIV⁺ individuals susceptible to a number of opportunistic infections and malignancies including *pneumocystis carinii* pneumonia (PCP), cytomegalovirus retinitis (CMVR), *mycobacterium avium* complex (MAC), toxoplasmosis, tuberculosis as well as AIDS-related lymphoma and Kaposi sarcoma. In addition to any role in the control of HIV viremia, NK cells may also be important in the immune response to opportunistic infections and transformations.

Dysregulation of the immune system during HIV infection extends to NK cells, with reports of altered NK cell phenotype and function in acute and chronic infection. Acute HIV infection is associated with an increase in NK cell (CD56⁺CD3⁻) numbers, together with a depletion of CD56^{bright}CD16⁺ NK cells (Alter et al, 2007b). Continued viral infection leads to a decrease in the number of CD56⁺ cells, with an increase in the functionally anergic CD56⁻CD16⁺ subset of NK cells. There are several reports of alterations in the expression of a number of cell surface receptors on NK cells during HIV infection

including the expression of KIR, CD94/NKG2, LILRB1, NCRs (Costa et al, 2001; De Maria et al, 2003; Kottlil et al, 2004; Mavilio et al, 2003).

The importance of the role of NK cells in HIV infection is supported by studies of rare individuals who remain HIV⁺ despite repeat exposure to the virus (exposed uninfected; EU) and individuals whose immune system is naturally capable of controlling the virus infection (long term non progressor; LTNP). High levels of constitutive NK cell activation and the presence of the activatory 3DS1 gene has been associated with EU and LTNP patients respectively (see Chapter 5.1.3).

1.4 NK Cell Receptors

NK cells express a large number of inhibitory and activatory cell surface receptors that control the activation status of the cell (Table 1.1, reviewed in (Lanier, 2005)). These receptors include members of a number of different families of receptors including the KIR, C-type lectin, NCR and LILR family of receptors. Many of these receptors are expressed only on a subset of NK cells and can also be expressed on other cell types, particularly T cells. A number of NK cell receptors are specific for HLA molecules; these include members of the lectin family (NKG2A/C/E in association with CD94) and the Ig superfamily (LILR and KIR family of receptors).

Receptor	Ligand	Function
Immunoglobulin-like receptors		
KIR3DL1	HLA-Bw4	inhibitory
KIR3DS1	HLA-Bw4 ?	activatory ?
KIR2DL1	HLA-C2	inhibitory
KIR2DS1	HLA-C2	activatory
KIR2DL2/3	HLA-C1	inhibitory
KIR2DS2	HLA-C1	activatory
KIR2DL4	HLA-G	activatory ?
KIR3DL2	HLA-A3, - A11	inhibitory
KIR2DS4	HLA-Cw4 + ?	activatory
LILR family (ILT)	HLA	some inhibitory, some activatory
Lectin like receptors		
CD94/NKG2A	HLA-E	inhibitory
CD94/NKG2C	HLA-E	activatory
Cytotoxicity Receptors		
NKG2D	MICA, MICB, ULPB	activatory
NKp30	Unknown cellular ligand, BAT3	activatory
NKp44	Unknown cellular ligand, viral HA	activatory
NKp46	Unknown cellular ligand, viral HA	activatory
CD16	IgG	activatory
DNAM-1	PVR , Nectin-2	activatory
NKp80	AICL	activatory, coreceptor
CD59	unknown	activatory, coreceptor
NTB-A	NTB-A	activatory, coreceptor
2B4 (CD244)	CD48	activatory, coreceptor
CD2	LFA-3	activatory, coreceptor
LFA-1	ICAM	activatory
TLR	PAMPs e.g. dsRNA,	activatory

Table 1.1 NK cell receptors

NK cells can express a variety of different inhibitory and activatory receptors. Many of the inhibitory receptors recognize HLA molecules including the CD94/NKG2A heterodimer and members of the KIR and LILR family of receptors. Activatory receptors include receptors that recognize ubiquitous cellular ligands and stress-induced ligands.

1.4.1 Activatory Receptors

NK cells express a large number of activatory and co-activatory receptors (Bryceson et al, 2006a). Until recently, attempts to identify the triggering receptors for natural cytotoxicity were unsuccessful. The discovery of a range of activating receptors, including the NCRs and NKG2D has allowed dissection of the mechanisms of NK cell recognition of target cells (Pende et al, 2001; Pende et al, 2002). NK cell activatory receptor signals induce diverse signalling cascades in contrast to inhibitory receptors, which use the common ITIM-dependent mechanism. CD16, NKp30, NKp46 and short tailed KIR signal through ITAM containing adaptors including DAP12, FcεR1 chain and the CD3 ζ chain (Lanier et al, 1998; Vitale et al, 1998; Vivier et al, 1992). Activation of ITAM containing adaptors leads to the activation of tyrosine kinases Syk and ZAP-70 (ζ associated protein of 70 kDa) (Zoller et al, 1997). NKG2D associates with the DAP10 and ligation leads to the activation of PI3K and Grb2 (Upshaw et al, 2006; Wu et al, 1999).

In addition to the main triggering receptors NK cells express a number of receptors that act as a source of co-stimulatory signals. CD2 recognises the broadly expressed LFA-3 (leukocyte function-associated antigen-3) but the resultant signalling is largely unknown (Selvaraj et al, 1987). 2B4 recognises CD48 which is expressed on haematopoietic cells and recruits SAP (SLAM-associated protein) and Fyn tyrosine kinases through its cytoplasmic immunotyrosine-based switch motif (ITSM) (Brown et al, 1998; Eissmann et al, 2005). DNAM-1, is expressed on virtually all NK cells and recognises two closely related Nectin family members poliovirus receptor (PVR; CD155) and Nectin-2 (Bottino et al, 2003). DNAM-1 is reported to cooperate with NKp30 in the NK cell mediated killing of mature and immature DCs (Pende et al, 2006).

1.4.1.1 CD16

The expression of CD16 (FcγRIIIA), a low affinity Fc immunoglobulin receptor, enables NK cells to perform antibody dependent cell cytotoxicity (ADCC) in addition to natural cytotoxicity. Engagement of CD16 by the Fc portion of antibody leads to activation of NK

cell cytotoxicity and the killing of opsonised targets. Crosslinking of CD16 (but not NKp46, NKG2D, 2B4, DNAM-1, or CD2) results in an increase in cytotoxicity in resting NK cells (Bryceson et al, 2006b). Triggering of many receptors leads to activation of cytotoxicity in IL-2 stimulated NK cells but, excluding CD16, costimulation is required for the induction of cytotoxicity in resting NK cells.

1.4.1.2 NCRs

Human NK cells express three receptors, collectively known as the natural cytotoxicity receptors, NKp44, NKp46 and NKp30, which are directly involved in natural cytotoxicity (Moretta et al, 2000; Pessino et al, 1998; Sivori et al, 1999). All NK cells express NKp30 and NKp46, while NKp44 is expressed on IL-2 activated NK cells. Blocking of all three receptors leads to strong inhibition of killing of many target cell lines. Although the physiological ligands for these receptors are not known, NKp44 and NKp46 have been reported to recognize certain viral proteins (Arnon et al, 2001; Mandelboim et al, 2001). NKp30 recognises an unidentified ligand on dendritic cells (Ferlazzo et al, 2002) as well as the tumour and stress associated HLA-B associated transcript 3 (BAT3) (10th Meeting of the Society of Natural Immunity). Ligands for the NCRs are reported to be induced or up-regulated in disease conditions including HIV and vaccinia virus infection (Chisholm and Reyburn, 2006; Vieillard et al, 2005).

1.4.1.3 NKG2D

NKG2D is another important receptor in the triggering of NK cell cytotoxicity. This receptor is a lectin-like homodimeric protein expressed on NK cells, CD8+ T cells and $\gamma\delta$ T cells. NKG2D associates with the activating adaptor DAP10 and recognizes a variety of stress-induced self-ligands, such as MICA, MICB and ULBP molecules (Pende et al, 2001; Watzl, 2003). In addition to triggering cytotoxicity, engagement of NKG2D by its ligands has been shown to upregulate CD25 (IL-2R α) and causes the secretion of IFN- γ .

1.4.2 HLA

An important source of inhibitory signals for NK cells are MHC molecules expressed on target cells. MHC molecules were initially identified as the main target antigens in transplantation rejections. In humans these molecules are encoded on chromosome 6, are termed human leukocyte associated (HLA) antigens and fall into two categories – class I and class II. Expression of class II HLA is confined to specialised antigen presenting cells where it functions to present antigen to helper T cells. Class I HLA antigens are divided into classical (HLA-A, -B and -C) and non-classical (HLA-E, -F, and -G) HLA antigens.

HLA-A, -B and -C genes are expressed on virtually all nucleated cells. The purpose of these proteins is to present antigen to cytotoxic T lymphocytes (CTL) and to regulate NK cell function. The classical HLA class I genes are highly polymorphic with over 600 alleles of HLA-B, 300 alleles of HLA-A and 150 alleles of HLA-C reported (Marsh et al, 2005). Class I molecules consist of a single α chain (encoded in the HLA complex), which folds into three extracellular Ig domains ($\alpha 1$, $\alpha 2$ and $\alpha 3$) noncovalently associated with a small extracellular protein called $\beta 2$ -microglobulin (encoded by a single gene unlinked to the HLA complex). Classical class I antigens bind peptide fragments for presentation to cells of the immune system. A peptide-binding groove is formed between the α -helices of the $\alpha 1$ and $\alpha 2$ domains, with the base of the groove derived from eight β -strands from these domains (see Figure 4.1.1). Peptides derived from a sample of all of the proteins synthesised in the cells are generated by proteolytic cleavage by proteasomes. These peptides are then transported into the lumen of the endoplasmic reticulum by transporters associated with antigen processing (TAP) and loaded into the peptide-binding groove of class I HLA. These HLA/peptide complexes are then exported to the cell surface through Golgi transport for interaction with cells of the immune system. One important function of HLA class I molecules is presentation to $CD8^+$ T cells. Intracellular infection or transformation will alter the profile of expressed proteins within the cell. This will in turn lead to the presentation of foreign peptide by HLA on the cell surface. Recognition of an HLA/peptide complex by the T cell receptor (TCR) of cytotoxic T cells targets the infected

or transformed cell for destruction. Classical class I molecules also function as an important source of inhibitory (and possibly activatory) signals for NK cells due to interaction with KIR (see 1.5).

Non-classical HLA class I antigens are not as polymorphic as classical class I antigens and the function of some of these proteins is not clear. HLA-F is found predominantly intracellularly without bound peptide, although it may be expressed at the cell surface under certain conditions (Lepin et al, 2000; Wainwright et al, 2000). Similar to class I HLA, HLA-G is found associated with β 2-microglobulin with peptide bound in the antigen-binding groove (Kovats et al, 1990). HLA-G is normally expressed only on foetal trophoblast cells although its expression has also been reported elsewhere (Kirszenbaum et al, 1994; Yang et al, 1996). Allelic polymorphism, splice variants and membrane shedding gives rise to HLA-G that is expressed both as a membrane bound receptor and as a soluble molecule (Lee et al, 1995). HLA-E expression at the cell surface is dependent on the loading of a hydrophobic peptide derived from the leader sequence of other class I proteins in the ER. This peptide loading has been reported to involve TAP (Lee et al, 1998a), but TAP-independent mechanisms may also occur as HLA-E has been detected on the surface of PBMCs isolated from TAP-deficient patients (Furukawa et al, 1999). HLA-E requires the presence of a methionine residue at P2 of the nonamer peptide for efficient binding and cell surface expression; leader sequences which generate a peptide with threonine at P2 do not support HLA-E expression (Braud et al, 1997). An important function of non-classical HLA-E and HLA-G molecules is to provide signals to NK cells, including activatory signals from HLA-G (which may be of importance during pregnancy) and inhibitory and activatory signals from HLA-E due to interaction with CD94/NKG2 heterodimers.

In order to maintain surveillance on the expression of highly polymorphic molecules such as HLA antigens, NK cells have been required to evolve receptor systems to deal with a high level of complexity. To condense the level of complexity, KIR receptors recognise groupings of HLA allotypes, and simplify the hundreds of different allotypes into a handful of HLA specificities (see Table 1.2). The region around amino acid at position 80 of the HLA chain is of particular importance for the recognition of HLA-B and -C allotypes.

Class I HLA	Subgrouping	Specific KIR
HLA-A	A3	3DL2
	A11	3DL2
	> 20 other serotypes	no known NK receptor
HLA-B	Bw4	3DL1
	Bw6	no known NK receptor
HLA-C	C1	2DL2/3
	C2	2DL1

Table 1.2 KIR recognise broad grouping of HLA Class I molecules

KIR molecules are not specific for individual HLA allotypes, instead they recognise broad grouping of HLA that contain many different allotypes. All HLA-C allotypes are recognised by a KIR.

1.4.3 Non-HLA –specific inhibitory receptors

Although HLA expression is the dominant source of inhibitory ligands, inhibitory receptors for non-HLA ligands have been identified (Lebbink and Meyaard, 2007). These include KLRG1, a lectin-like receptor expressed on a subset of NK cells that recognises constitutively expressed cell junction proteins of the cadherin family (Ito et al, 2006). NK cells also express members of the Siglec family of inhibitory receptors that recognise terminal sialic acid residues on glycoconjugates (Nicoll et al, 1999; Zhang et al, 2000). Activation of NK cells via “non-MHC missing self “ may be possible upon the loss of ligands for these receptors e.g. the loss of E-cadherin expression in epithelial tumours may result in the loss of an inhibitory signal for KLRG1⁺ NK cells and lower the threshold of activation for those NK cells (Colonna, 2006).

1.4.4 Non-KIR HLA-specific receptors

In addition to KIR family members, NK cells express HLA-specific receptors from the LILR and C-type lectin family of receptors. These include both inhibitory and activatory receptors.

1.4.4.1 CD94/NKG2A/C/E

The CD94/NKG2 receptors are lectin-like disulphide linked heterodimers expressed as type II membrane proteins on NK cells and a subpopulation of T cells. Association of the invariant CD94 subunit with the immunotyrosine inhibitory motif (ITIM)-bearing NKG2A forms an inhibitory receptor, while association with NKG2C (and possible NKG2E) form an activatory receptor due to association with the activatory adaptor molecule DAP12. The ligand for CD94/NKG2 dimers is the non-classical MHC molecule HLA-E (Braud et al, 1998; Lee et al, 1998b). HLA-E binds and presents peptide derived from the leader sequence of MHC class I heavy chain (Vales-Gomez et al, 1999).

1.4.4.2 LILR

Leukocyte immunoglobulin-like receptors (LILR) are a family of receptors containing both inhibitory and stimulatory members, encoded within the leukocyte receptor complex (LRC) on chromosome 19, and are expressed by cells of both myeloid and lymphoid lineage (reviewed in Brown, 2004). Several members of the family are known to recognise a broad range of HLA molecules. LILRB1 (LIR-1, ILT-2) is an inhibitory member of the family expressed on NK cells, which signals via cytoplasmic ITIMs. LILRB1 recognises some but not all HLA-A, -B, -C, -E, -F and -G molecules (Vitale et al, 1999). LILRB1 also binds with high affinity to the CMV-encoded MHC homologue UL18, and UL18 inhibits LILRB1⁺ NK cells (Cosman et al, 1997).

1.4.5 MHC Receptors in Mice

Similar to humans, mice express CD94 in association with members of the NKG2 family. These receptors recognise the non-classical MHC molecule termed Qa-1 in mice. Mice do not possess KIR genes and the Ly49 family of receptors performs an analogous function to the KIR in humans (Takei et al, 1997). While the Ly49 and KIR families are structurally very different - Ly49 receptors are C-type lectin proteins, while KIR are members of the Ig superfamily they share many features. The Ly49 gene cluster forms part of the NK gene complex on chromosome 6 and contains 11 genes and 5 pseudogenes in the C57BL/6 strain of mice. The number and type of functional Ly49 genes is believed to differ between mouse strains. Ly49 receptors are type II glycoproteins that are expressed as disulfide-linked homodimers at the cell surface (Yokoyama and Seaman, 1993). Ly49 receptors are expressed on NK1.1⁺ and NK1.1⁻ T cells as well as on NK cells and each NK cell expresses only a subset of the available Ly49 receptors. Ly49 receptors are polymorphic and expression of these receptors occurs in a monoallelic fashion (Rouhi et al, 2006). Similar to the KIR, the Ly49 family includes both inhibitory and activatory members that recognise polymorphic MHC antigens. Inhibitory receptors contain ITIMs that are phosphorylated upon ligation leading to the recruitment and activation of the phosphatase SHP-1 (Nakamura et al, 1997). Expression of the class I ligands for the inhibitory Ly49 receptors (Ly49A, C, G and I) protects target cells from NK cell lysis. Activatory Ly49 receptors do not contain signalling domains but associate with the activatory adaptor DAP12 (McVicar et al, 1998).

The best characterised Ly49 receptor is the inhibitory Ly49A, which recognises mouse MHC class I H-2D^d (Held et al, 1996a). Expression of H-2D^d protects cells from lysis by Ly49A⁺ NK cells, and does so independently of the sequence of the bound peptide (Correa and Raulet, 1995), although peptide dependency has been described for other Ly49 receptors (Franksson et al, 1999). Expression of the H-2D^d ligand reduces the cell surface expression of Ly49A (Held et al, 1996b). This is due both to lower expression of the receptor and a reduction in the availability of the receptor due to interaction in *cis* with its MHC ligand. It is estimated that 75% of Ly49A on H-2D^d-expressing NK cells is occupied in *cis*, with the remaining 25% available for interaction with H-2D^d in *trans* (Andersson et

al, 2007). It has been suggested that a dynamic interaction in *cis* would provide the NK cell additional regulatory control in the balance between inhibitory and activatory signals.

1.5 KIR

The discovery of KIR has revolutionised the NK cell field and allowed the definition of many of the molecular events involved in target cell recognition. Prior to the characterisation of these receptors it was known that NK cells displayed distinct allospecificities when exposed to allogenic cells (Ciccone et al, 1992). Identification of the KIR family of receptors and analysis of their HLA-specificity allowed for dissection of the events controlling the observed allospecificity and an understanding of the events controlling NK cell activation in health and disease.

Encoded on chromosome 19, the killer cell immunoglobulin receptors (KIR) are a family of Ig receptors that include both inhibitory and activatory members that are expressed on NK cells and a subset of CD8⁺ T cells (reviewed by (Vilches and Parham, 2002)). Members of the KIR family recognise various HLA-A, -B, -C and -G molecules (see Table 1.1). The most commonly used nomenclature system used to describe KIR is based on receptor protein structure. KIR receptors are termed 2D or 3D depending on the number of Ig domains present in the extracellular domain and further divided into S or L, depending on the length (short or long) of their cytoplasmic tail. In 3D KIR, the Ig domains are termed D0, D1 and D2, with D0 being the most distal from the membrane. Most 2D KIR (type I) contain two Ig domains that most resemble D1 and D2, while type II KIR, 2DL4, 2DL5A and 2DL5B, contain Ig domains that most closely resemble the D0 and D2 domains (Figure 1.5). They have also been given CD designations as CD158a, CD158b, etc. based on an approximate centrometric-telomeric order of the genes.

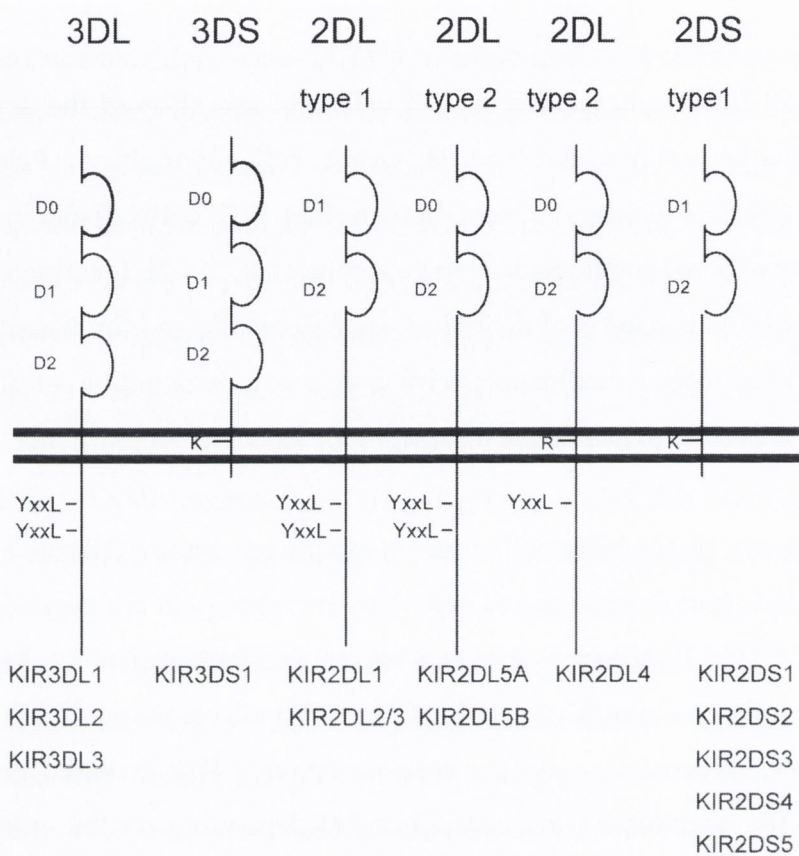


Figure 1.5 The domain structure of the KIR.

3D KIR possess three extracellular Ig domains – D0, D1, D2. Type 1 2D KIR possess 2 Ig domains most closely resembling D1 and D2 while type 2 2D KIR possess 2 Ig domains most closely resembling D0 and D2. Activating KIR, including 2DL4, contain a positively charged residue in the transmembrane domain to allow association with activatory adaptors; inhibitory KIR possess ITIMs to transduce inhibitory signals.

1.5.1 KIR Gene Complex

The KIR family of receptors is encoded within the LRC on human chromosome 19q13.4. In addition to the KIR gene family, this portion of the genome encodes a number of related sequences, including the leukocyte Ig-like receptor family (LILR) [also known as the Ig-like transcript (ILT) family] and the leukocyte-associated inhibitory receptor (LAIR) genes as well as the FcαR gene, the NK cell activatory receptor NKp46 and the collagen-binding receptor GPV1 (Figure 1.6).

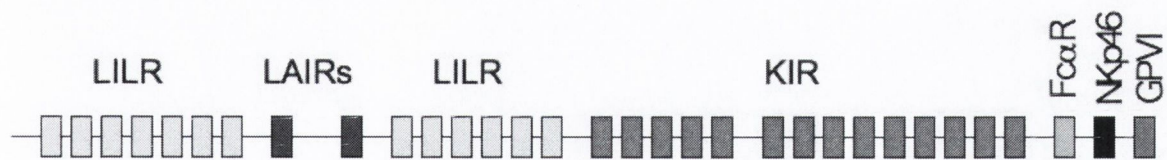


Figure 1.6 The Leukocyte receptor complex

The leukocyte receptor complex (LRC) on chromosome 19 encoded KIR molecules (up to 14 genes plus 2 pseudogenes) as well as leukocyte immunoglobulin-like receptor (LILR, also known as ILT) and leukocyte-associated inhibitory receptor (LAIR) families of receptors. The FcαR gene, the NK cell activatory receptor NKp46 gene and the collagen-binding receptor GPV1 are also present.

The KIR gene complex contains 14 genes and 2 pseudogenes, arranged in head to tail fashion with each gene covering approximately 10-16 kb. 2 Kb of sequences separates genes along the chromosome, except for a unique 14 kb stretch upstream of 2DL4 (Martin et al, 2000). The exon-intron structure of each KIR gene is similar (Figure 1.7). In 3D KIR there are nine exons; the first two encode a leader sequence, each Ig domain is encoded in a separate exon, the stem and transmembrane region are encoded by a single exon and the cytoplasmic domain is encoded by exon 8 and 9 (Wilson et al, 1997). In 2D KIR, with the exception of 2DL4 and 2DL5, exon 3 encoding D0 is a pseudoexon, leading to a D1D2 configuration (Vilches et al, 2000a). Type II 2D KIR (2DL4 and 2DL5) lack exon 4 and thus have a D0D2 configuration (Selvakumar et al, 1997; Vilches et al, 2000b).

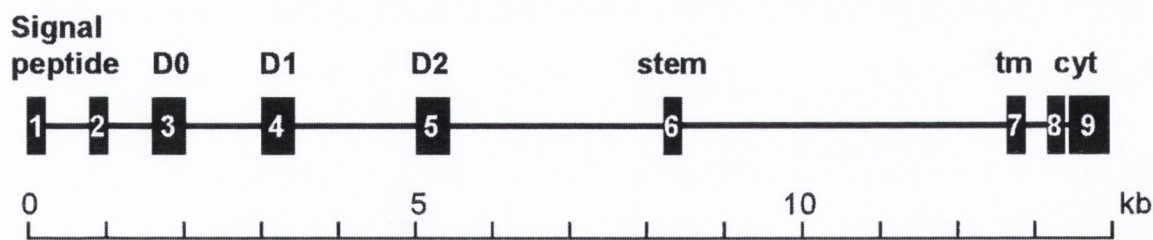


Figure 1.7 Intron-exon structure of a typical KIR gene (3DL1)

3DL1 is encoded by 9 exons. Exon 1 and 2 encode a leader sequence, each Ig domain is encoded in a separate exon, the stem and transmembrane region are each encoded by a single exon and the cytoplasmic domain is encoded by exon 8 and 9. Taken from (Vilches and Parham, 2002).

1.5.2 Variation of KIR gene

Individuals vary greatly at the KIR gene cluster; this variation is a function both of gene number, gene content and allelic polymorphism.

1.5.2.1 Haplotypic Variation

Individuals vary in the number of KIR genes encoded on their chromosomes. The number of expressed KIR genes present on a haplotype can vary from 7-12 with three framework genes, 3DL3, 2DL4 and 3DL2 common to all haplotypes. KIR haplotypes can be roughly divided into two groups A and B (Figure 1.8), originally differentiated on the presence of a 24 kb HindIII fragment on Southern blot analysis. Haplotype A encodes 7 genes, 2DL1, 2DL3, 2DL4, 2DS4, 3DL1, 3DL2 and 3DL3, while haplotype B, which encompasses many more subtypes, contains various combinations of 2DS1, 2DS2, 2DS3, 2DS5, 3DS1 and 2DS4 (Uhrberg et al, 2002). The frequency of the A haplotype is approximately 50% in Caucasian population, but represents 75% of haplotypes in the Japanese population and 15% in Australian Aborigines (Toneva et al, 2001; Yawata et al, 2002).

The striking difference between the two haplotypes is the number of activating (short-tailed) KIR present. While B haplotypes contains several activating receptor genes, the A haplotype contains just two – 2DL4 and 2DS4. However many individuals, homozygous for the A haplotype, are believed to express no functional activating KIR at the cell surface as 2DL4 is localised to endosomes (Rajagopalan et al, 2006) and a common allele of 2DS4 contains a frameshift mutation that is predicted to lead to premature truncation and a secreted non-functional receptor (Maxwell et al, 2002).

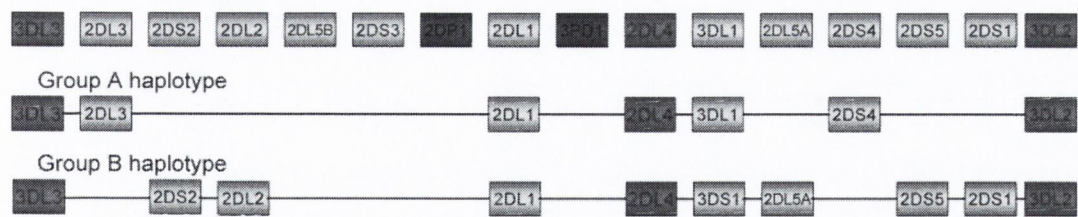


Figure 1.8 KIR haplotypes can be divided into group A and B haplotypes

Based on the presence of absence of KIR genes, KIR haplotypes can be classified as group A or B haplotypes. Group A is the simplest haplotype containing only 7 expressed KIR genes, mostly inhibitory receptors. Group B haplotypes is more variable and contains many more subgroups. Group B haplotypes differ mainly in the number of type of activatory KIR genes present.

1.5.2.2 Allelic polymorphism

In addition to variation in the number of KIR genes, individuals also show marked allelic polymorphism at most KIR loci (Table 1.3). Nucleotide variation occurs throughout KIR genes, in contrast to HLA alleles where differences are localised to one or two exons. 3DL1 and 3DL2 both show a high level of polymorphism, although the source of the variability is different for these two receptors. Allelic variation of 3DL1 seems to arise mainly from recombination at that locus, while 3DL2 appears to be resistant to recombination and point mutations are responsible for allelic differences (Gardiner et al, 2001).

KIR gene	Alleles
2DL1	14
2DL2	5
2DL3	7
2DL4	26
2DL5	7
2DS1	4
2DS2	8
2DS3	5
2DS4	8
2DS5	5
3DL1	47
3DS1	12
3DL2	23
3DL3	54

Table 1.3 Allelic polymorphism of KIR genes.

Many KIR genes show high levels of polymorphism. Over 40 alleles of 3DL1 have been recorded. Data is taken from IPD (Immuno Polymorphism Database)-KIR Database (Last accessed June 2007, <http://www.ebi.ac.uk/ipd/kir/alleles.html>).

1.5.3 Recognition of HLA by KIR

Various KIR molecules recognise class I HLA-A, -B and -C allotypes as well as the non-classical HLA-G receptor. HLA-C allotypes can be divided into two mutually exclusive groupings based on the amino acid sequence – HLA-C1 allotypes contain serine at position 77 and asparagine at position 80, HLA-C2 allotypes contain asparagine at position 77 and lysine at position 80. Inhibitory 2D KIR have been identified that recognise both of these HLA-C grouping with 2DL1 recognising HLA-C2 molecules while 2DL2 and 2DL3 recognise HLA-C1 allotypes (Biaassoni et al, 1995; Moretta et al, 1993). Short-tailed

activatory 2D KIR (2DS1 and 2DS2) also recognise HLA-C2 and -C1 respectively ((Moretta et al, 1993), Chapter 5). HLA-B allotypes are similarly divided into HLA-Bw4 and Bw6 grouping. 3DL1 recognises Bw4 allotypes and is discussed below; no NK receptor has been reported to directly bind Bw6 allotypes. The ligand of 3DL2 has been an issue of dispute, with difficulty repeating initial observations that it bound to HLA-A3 and HLA-A11 (Pende et al, 1996). This discrepancy has been recently resolved with the finding that the interaction between KIR3DL2 and these HLA-A alleles is highly peptide dependent (Hansasuta et al, 2004). Indeed, of a panel of eight peptides tested in tetramer binding assay only one, an EBV-derived peptide, allowed recognition. Initial experiments that observed protection of HLA-A3 or HLA-A11 from lysis by KIR3DL2⁺ NK cells used EBV transformed B cells lines as target cells. 2DL4 recognises the nonclassical HLA-G molecule (Rajagopalan et al, 2006). In contrast to other KIR, 2DL4 is mainly expressed in endosomes and encounters its ligand after endocytosis of soluble HLA-G leading to NK cell activation and cytokine secretion but not NK cell cytotoxicity (Kikuchi-Maki et al, 2003).

1.5.4 KIR Signalling

The signalling potential of KIR molecules is dictated by the cytoplasmic domain. Long-tailed KIR (2D and 3D) contain inhibitory tyrosine based immunoreceptor motifs (ITIMs) in this domain and transmit inhibitory signals to the NK cells (Bruhns et al, 1999). Short tailed KIR possess a basic residue in the transmembrane region, which allows them to associate with the activatory adaptor DAP12 and transmit positive signals (Lanier et al, 1998). 2DL4 is a unique KIR, containing both a positively charged (arginine) residue in its transmembrane domain and a single ITIM in its cytoplasmic domain. In line with these signalling elements, both inhibitory and activatory roles for 2DL4 have been reported, although in cases where the full-length receptor was used, activatory signals dominated (Faure et al, 2002). In resting NK cells engagement of 2DL4 leads to IFN- γ production, without a concomitant increase in cytotoxicity (Kikuchi-Maki et al, 2005; Rajagopalan et al, 2001).

1.5.4.1 Activating Signals

Activating KIR do not contain any signalling moiety in their cytoplasmic domain, rather they associate with an ITAM containing adaptor molecule, DAP12. This association is based on the interaction between the charged lysine residue in the transmembrane domain of the KIR protein and a negatively charged aspartic acid residue in DAP12 (Campbell and Colonna, 1999). While 2DL4 contains a positively charged amino acid in its transmembrane domain (in this case arginine), it does not signal through DAP12 but instead associates with Fc receptor γ protein (Kikuchi-Maki et al, 2005). DAP12, which exists as a disulfide linked homodimer, is also known to associate with the activating CD94/NKG2C dimer and the natural cytotoxicity receptor NKp44 (Lanier et al, 1998). When the receptor/adaptor molecule complexes are clustered by interactions between KIR and their cognate ligand, the tyrosine residues in the ITAM are phosphorylated, often by kinases from the Src family. The phosphorylated ITAM provides a docking site for the SH2 domains of ZAP-70 and Syk kinases (McVicar et al, 1998). A key mediator of the downstream effects of activating KIR ligation is PI3 kinase, which leads to activation of extracellular regulatory kinase (ERK) and the mobilisation of lytic granules and target cell death (Leibson, 1997).

1.5.4.2 Inhibitory Signals

Inhibitory KIR contain ITIM motifs (YxxL) through which they transmit their inhibitory signal upon ligation (Figure 1.9). Engagement of inhibitory KIR leads to receptor clustering and ITIM phosphorylation, possibly by a member of the Src kinase family. A common feature of all the HLA-specific receptors (including KIR, LAIR and CD94/NKG2A) is the recruitment of Src homology region 2-containing protein tyrosine phosphatase-1 (SHP-1). SHP-1 is a cytosolic protein containing two SH2 domains, which interact with phosphorylated tyrosine residues of the ITIM, and a C terminal catalytic domain (Burshtyn et al, 1999; Burshtyn et al, 1996). Over-expression of a catalytically inactive SHP-1 in NK cells prevents the inhibition mediated by KIR (Yusa and Campbell, 2003). Although the exact targets of SHP-1 dephosphorylation are unclear, they are

believed to include targets phosphorylated due to ligation of activatory receptors, including Syk kinase (Chiang and Sefton, 2001; Plas et al, 1996) and linker for activation of T cells (LAT) (Valiante et al, 1996).

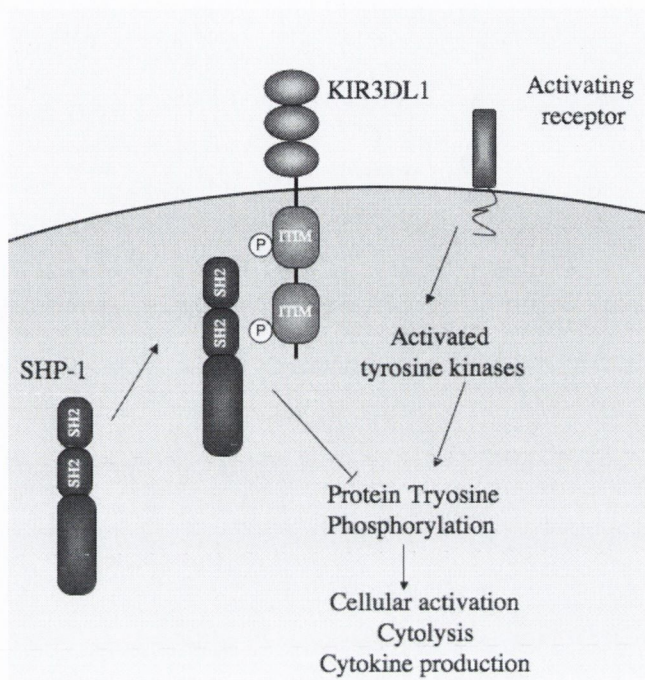


Figure 1.9 Signalling of inhibitory KIR

Inhibitory KIR signal through ITIMs present in the cytoplasmic domain of the protein. Recruitment and activation of the phosphatase SHP-1 leads to the termination of activation signals.

1.5.5 KIR expression on NK cells

All KIR genes (with the possible exception of 3DL3) present in the genome of an individual are expressed in the polyclonal NK cell population. However, a single NK cell may express only some of the KIR genes encoded within an individual's genome, and this expression is stable over time (Shilling et al, 2002). Expression of KIR genes is dictated by the methylation status of the promoter – expressed genes are hypo-methylated (Chan et al, 2003; Santourlidis et al, 2002). This variegated pattern of expression allows NK cells to detect alterations in the expression of a single HLA locus, as this will result in a strong

response from the subset of NK cells whose only self-specific inhibitory receptor recognises that particular molecule. The frequency of NK cells co-expressing a given combination of receptors can usually be well estimated as the product of individual frequencies, suggesting that different receptor genes are expressed independently. Similarly for alleles, individual NK cells may express no, one or two alleles of 3DL1, there being no allelic exclusion (Gardiner et al, 2001).

1.5.6 KIR expression on T cells

In addition to their expression on NK cells, KIR are also expressed on CD4⁺ and CD8⁺ $\alpha\beta$ T cells and $\gamma\delta$ T cells. Within the $\alpha\beta$ T cell subset KIR are predominantly expressed by CD8⁺ T cells, although KIR⁺ CD4⁺ T cells have been described (van Bergen et al, 2004). KIR expression is induced on T cells after TCR rearrangement as evidenced by the co-existence of KIR⁺ and KIR⁻ T cell clones with identical TCR (Vely et al, 2001). KIR⁺ T cells are believed to represent a memory cell population due to their cell surface phenotype (CD45RO⁺, CD18⁺, CD44⁺, CD29⁺, CD57⁺, CD28⁻, CCR7⁻) (Mingari et al, 1996). In line with their expression on memory T cells the size of the KIR⁺ T cell population increases with age, up to 30% in elderly individuals (Anfossi et al, 2001).

KIR expression on both CD8⁺ and CD4⁺ T cells has an inhibitory effect on cytokine production. The level of IFN- γ and TNF- α production from 3DL1⁺ T cell clones stimulated with bacterial superantigen was increased in the presence of a blocking KIR or HLA antibody (D'Andrea et al, 1996). Analysis of 2DL2⁺ CD4⁺ T cells revealed that recruitment of the KIR to the signalling complex disrupted late, but not early T cell signalling events and thereby prevented TCR-induced transcriptional activation and thus cytokine production. In this case, T cell cytotoxicity, mediated by preformed cytotoxic granules and therefore independent of transcriptional activity, was not affected by KIR (Henel et al, 2006), although others have reported an inhibitory effect on cytotoxicity (Bakker et al, 1998; De Maria et al, 1997; Phillips et al, 1995). The proliferative capacity of KIR⁺ T cells is also impaired (Arlettaz et al, 2004).

KIR are also believed to play a role in protecting memory T cells from activation-induced cell death (AICD). Following stimulation and clonal expansion, the bulk of effector T cells undergo programmed death to maintain homeostasis. Resistance to AICD allows surviving cells to develop into a memory pool of T cells. KIR expression, independent of ligation, has been shown to inhibit the TCR-induced activation of PKC and up-regulation of FasL on Jurkat T cells (Chwae et al, 2002). Analysis of tumour-specific CTLs showed that expression of 2D KIR on these cells provided protection from AICD through the induction of c-FLIP-L (Gati et al, 2003). cFLIP-L is a caspase 8 homologue that inhibits the activation of pro-caspase 8 downstream of Fas ligation. KIR signalling can also lead to the activation of phosphoinositol 3-kinase (PI3K) and in turn the activation of the anti-apoptotic AKT kinase in T cells (Marti et al, 1998). Memory T cells expressing TCR show increased avidity for their ligand and are primed to respond rapidly to a second exposure to antigen (Fahmy et al, 2001). KIR expression by these cells may function to increase the threshold of activation and thus help to prevent inappropriate immune response to structurally similar antigens. Variable KIR expression on T cells expressing identical TCR may also contribute to heterogeneity of the T cell response.

1.5.7 KIR Structure

The crystal structure of 2DL1, 2DL2 and 2DL3, either alone or in association with their HLA-C ligand has been determined (Boyington et al, 2000; Fan et al, 1997; Maenaka et al, 1999; Snyder et al, 1999). The first crystal structure determined was that of 2DL2 bound to HLA-Cw3 and a nonamer self peptide GAVDPLLAL (Boyington et al, 2000). The structures of the 2D KIR are similar; each Ig domains adopt a conformation similar to the C2-type Ig fold observed in the haematopoietic receptor family (Fan et al, 1997). The two Ig domains are positioned at the acute angle that is different for each receptor varying from 66° in 2DL1 to 78° in 2DL3 and 81° in 2DL2. The hinge region between the D1 and D2 domain is stabilized by a highly conserved hydrophobic core - a conserved inter-domain salt bridge further restricts the hinge angle. The structure revealed a 1:1 stoichiometry, and a binding orientation that is analogous to TCR/HLA binding. KIR is bound across both the $\alpha 1$ and $\alpha 2$ helices of HLA and the C terminal end of the bound peptide with its long axis nearly orthogonal to the peptide binding cleft (Figure 1.10). There are six surface loops

(three from D1, three from D2) on the 2DL2 molecule that interact with the HLA-Cw3 molecule, three of these loops interact with the $\alpha 1$ helix of HLA while the other three contact the $\alpha 2$ helix of HLA. Interaction is mainly through strong charge complementarity, with KIR providing six acidic residues and HLA providing six basic residues to the interface.

Although the crystal structure of 3DL1 has not yet been elucidated, the structure of the 2D KIR offers insights into the possible interactions involved in 3DL1 binding to HLA-Bw4. Although 3DL1 contains three extracellular Ig domains (D0, D1 and D2), D1 and D2 are believed to play the primary role in ligand binding in a homologous manner to D1 and D2 in the 2D KIR, with D0 acting as a binding enhancer (Khakoo et al, 2002). Sequence comparison shows that many of the 2DL2 and HLA-Cw3 interface residues are conserved in 3DL1 and HLA-B Bw4 respectively (Boyington et al, 2001).

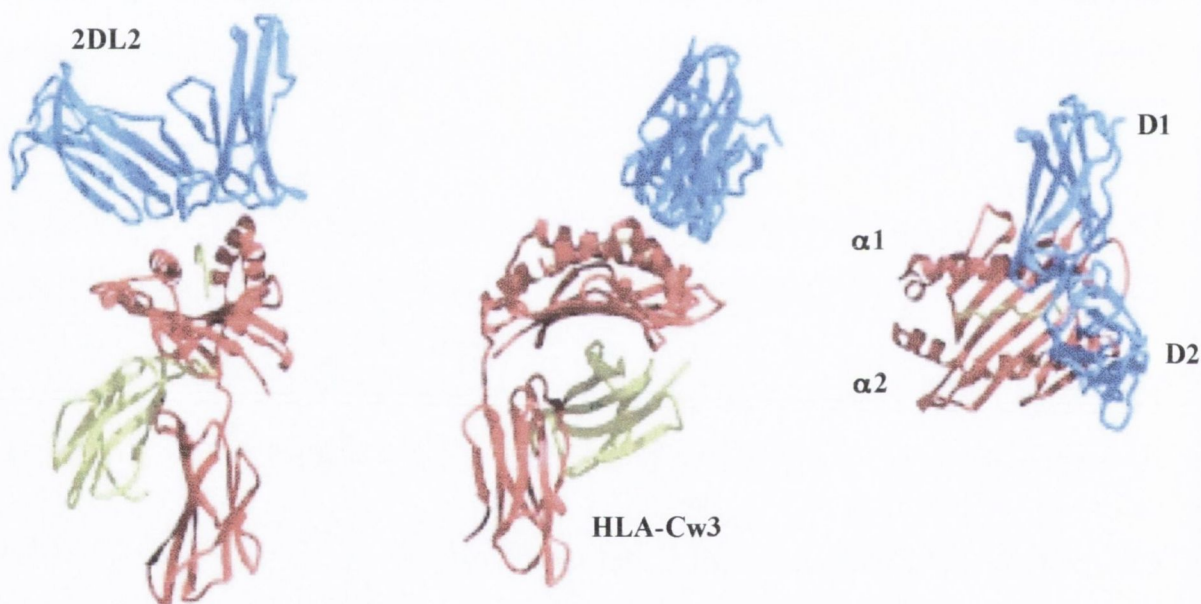


Figure 1.10 2DL2 in complex with HLA-Cw3/GAV

Front, side and top views of 2DL2/HLA-Cw3/GAV complex. The class I HLA molecules are red and yellow ribbons with yellow peptide. 2DL2 is in blue. Taken from Boyington et al, 2002

1.5.8 KIR and disease

NK cells are known to be important in the control and resolution of a number of disease conditions, but the functional role of KIR⁺ NK (or T) cells is less clear. A range of genetic association studies supports the importance of the KIR family of receptors in the response to a number of pathogenic challenges, as well as in autoimmunity and pregnancy (Table 1.4). The presence or absence of individual KIR genes, as well as the balance in the number of activatory or inhibitory genes present have been correlated with clinical parameters. A recurring theme in the associations seen is that the presence of activatory KIR receptors can predispose to autoimmune conditions. Interpretation of the genetic studies of the influence of KIR on disease are complicated by a number of factors including the strong linkage disequilibrium between individual KIR genes and the requirement of co-expression with their HLA ligands (themselves capable of influencing the immune response due to presentation to T cells) for functional interaction.

Disease	KIR Association	Observation
Infectious		
HIV-1	3DS1/HLA-Bw4 Ile80	Slows progression
	3DL1/ B*57 Ile 80	Slows progression
HCV	2DL3/HLA-C1 homozygosity	Resolution of infection
	3DS1/HLA-Bw4	Resolution of infection
	3DS1/HLA-Bw4 Ile80	Protection from hepatocellular carcinoma
CMV reactivation in bone marrow recipient	> 1 activating KIR in donor	Reduced risk
<i>P. Falciparum</i>	3DL2*002	Strong response to infected RBCs
Cancer		
Malignant melanoma	2DL2/3 with HLA-C1	Susceptibility
Leukemia	2DL2	Susceptibility
	AB1 (AML) and AB9 (CML) Kir genotypes	Susceptibility
	3DL1/3DL1 + Bw4 (CLL)	Susceptibility
	3DL1/3DL1 - Bw4 (CLL)	Protection
	2DL2/3 +Cw1 (myeloid leukemia)	Susceptibility
	2DL3/3 +Cw1 (myeloid leukemia)	Protection
	3DS1/absence of C2 and/or Bw4	Susceptibility
Cervical cancer	Genotype 10 (2DL1/2/3/4, 3DL1/2/3, 2DS4	Susceptibility
	2DL5*002	Protection
	≥ 5 activating KIR (EBV+)	Susceptibility
Nasopharyngeal carcinoma	≥ 5 activating KIR (EBV+)	Susceptibility
Lymphoproliferative disease of large granular	abnormal KIR expression	May contribute to disease pathogenesis
	KIR/HLA mismatch	May contribute to disease pathogenesis
Cutaneous T-cell lymphoma	Expression of 3DL2 on malignant cells	May contribute to disease pathogenesis, diagnostic marker
Autoimmune/inflammatory		
Psoriatic arthritis	2DS1/2DS2; HLA-Cw group homozygosity	Susceptibility
psoriasis vulgaris	2DS1/HLA-Cw*06	Susceptibility
	2DS1; 2DL5; KIR haplotype B	Susceptibility
Rheumatoid vasculitis	2DS2/HLA-Cw*03	Susceptibility
Scleroderma	2DS2+ /2DL2-	Susceptibility
IDDM	2DS2/HLA-C1	Susceptibility
	2DS2/2DL2	Susceptibility
Behcet's disease	Abnormal 3DL1 expression	Associated with severe eye disease
Idiopathic bronchiectasis	2DS1 and/or 2DS2/HLA-C1 homozygosity	Susceptibility
Spondylarthritides	Increased 3DL2 expression	May contribute to disease pathogenesis
Acute coronary syndromes	de novo expression of 2DS2 in CD4+ T cells	May contribute to disease pathogenesis
Reproduction		
pre-eclampsia	Maternal AA KIR genotype, C2 in fetus	Susceptibility
Recurrent spontaneous abortion	Mothers lacking inhibitory KIR for fetal HLA-C	Susceptibility

Table 1.4 Association of KIR and disease

A summary of the data generated from genetic studies that showed association of KIR genes with disease. Adapted from (Boyton and Altmann, 2007; Rajagopalan and Long, 2005).

1.6 KIR3DL1

3DL1 was first identified as a 70 kDa glycoprotein that when expressed on NK cell clones prevented lysis of targets expressing some HLA-B molecules (D'Andrea et al, 1995). Using class I alleles HLA-B*1502 and B*1513, which differ only at residues 77-83 which define the Bw4 and Bw6 serological epitopes, the ligand of 3DL1 was later identified as HLA-B molecules that carry the Bw4 epitope (Gumperz et al, 1995). HLA-B alleles can be divided into two subclasses (Bw4 and Bw6) depending on the sequence motif at position 77-83 of the HLA-B $\alpha 1$ domain. Bw6 is present on about two thirds of known HLA-B alleles, while the Bw4 motif is found on the remaining third and on some HLA-A alleles. HLA-Bw4 alleles are represented by at least five different sequences, while Bw6 alleles are composed of two different sequence motifs. 3DS1 segregates as an allele of 3DL1 but, unlike other alleles of 3DL1, displays all the characteristic of an activatory receptor. The specificity of the 3DS1 receptor is unknown.

The 3DL1 gene is highly polymorphic, with 47 alleles reported to date (<http://www.ebi.ac.uk/ipd/kir/alleles.html>). In one study, which looked at a panel of 19 donors, seven new alleles of 3DL1 were identified, suggesting that there is significant polymorphism of the gene within the population (Gardiner et al, 2001). Generation of allelic variation of 3DL1 appears to be due mainly to recombination, a mechanism that is also likely to have produced 3DS1. Some haplotypes lack a 3DL1 allele; the frequency of the null haplotype is estimated at approximately 7%, with individuals lacking 3DL1 accounting for approximately 0.5% of the population (Gardiner et al, 2001). Comparison of the alleles showed that the polymorphisms occur in loop areas of the receptor predicted to interact with its HLA ligand. It is therefore speculated that different alleles of 3DL1 will have different ligand specificities.

1.6.1 The role of presented peptide

HLA class I molecules bind to and present peptides derived from the pool of proteins made within the cell for inspection by the immune system. The observation that certain HLA-

bound peptides affect recognition of HLA by KIR raised the possibility that NK cells could discriminate between self and non-self peptides. More detailed analysis showed that certain peptides did prevent recognition by KIR, but that this was not a truly peptide-specific recognition as KIR could recognise that same HLA molecule presenting very different peptides (Malnati et al, 1995). Single amino acid substitutions in both a protective and a nonprotective peptide presented by HLA-B*2705 revealed the importance of residues 7 and 8 in the peptide for recognition by 3DL1 (Peruzzi et al, 1996). Based on inferences made from the crystal structure of 2D KIR in association with their ligand, these peptide residues are predicted to interact with 3DL1. The crystal structure of the 3DL1 ligand B*2705 complexed with three different immunodominant viral peptides was solved and the tetrameric complexes of these B*2705 peptide complexes were tested for their ability to bind to 3DL1 (Stewart-Jones et al, 2005). 3DL1 bound strongly to two of these complexes (B*2705 with flu-derived nonamer and HIV-derived decamer) but not to B*2705 complexed with an EBV-derived peptide (RRIYDLIEL). Mutation of the glutamate residue at P8 of the EBV peptide to threonine restored binding to 3DL1. Only the EBV peptide exhibits a region of high negative charge at P8, a region that projects into the solvent in the EBV-derived peptide complex. This residue is also larger than the equivalent residues (asparagine or threonine) in the complexes that show binding, suggesting that steric hindrance may prevent 3DL1 binding.

The potential role for the ability of KIR to discriminate between peptide was illustrated by a serendipitous discovery using a transfected vector encoding the neomycin resistance gene. Transfection of T cells with this plasmid skewed the profile of peptide bound to HLA-B to an extent that prevented recognition by 3DL1, and allowed the T cells to be lysed by autologous natural killer cells (Liberatore et al, 1999). One theory to explain the peptide influence on KIR recognition is that a normal cell contains a proportion of peptides that permit recognition, tuned just above the threshold that permits inhibition of NK cells; even subtle changes in that pool might then affect NK cell inhibition. Peptide specificity is also seen in the recognition of HLA-C by 2D KIR (Rajagopalan and Long, 1997) and in the recognition of HLA-A3 and A11 by 3DL2 (Hansasuta et al, 2004)

The recognition of HLA-Bw4 by 3DL1 is a complex interaction of two highly polymorphic proteins, with the presentation of a large range of peptides by the Bw4 molecule adding a further layer of complexity. The pattern of amino acid substitutions within 3DL1 allotypes, together with the variation in the prevalence of different alleles across populations suggests that the polymorphism of the 3DL1 gene may have functional consequences. Similarly, sequence variability within Bw4 allotypes may influence this interaction. An understanding of the influence of the genetic polymorphism on the interaction of these proteins will allow us greater insight into the control of NK cell activation. The 3DL1 gene is unusual among the KIR genes in that both inhibitory and activatory receptors are believed to be encoded at the same locus. The 3DS1 allele of 3DL1 is of particular interest as it has been linked to a slower progression to AIDS in HIV infection. Despite much interest in this 3DL1 allele, much of the fundamental characterisation of this receptor, including expression profile and ligand specificity is unknown.

1.7 Aims

The aims of the research undertaken for this thesis were

- To characterise the role of polymorphism on the functional interaction between 3DL1 and HLA-Bw4
- To investigate the expression, ligand specificity and function of 3DS1
- To investigate the role of Natural Killer cells in HIV Long term non progression.

Chapter Two

Materials and Methods

2.1 Materials

PCR

PCR was carried out using recombinant *Taq* DNA polymerase, with dNTPs and reaction buffer from Invitrogen. High fidelity PCR was carried out using Bio-X-Act DNA polymerase from Biorun. Reverse transcription was carried out using ImProm-II™ RT kit from Promega. Oligonucleotide primers were obtained from Sigma-Genosys. 1 kb DNA ladder was obtained from Promega, low DNA mass ladder was purchased from Invitrogen. DNA electrophoresis was carried out using ThermoEC Midicell® Primo™ Electrophoretic Gel System. Agarose and ethidium bromide were both purchased from Sigma. MetaPhor agarose was purchased from Cambrex. DNA was visualised using the Epi Chemi II Darkroom from UVP Bioimaging Systems.

Flow Cytometry

Anti-CD56 FITC (clone NCAM16.2), anti-CD56 PE (clone B159), anti-CD69 FITC (clone FN50), anti-CD3 PerCP (clone SK7), anti-IFN γ FITC (clone 25723.11), anti-CD107a FITC (clone H4A3), anti-3DL1 PE (clone DX9), anti-CD25 FITC (clone M-A251) and their isotype-matched controls were purchased from BD. Anti-NKp30 PE (clone Z25), anti-NKp44 PE (clone Z231), anti-NKp46 PE (clone BAB281), anti-NKG2A PE (clone Z199), anti-LILRB1 PE (clone HP-F1) and anti-3DL1 PE (clone Z27.3.7) were purchased from Beckman Coulter. Anti-HLA Class I PE (clone W6/32) was purchased from DAKO Cytomation. Anti-2DL1/S1 PE (clone EB6) and anti-2DL2/3/S2 (clone GL183) were purchased from Immunotech. Biotinylated HLA-B*2705 monomers were a gift from Dr. Simon Kollnberger MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, Oxford, UK.

Flow cytometry was carried out on a BD FACSCalibur™. Cell sorting was carried out on a BD FACSria and a DAKO MoFlo cell sorter.

Cell Culture

RPMI-1640, IMDM, L-glutamine, penicillin and streptomycin were obtained from Gibco™. Yssel's medium was obtained from Gemini Bio-Products, CellGro® Stem Cell

Growth medium was obtained from CellGenix. FCS was from PAA Laboratories and human AB serum was from Sigma. Geneticin was purchased from Sigma.

The 721.221 cell line and its transfectants (721.221 A*0101, 721.221 B*0702, 721.221 B*1513, 721.221 B*2705, 721.221 B*3801, 721.221 B*5101 and 721.221 B*5801), the B cell line JY and the K562 cell line were a gift from Peter Parham, Department of Structural Biology, Stanford University, Stanford, CA 94305. The B cell line RPMI 8866 cell line was a kind gift from Dr Salim Khakoo, University Medicine, Southampton General Hospital, Southampton SO16 6YD. Jurkat cell lines stably expressing 3DL1*001, 3DL1*002 and 3DS1 were generated by Dr Clair Gardiner. Human peripheral blood mononuclear cells (PBMCs) were obtained from normal healthy volunteers with informed consent.

Recombinant human IL-2 was a gift from the Biological Resources Branch of the National Cancer Institute. Recombinant human IL-15 was purchased from Strathman Biotec; anti-CD3 (OKT3) antibody was obtained from eBiosciences.

Cytotoxicity Assays

⁵¹Cr was purchased from Amersham. Optiphase Supermix scintillation cocktail and Flexiplates were obtained from Perkin Elmer. Samples were read on a Perkin Elmer Microbeta Trilux. Purified anti-3DL1 antibody (clone DX9) was purchased from BD Biosciences, anti-3DL1 antibody (clone Z27.3.7) was purchased from Immunotech and anti-CD94 antibody (clone HP-3B1) was purchased from Serotec.

General laboratory chemicals were purchased from Sigma, with the exception of Coelenterazine (Argus Fine Chemicals), passive lysis buffer (Promega) and Luciferin (Biosynth).

2.2 Methods

2.3 Growth and Maintenance of cell lines

The K562, JY, 721.221 and Jurkat cell lines were maintained in RPMI-1640 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The 721.221 cell line transfectants and the Jurkat cell line transfectants were maintained in RPMI supplemented with 10% (v/v) FCS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.5 mg/ml geneticin. Cells were maintained in a humidified environment at 37 °C and at 5% CO₂.

2.4 Cryopreservation of cells

Cells were grown to a concentration of approximately $4\text{-}5 \times 10^5$ cells/ml and counted using trypan blue exclusion dye to measure viability. Cells were resuspended at a concentration of 2×10^7 cell/ml in ice-cold RPMI. An equal volume of freezing solution (80% FCS, 20% DMSO) was then added slowly to the cells to give a final concentration of 1×10^7 cells/ml. This solution was aliquoted at 1 ml/vial into cryotubes. The cryotubes were transferred to -80 °C overnight and moved to liquid nitrogen storage the following day for long-term storage.

2.5 PBMC Isolation

Peripheral blood from donors was collected into tubes containing the anticoagulant EDTA and PBMCs were isolated by density gradient centrifugation. In brief, blood was diluted 1 in 2 in PBS and then layered gently onto lymphoprep™, taking care to ensure a sharp interface was maintained. Tubes were centrifuged for 30 minutes at 300 g; the brake was not applied to ensure gradient was not disturbed. The lymphocyte layer was carefully removed and washed in PBS. PBMC isolation from the blood of HIV positive patients was carried out in a level 3 bio-containment unit.

2.6 NK cell isolation

NK cells were isolated from PBMCs using magnetic bead separation. Four different kits were used – CD56 microbead kit (Miltenyi Biotech, positive isolation), EasySep™ CD56 Positive Selection (Stem Cell Technologies, positive selection), StemSep™ NK Cell Enrichment (Stem cell Technologies, negative selection) and NK Cell Isolation Kit II (Miltenyi Biotech, negative selection). All protocols were performed according to the manufacturer's instructions.

CD56 microbeads (Miltenyi Biotech, positive isolation)

In brief, PBMC were incubated with microbeads conjugated to anti-CD56 antibody at 4 °C, washed and then passed down a column placed in a magnetic field. The column was washed, removed from the magnetic field and the CD56⁺ cells eluted from the column.

EasySep™ CD56 Positive Selection (Stem Cell Technologies, positive selection)

PBMCs were incubated with a bispecific antibody complex directed against CD56 and dextran in a Falcon® 5 ml polystyrene round-bottomed tube (Becton Dickinson). Magnetic dextran iron particles were then added to the tube, which was placed in a magnetic field and the negative cells were washed off. The tube was removed from the magnetic field and the positive cells were eluted.

StemSep™ NK Cell Enrichment (Stem cell Technologies, negative selection)

PBMCs were incubated with a mixture of bispecific antibodies directed against dextran and a variety of cell surface antigens (CD3, CD4, CD14, CD19, CD66b and glycophorin A). Magnetic dextran particles were added, the cells were passed down a column held within a magnetic field and the negative cells were collected.

NK Cell Isolation Kit II (Miltenyi Biotech, negative selection)

PBMCs were incubated with a cocktail of biotin-labelled antibodies (anti- CD3, CD4, CD14, CD15, CD19, CD34, CD123 and glycophorin A) and then with anti-biotin microbeads. Cells were loaded onto a column within a magnetic field and unlabelled cells were collected from the column.

In all cases purity was determined by flow cytometry using conjugated antibodies directed against CD16, CD56 and CD3.

2.7 NK cell cloning

NK cells were cloned using the technique of limiting dilution in cloning medium; three different protocols were tested, which differed mainly in the cell culture medium and feeder cells used.

2.7.1 Yssel's Medium

Cloning medium consists of Yssel's medium supplemented with two types of irradiated feeder cell – allogenic PBMCs from 3 donors ($1 \times 10^6/\text{ml}$) and the B cell line JY ($1 \times 10^5/\text{ml}$). Yssel's medium was prepared using IMDM (Iscoe's Modified Dulbecco's Medium) as a base. A number of additions were then made to this medium – 3 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.1 $\mu\text{g}/\text{ml}$ PHA, 5 $\mu\text{g}/\text{ml}$ insulin, 35 $\mu\text{g}/\text{ml}$ transferrin, 20 μM ethanolamine, 2.5 $\mu\text{g}/\text{ml}$ BSA, 1 $\mu\text{g}/\text{ml}$ palmitic acid, 1 μg linoleic acid and 1 $\mu\text{g}/\text{ml}$ oleic acid. Commercially available Yssel's medium was also purchased from Gemini Bioproducts. PBMCs were isolated from three different donors as described above and irradiated at 5,000 rads. The JY cells line was irradiated at 12,000 rads.

Purified NK cells were diluted to a concentration of 25 cells/ml in cloning medium and plated into tissue culture plates in a volume of 20 μl (0.5 cell/well). Cells were cultured for one week at 37 °C at 7.5% CO_2 . After one week, 20 μl of cloning medium supplemented with 200 U/ml hrIL-2 was added to each well. Wells that showed growth after an additional week were transferred to 1 ml of cloning medium with 200 U/ml hrIL-2 in a 48-well plate. If growth continued, these cells were transferred to 2 ml of medium in a 24-well plate. After this, clones were supplemented once a week with cloning medium containing 200 U/ml hrIL-2 by replacing half of the well volume with fresh medium. Clones were expanded to no more than 4 wells of a 24-well plate.

2.7.2 RPMI 8866

Cloning medium consists of RPMI 1640 supplemented with 10% FCS, 5% human AB serum (not heat-treated, filtered through a 0.8 μ m filter), L-glutamine (2mM), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (5×10^{-5} M) and hrIL-2 (500 U/ml). This medium was supplemented with irradiated (5,000 rads) allogenic PBMCs from 3 donors (1×10^6 /ml) and 1×10^5 /ml irradiated RPMI8866 cells (5,000 rads). Purified NK cells were diluted to a concentration of 10 cells/ml in RPMI and then an equal volume of cloning medium was added. 100 μ l of cells were plated into 96-well round-bottomed tissue culture plates (0.5 cell/well). Cells were cultured for 10-14 days at 37 °C at 7.5 % CO₂. Wells that showed growth were transferred to 1 ml of cloning medium in a 48-well plate. Clones were expanded as outlined above (Yssel's medium).

2.7.3 Stem Cell Growth Medium

Cloning medium consists of Stem Cell Growth Medium (CellGenix) supplemented with 5 % heat-inactivated human AB serum, hrIL-2 (250 U/ml) and anti-CD3 antibody OKT3 (50 ng/ml). This was supplemented with 5×10^5 /ml irradiated PBMCs (5,000 rads) from three allogenic individuals.

Purified NK cells were diluted to a concentration of 5 cells/ml in cloning medium and plated at 100 μ l in 96-well V-bottomed tissue culture plates (0.5 cells/well). Cells were cultured for 4 days at 37 °C at 7.5 % CO₂. On day 4 half the medium was removed and replaced with fresh cloning medium containing 1,000 U hrIL-2/ml. On day 7 wells that showed growth were transferred to 1 ml of cloning medium in a 48-well plate. Clones were expanded as outlined above (Yssel's medium).

A comparison of the three protocols used for NK cell cloning is given in Table 2.1.

	RPMI 8866	Yssels	StemCell
Basal Medium	RPMI	Iscoe's modified Dulbecco's medium	Stem Cell Growth Medium
Medium Supplements	foetal calf serum, human AB serum, L-glutamine, non essential amino acids, sodium pyruvate, mercaptoethanol	foetal calf serum, human AB serum, L-glutamine, PHA, insulin, transferrin, ethanolamine, palmitic acid, linoleic acid, oleic acid	human AB serum, L-glutamine, anti-CD3 antibody
Feeder cells	allogenic PBMCs, RPMI8866	allogenic PBMCs, JY	allogenic PBMCs
Cytokines	IL-2	IL-2 from day 7	IL-2

Table 2.1 A comparison of the NK cell cloning protocols used.

2.8 Polyclonal NK cell expansion

Purified polyclonal NK cells were expanded in medium for four weeks to generate sufficient numbers for experiments. Expansion was performed in both Yssel’s cloning medium (supplemented with irradiated PBMCs and JY cells as for cloning, see above) and RPMI cloning medium supplemented with irradiated PBMCs and RPMI 8866 cells. Purified NK cells were suspended at 2×10^5 cells/ml and expanded in 24 well plates.

2.9 Transfection

2.9.1 GeneJuice® transfection

GeneJuice® transfection reagent (Invitrogen) was used with the Jurkat cell line according to the manufacturer’s instructions. In brief, cells were plated at a concentration of 1×10^5 - 1×10^6 cells/ml in 100 µl in a 96-well plate. Genejuice® (0.2 µl-1 µl) was added to serum-free RPMI-1640 medium (total volume of 10 µl per transfection), and allowed to stand at

room temperature for 5 minutes. GeneJuice®/Serum-free medium mixture was added to DNA (typically 100-300 ng) in a 96-well V-bottomed plate and incubated for 10 minutes. This mixture was then added to the cells, which were allowed to recover for 6-30 hours at 37 °C before stimulation. Each transfection was done in triplicate.

2.9.2 Lipofectamine 2000® transfection

Jurkat cells were plated at a concentration of $5 \times 10^5 - 5 \times 10^6$ cells/ml in 100 μ l in a 96-well plate. For each transfection, 2 μ l of Lipofectamine 2000® was diluted in 50 μ l serum-free RPMI and incubated for 5 minutes. The diluted Lipofectamine 2000® was added to DNA (200 ng DNA in 25 μ l), mixed and incubated for 20 minutes. This mixture was then added to the cells and rocked gently to mix. The cells were allowed to recover for 24 hours before stimulation.

2.9.3 Electroporation

Two different systems of electroporation were investigated; one using a 2 mm gap cuvette and the other using a 4 mm gap cuvette.

2 mm gap cuvette system

10^7 Jurkat cells were washed and resuspended in 250 μ l of cytomix electroporation buffer (120 mM KCl, 0.15 mM CaCl_2 , 10 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, 25 mM Hepes, 2 mM EGTA, 2 mM ATP, 5 mM glutathione, pH 7.6). Reporter plasmid DNA (typically 15-30 μ g) was added, cells were mixed and transferred to a 2 mm gap sterile electroporation cuvette on ice. The cuvette was placed in a BioRad Gene Pulser II electroporator and subjected to 2 pulses, 30 seconds apart, each 240 V and 100 μ F.

4 mm gap cuvette

10^7 Jurkat cells were washed and resuspended in 300 μ l of cytomix electroporation buffer (120 mM KCl, 0.15 mM CaCl_2 , 10 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, 25 mM Hepes, 2 mM EGTA, 2 mM ATP, 5 mM glutathione, pH 7.6). Reporter plasmid DNA (typically 15-30 μ g) was

added, cells were mixed and transferred to a 4 mm gap cuvette on ice. The cuvette was then pulsed once in a BioRad Gene Pulser II electroporator at 250 V and 950 μ F.

After electroporation cells were allowed to come to room temperature for 10 minutes and then transferred to a T25 tissue culture flask in 10 ml of RPMI supplemented with 20% FCS. Cells were allowed to recover for 24 hours before stimulation.

2.10 Cell stimulation

Transfected Jurkat cells were plated at a concentration of 1×10^6 cells/ml in 100 μ l in a 96-well U-bottomed plate. Stimulator cells (721.221 cell line and its transfectants) were counted, resuspended in complete medium and 100 μ l added to each well to give the desired stimulator to responder ratio. CD3/CD28 Dynabeads® (3 beads/cell) were added to positive wells and volume adjusted to 200 μ l with complete medium. 100 μ l of complete medium was added to control wells. Cells were incubated for 18 hours at 37 °C and 5% CO₂, after which the cells were harvested and reporter enzyme activity was measured.

2.11 Measurement of luciferase activity

18 hours post-stimulation medium was removed from the cells and the cells were lysed for 15 minutes on a shaker at room temperature with 50 μ l 1x Passive Lysis Buffer (Promega). In the case of GeneJuice® and Lipofectamine® mediated transfection, where transfection efficiency was normalised using a *Renilla* luciferase, the supernatant was split - half was used to measure the NFAT controlled Firefly luciferase activity and the remainder was used to measure the constitutive *Renilla* luciferase activity. When cells were transfected via electroporation, only the Firefly luciferase construct was used.

Firefly luciferase activity was assayed by the addition of 40 μ l of luciferase assay mix (20 mM tricine, 0.26 mM (MgCO₃)₄Mg(OH)₂.5H₂O, 2.67 mM MgSO₄, 0.1 M EDTA, 33.3 mM DTT, 270 μ M coenzyme A, 470 mM luciferin, 530 μ M ATP) to the sample and *Renilla* luciferase was read by the addition of 40 μ l Coelentrastazine (2 μ g/ml).

Luminescence was read using the Reporter microplate luminometer (Turner designs). Firefly luminescence readings were corrected for *Renilla* activity where necessary and expressed as fold stimulation over control wells.

2.12 Measurement of SEAP activity

18 hours after stimulation the assay plate was wrapped in cling film and placed into a waterbath at 68 °C for 2 hours. After this time, 100 µl of supernatant was added to 100 µl DEAB mix (2 M Diethanolamine pH 10, 0.1 M L-homoarginine, 0.1 mM methylumbeliferol phosphate) in a 96-well flat-bottomed plate. The plate was again wrapped in cling film and incubated overnight at 37 °C. Fluorescence was read, with excitation wavelength of 355 nm and an activation wavelength of 460 nm. SEAP activity was expressed as fold stimulation over control wells.

2.13 FACS analysis

Cells were resuspended at approximately $0.5 - 2 \times 10^6$ cells/ml in PBS and aliquoted in 100 µl into Falcon® 5ml polystyrene round-bottomed tubes (Becton Dickinson). Antibodies were added at an optimal concentration determined by titration (typically 2-5 µl) and incubated at room temperature for 15 minutes. Cells were washed in PBS, resuspended in a final volume of 150 µl PBS and analysed using a BD FACSCalibur™ machine. FACS analysis of PBMC isolated from HIV positive patients was performed on a dedicated BD FACSCalibur™ machine in St. James Hospital, Dublin.

2.14 Tetramer formation and staining

HLA tetramers consist of four B*2705 molecules refolded in the presence of $\beta 2m$ and specific peptides (Table 2.2) and were formed from the tetramerisation of biotinylated HLA monomers. Monomers (typically 30-40 µg protein) were pulsed with 5 equal aliquots of extravidin PE (Sigma) over 10-15 hours at 4 °C in the dark. In total 1 mole of extravidin PE is added per 4 moles of monomer.

$0.5 - 1 \times 10^6$ cells were washed and re-suspended in 50 µl of FACS buffer (PBS, 2% FCS, 0.1 % NaN_3), tetramer (typically 1 µg, determined by titration) was added and cells were incubated at 37 °C for 30 minutes in the dark. Cells were washed twice, re-suspended and analysed on a BD FACSCalibur™ machine.

Peptide Name	Sequence	Source
AGG	SRHHAFLFR	human aggrecan
ARK	ARKLLLDNL	Chlamydia 138
Flu	SRYWAIRTR	Influenza
RKSTEL	RRYQKSTEL	Human histone H3.3

Table 2.2 B*27 Tetramers Peptide Sequence

2.15 IFN- γ intracellular staining

Cells were incubated with the appropriate stimuli and BD GolgiStop™ (1 μ g/ml) was added 4-6 hours before the stimulation was stopped. The cells were harvested into a 96-well plate and washed with PBS. Cells were resuspended in 50 μ l of blocking buffer (10% human AB serum, 40 μ g/ml mouse IgG, 40 μ g/ml rabbit IgG) and incubated at 4 °C for 20 minutes. The cells were washed twice in staining buffer (1% FCS, 0.09% NaN₃ in PBS), resuspended in 50 μ l/well of extracellular antibody (typically anti-CD56, anti-3DL1) in PBS and incubated at 4°C for 30 minutes. The cells were washed twice in staining buffer, resuspended in 100 μ l of BD Cytotfix/Cytoperm and incubated at 4 °C for 20 minutes covered with foil. The cells were washed twice in BD Perm/Wash, resuspended in 0.25 μ g of anti-IFN- γ (prepared in 50 μ l of BD Perm/Wash) and incubated at 4 °C for 30 minutes. The cells were washed twice in BD Perm/Wash and resuspended in staining buffer. Cells were then analysed using a BD FACSCalibur™ machine. Cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μ g/ml) for 4 hours in the presence of BD GolgiStop™ (1 μ g/ml) as a positive control.

2.16 CD107a staining

Cells were incubated with the appropriate stimuli in the presence of anti-CD107a FITC (20 μ l/ml). Monensin (6 μ g/ml) was added 1 hour post stimulation and the cells were incubated

for a further 5 hours. The cells were harvested, washed with PBS, stained with extracellular antibodies and analysed using a BD FACSCalibur™ machine.

2.17 ⁵¹Cr release assay

Target cells (K562 or 721.221 cell lines, 1×10^6) were resuspended in 1 ml of complete medium. $\text{Na}_2^{51}\text{CrO}_4$ (1.85 MBq) was added to the cells, which were then incubated at 37 °C for 1 hour. After this time, 10 ml of complete medium was added to the cells and they were incubated for a further 10 minutes at 37 °C. The cells were then centrifuged at 300 g for 10 minutes. The cells were washed twice in complete medium. The cells were resuspended in 10 ml of complete medium and plated at 100 μl (1×10^4 cells)/well in a 96-well U-bottomed plate. Effector cells were resuspended to a suitable concentration in complete medium and 100 μl of cells was added to wells containing target cells to give the desired effector to target ratio. Samples were tested in quadruplicate. 100 μl of complete medium was added to 4 wells to give background reading. 100 μl of 10% Triton X-100 was added to 4 wells to give a value for maximum release. The plate was centrifuged at 150 g for 5 minutes and then incubated for 4 hours at 37 °C. After this time the plate was centrifuged at 300 g for 10 minutes. 25 μl samples of the supernatants were taken and added to Optiphase Supermix scintillation cocktail (150 μl) into 96-well sample plates. The plate was read using a Microbeta Trilux scintillation counter.

The % cytotoxicity was calculated according to the formula:

$$\% \text{ cytotoxicity} = \frac{\text{Experimental-spontaneous}}{\text{Maximum-spontaneous}} \times 100$$

where experimental = average value of sample wells, spontaneous = average value of well containing complete medium, maximum = average value of wells containing Triton X-100.

Anti-3DL1 antibodies (DX9 and Z27) and anti-CD94 antibody (HP-3B1) were used as blocking antibodies in cytotoxicity assays at 10 $\mu\text{g/ml}$.

2.18 Plasmid transformation and Purification

Plasmids were transformed into chemically competent *E. coli* (One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen) according to the manufacturer's instructions. In brief, competent cells were thawed on ice, plasmid DNA was added and cell were incubated on ice for 30 minutes. Cells were then incubated at 42 °C for 30 seconds and 250 µl of pre-warmed SOC medium was added. The cells were shaken horizontally for 1 hour, plated on pre-warmed LB agar plates containing the appropriate antibiotic and incubated overnight at 37 °C. Single colonies were selected and grown in LB broth for plasmid purification. Plasmids were purified using QIAfilter Plasmid Midi Kit (Qiagen) according to manufacturer's instructions. In brief, the bacterial cells were lysed; cellular protein, genomic DNA and cell debris were then removed after precipitation. The remaining lysate was applied to a QIAGEN-tip 100, the tip was then washed and the DNA was eluted. DNA was precipitated in 0.7 volumes of isopropanol, centrifuged at 15,000 g for 30 minutes at 4 °C. The concentration of the plasmid was determined by UV spectrophotometry, using the formula:

$$\left(\begin{array}{c} \text{DNA concentration} \\ (\text{ng}/\mu\text{l}) \end{array} \right) = A_{260} \times 50 \times \text{dilution factor}$$

2.19 DNA isolation

DNA was isolated from blood using a "salting out" procedure. 500 µl of blood was added to 900 µl of red blood lysis solution (0.32 M sucrose, 1 % (v/v) Triton X-100, 5 mM MgCl₂, 12 mM Tris-HCL, pH 7.5) and mixed. Tubes were centrifuged at 11,000 g for 1 minute and the supernatant was discarded. 1 ml of deionised water was added to the cell pellet and resuspended by vortexing. The tube was spun as above and the supernatant discarded. 80 µl DNA buffer (0.375 M NaCl, 1.2 M Na₂EDTA, pH 8), 30 µl 5X proteinase K (10 mg/ml proteinase K in 1 % SDS, 0.2 M Na₂EDTA, pH 8) and 260 µl SDS was then added to the tube and mixed by vortexing. The tube was then incubated at 55 °C for 30 minutes, vortexing occasionally. 100 µl 6M NaCl was added and the tube was shaken

vigorously for 15 seconds. The sample was centrifuged for 5 minutes at 11,000 g. The supernatant was removed; 1 ml of ice-cold 100% ethanol was added and mixed gently by inversion for 10 seconds. The tube was centrifuged for 2 minutes at 11,000 g and the supernatant was discarded. 1 ml of 70 % ethanol was added to the pellet and the tube was centrifuged at 4 °C at 11,000 g for 2 minutes. The supernatant was removed carefully, the pellet was allowed to air-dry for approximately 5 minutes and was then resuspended in 100 µl molecular biology grade water (Sigma). DNA was quantified by spectrometry, measuring absorbance at 260 nm and 280 nm.

2.20 RNA isolation

1-10 x 10⁶ cells were resuspended in 1 ml of Trizol® and RNA was isolated according to the manufacturers guidelines. Briefly, cells were lysed in Trizol® and the lysate was separated into aqueous and organic phases by chloroform addition and centrifugation. RNA remained exclusively in the aqueous phase, DNA in the interphase and protein in the organic phase. RNA was precipitated from the aqueous phase by addition of 70% (v/v) isopropanol in DEPC-treated H₂O, washed with 100% ethanol and solubilised in molecular biology grade H₂O. Isolated RNA was stored at -80°C.

2.21 cDNA synthesis

cDNA synthesis was performed using ImProm-II™ Reverse Transcription System kit (Promega) according to the manufacturer's instructions. Briefly, target RNA and random primers were combined, heated to denature the RNA and quickly chilled to allow primers to anneal. Reaction mix was set up in a volume of 30 µl with 1x ImProm-II™ Reaction Buffer, 2.5 mM MgCl₂, 0.5 mM dNTPs, 40 U ribonuclease inhibitor and 2 µl ImProm-II™ Reverse Transcriptase. The reaction mix was combined with the template RNA and primers, annealed at 25°C, and first-strand cDNA synthesis carried out at 42 °C. The reverse transcriptase was then inactivated at 70 °C for 15 minutes. The quality of cDNA produced was checked by β-actin amplification.

2.22 PCR

2.22.1 3DL1 allelic gDNA typing

PCR was preformed in a final volume of 25 μ l in 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCL), 1.5 mM MgCl₂ and 0.2 mM dNTPs. Primers were used at a concentration of 0.5 pM each, 200-300 ng DNA was used per reaction. Table 2.3 contains a list of primer pairs used to amplify the alleles of 3DL1. The quality of the DNA was assessed by amplification of β -actin (forward: 5'-cgcgagaagatgacccagatc-3', reverse: 5'-ttgctgatccacatctgctgg-3'). PCR amplifications were carried out using a Hybaid PX2 PCR machine using the cycling parameters described in Table 2.4.

2.22.2 3DL1 cDNA typing

PCR was preformed as with gDNA typing above using the following primer sets (Table 2.5) and amplification conditions (Table 2.6).

2.22.3 High Fidelity PCR

High fidelity PCR was performed in a final volume of 25 μ l in 1X reaction buffer (20 mM Tris-HCl pH 8.4, 50 mM KCL), 1.5 mM MgCl₂ and 0.1 mM dNTPs. Primers were used at a concentration of 0.5 pM each, 200-300 ng DNA was used per reaction. Full length 3DS1 sequence was amplified from cDNA using the primers and PCR conditions outlined in Table 2.7 and Table 2.8. The FLAG sequence was added to the leader and D0 sequences of 3DS1 by primer additions using the primers and PCR conditions outlined in Table 2.7 and Table 2.9. The leader-FLAG and FLAG-D0 PCR products were used as templates in a recombinant PCR using the primers and PCR conditions outlined in Table 2.7 and Table 2.9.

Sequence Recognised		Primer Sequence 5'-3'	Expected Size (bp)
3DL1	<i>F</i>	CCATCGGTCCCATGATGCT	1600
	<i>R</i>	AGAGAGAAGGTTTCTCATATG	
3DL1*001	<i>F</i>	TACAAAGAAGACAGAATCCACA	1945
	<i>R</i>	GGAGCTGACAACTGATAGGG	
3DL1*002	<i>F</i>	TCCCATCTTCCATGGCAGAT	1848
	<i>R</i>	TAGGTCCCTGCAAGGGCAA	
3DL1*004	<i>F</i>	CAGACACCTGCATGTTCTC	939
	<i>R</i>	GTACAAGATGGTATCTGTAG	
3DL1*005	<i>F</i>	TCTTCGGTGTCACTATCG	1987
	<i>R</i>	GAGCTGACAACTGATAGGA	
3DS1	<i>F</i>	GGCAGAATATTCCAGGAGG	1800
	<i>R</i>	AGGGGTCCTTAGAGATCCA	

Table 2.3 gDNA 3DL1 allele typing primers

The presence of 3DL1 alleles in donors were examined by primer specific PCR using the above primers.

Temp °C	Time (mm:ss)	Cycle No.	Temp °C	Time (mm:ss)	Cycle No.
95	05:00	1	95	05:00	1
96	00:20	5	96	00:20	5
62	01:00		68	01:00	
72	00:45		72	00:45	
95	00:20	28	95	00:20	28
60	00:40		64	00:40	
72	01:30		72	01:30	
72	07:00	1	72	07:00	1
4	hold	1	4	hold	1

A, All 3DL1 alleles except *001

B, 3DL1*001

Table 2.4 PCR conditions for 3DL1 gDNA allele amplification

The presence of 3DL1 alleles in donors were examined by primer specific PCR using the primers detailed in Table 2.3 and the cycling parameters outlined above for *001 (B) and all other alleles (A).

Sequence Recognised		Primer Sequence 5'-3'	Expected Size (bp)
3DL1	<i>F</i>	ACATCGTGGTCACAGGTCC	556
	<i>R</i>	TGCGTATGTACCTCCTC	
3DL1*001	<i>F</i>	TACAAAGAAGACAGAATCCACA	550
	<i>R</i>	GGAGCTGACAACTGATAGGG	
3DL1*002	<i>F</i>	TCCCATCTTCCATGGCAGAT	374
	<i>R</i>	TAGGTCCCTGCAAGGGCAA	
3DL1*004	<i>F</i>	TCTTCGGTGTCACTATCA	487
	<i>R</i>	GAGCTGACAACTGATAGGA	
3DL1*005	<i>F</i>	TCTTCGGTGTCACTATCG	487
	<i>R</i>	GAGCTGACAACTGATAGGA	
3DS1	<i>F</i>	GGCACCCAGCAACCCCA	246
	<i>R</i>	AAGGGCACGCATCATGGA	
2DL4	<i>F</i>	CTGTCCCTGAGCTCTACAA	374
	<i>R</i>	CACTGAGTACCTAATCACAG	

Table 2.5 cDNA 3DL1 allele typing primers

The expression of 3DL1 alleles and 2DL4 in donor PBMC and NK cell clones was examined by primer specific PCR amplification of cDNA using the above primers.

Temp °C	Time (mm:ss)	Cycle No.
95	05:00	1
96	00:20	5
62	01:00	
72	00:45	
95	00:20	25
60	00:40	
72	01:30	
72	07:00	1
4	hold	1

Table 2.6 PCR conditions for 3DL1 cDNA allele amplification

The expression of 3DL1 alleles was examined by primer specific PCR amplification of cDNA using the primers detailed in Table 2.5 and the cycling parameters outlined above.

Sequence Amplified		Primer Sequence 5'-3'	Expected Size (bp)
3DS1	F	CCGAAT GCGGCCG CACCGGCAGCACCATGT	1168
	R	ATGCATGAATTCTTTCTCTGTGTGAAAACACAGTGTCCAATTA	
Leader	F	CCGAAT GCGGCCG CACCGGCAGCACCATGT	116
	R	<u>CTTATCGTCGTCGTCATCCTTGTAA</u> TCGGACCGGCCCTCTGGACCAA	
D0	F	<u>GATTACAAGGATGACGACGACGATAAG</u> CACATGGGTGGTCAGGACAA	81
	R	CACCACAGCGCTGGGCCAGG	
LeaderD0	F	CCGAAT GCGGCCG CACCGGCAGCACCATGT	170
	R	CACCACAGCGCTGGGCCAGG	

Table 2.7 Primers for the generation of FLAG-tagged 3DS1

Full length 3DS1 sequence was amplified from cDNA using the above primers. The leader and D0 sequences of 3DS1 were amplified and FLAG sequence was added by primer addition (FLAG sequence is underlined). LeaderD0 sequence (containing the inserted FLAG sequence) was generated by recombinant PCR using primers detailed above. A *NotI* restriction site was added to the 5' end of the 3DS1 and leaderD0 sequences by primer addition (sequence in bold).

Temp °C	Time (mm:ss)	Cycle No.
95	05:00	1
96	00:20	5
64	00:45	
72	01:30	
95	00:20	15
62	00:45	
72	01:30	
72	07:00	1
4	hold	1

Table 2.8 PCR conditions for 3DS1 amplification

The full length 3DS1 sequence was amplified from cDNA using the primers detailed in Table 2.7 and the cycling parameters outlined above.

Temp °C	Time (mm:ss)	Cycle No.
95	05:00	1
96	00:20	5
60	01:00	
72	00:45	
95	00:20	15
55	00:40	
72	01:00	
72	07:00	1
4	hold	1

Table 2.9 PCR conditions for leader, D0 and leaderD0 amplification

3DS1 leader and D0 sequences were amplified from cDNA using the primers detailed in Table 2.7 and the cycling parameters outlined above. The recombinant leaderD0 sequence was amplified from the leader and D0 PCR products using the primers outlined in Table 2.7 and the cycling parameters above.

PCR products were resolved on 1% agarose gels with 1 µg/ml ethidium bromide in TAE at 40 V for 90 minutes and visualised under UV light. Small DNA fragments were resolved on 2% MetaPhor® agarose gels.

2.23 Generation of FLAG-tagged 3DS1

To generate a mature 3DS1 protein with a FLAG tag at the N terminus, the FLAG sequence was inserted between the leader sequence and the D0 domain sequence of the protein. Full length 3DS1 and leader-FLAG-D0 sequences were amplified as outlined above. PCR products were excised from agarose gel and extracted (3DS1, see 2.25) or underwent PCR clean up (leader-FLAG-D0, see 2.24). TOPO TA cloning (see 2.29) was used to insert both PCR products into the sequencing vector pCR®4-TOPO after the addition of a 3' A overhang (see 2.26). Constructs were prepared for sequencing by MWG Biotech after a miniprep plasmid preparation. Plasmid based vectors (T3 and T7) were used for sequencing as well as the internal primer Kirseq 3'-3 (5'- GGGAGCTGACAACTGATA - 3') and error free clones were selected. 3DS1 full-length sequence was subcloned from the sequencing vector pCR®4-TOPO to the eukaryotic expression vector pcDEFIII using directional cloning with *NotI* and *EcoR1* restriction enzymes (see 2.26, 2.27). The leaderD0 sequence was excised by digestion with *NotI* and *Eco47III* (an intrinsic restriction site). The leader-FLAG-D0 amplicon was also digested with *NotI* and *Eco 47III* and ligated into the 3DS1 digested construct giving a full-length construct with a FLAG sequence directly after the leader sequence.

2.24 PCR CleanUp

PCR clean up was performed using a MiniElute kit purchased from Qiagen as per the manufacturer's instructions. In brief, buffer PB was added to the PCR reaction and this was loaded onto a MiniElute column and centrifuged for 1 minute at 10,000 g. The flow through was discarded and the column was washed with buffer PE. The flow through was again discarded and the column was centrifuged, and then placed in a 1.5 ml micro-centrifuge tube. 10 µl of H₂O was added to the membrane and allowed to stand for 1 minute. DNA was eluted by centrifugation for 1 minute at 10,000 g.

2.25 Gel extraction

DNA extraction from agarose gels was performed using QIAEXII kit from Qiagen, as per the manufacturer's instructions. In brief, the band of interest was excised from the agarose gel. 3 volumes of Buffer QX1 were added to 1 volume of gel and 10 μ l of QIAEXII was added. The mixture was incubated at 50 °C for 10 minutes with vortexing. The sample was then centrifuged at 10,000 g for 30 seconds, the supernatant was removed and the pellet was washed with buffer QX1. The pellet was then washed twice in buffer PE. The supernatant was then removed and the pellet was dried for 10-15 minutes. The pellet was re-suspended in 20 μ l of H₂O (pH 8) and allowed to stand at room temperature for 5 minutes. The sample was then centrifuged for 30 seconds at 10,000 g and the supernatant containing the DNA was removed.

2.26 3' A overhang addition

A 3' A overhang was added to Bio-X-Act amplified PCR products to enable TOPA-TA cloning. Purified DNA was resuspended in 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCL), 8 μ M dATP and 0.5 U Taq Polymerase. The mixture was heated to 72 °C for 15 minutes and then placed on ice.

2.27 Restriction digest

DNA (typically 1 μ g) was digested with 5-10 U of appropriate restriction enzymes at 37 °C for four hours in a reaction volume of 50 μ l. Double digestion with *Eco*R1 and *Not*I (Promega) was carried out in Promega buffer D (*Not*I 100% activity, *Eco*R1 50-75% activity). Digestion with *Eco*47III (Roche) was carried out in Roche buffer H. Digestion of plasmid with *Eco*R1 and *Eco*47III was carried out sequentially.

2.28 Ligation

Ligation of DNA fragment into vectors of interest was performed using TaKaRa DNA Ligation Kit Ver.2.1 according to the manufacturer's instructions. In brief, vector DNA and

DNA to be inserted was combined in 5 µl 100 mM Tris-HCl, pH 7.6, 5mM MgCl₂. 5 µl of Solution I (containing T4 DNA ligase) was added and the reaction mixed thoroughly. The sample was then held at 16 °C overnight. 1 µl of Solution III was then added to 9 µl of ligation reaction, mixed and used to transform *E. Coli* competent cells.

2.29 TOPO TA cloning

PCR products were cloned into the pCR®II-TOPO vector to allow for sequencing. 1 µl of salt solution (1.2 M NaCl, 0.06 M MgCl₂) was added to 4 µl of PCR product and 1 µl of cloning vector pCR®II-TOPO, incubated at room temperature for 5 minutes and then placed on ice. One Shot® Competent cells were then transformed using this mixture as per the manufacturer's instructions. 2 µl of the cloning reaction was added to a vial of competent cells, mixed gently without pipetting and placed on ice for 15 minutes. The cells were heat-shocked at 42 °C for 30 seconds and then transferred to ice. 250 µl of SOC medium (2 % Tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) was added and the tubes were shaken horizontally at 37 °C for 1 hour. 50 µl of the transformation reaction was plated on pre-warmed ampicillin agar plates and incubated overnight at 37 °C.

Colonies were screened for the presence of the correct insert by PCR using T3 (5'-ATTAACCCTCACTAAAGGGA-3') and T7 (5'-TAATACGACTCACTATAGGG-3') primers to amplify the insert. Colonies were picked at random from the plate and added to 20 µl of PCR cocktail (20 mM Tris-HCl pH 8.4, 50 mM KCL, 1.5 mM MgCl₂, 0.2 mM dNTPs). Colonies were patched to preserve for future work in 200 µl LB ampicillin broth and incubated at 37 °C. The PCR reaction was incubated at 94 °C for 10 minutes to lyse the bacterial cells and inactivate the nucleases. The PCR product was amplified 20 times (94 °C for 1 minutes, 55 °C for 1 minutes and 72 °C for 1 minute) with a final extension at 72 °C for 10 minutes and visualised by agarose gel electrophoresis as previously described. Colonies containing the correct insert were grown up and the plasmid was harvested as previously described (2.18).

2.30 Statistical Analysis

One-way ANOVA followed by Bonferroni's Multiple Comparison Test was used to test for statistically significant differences between groups using GraphPad Prism 4 software. Error bars represent standard deviation (SD) or standard error of the mean (SEM) as appropriate.

Chapter Three

Characterisation and cloning of 3DL1⁺ NK cells from normal donors

3.1 Introduction

NK cells are effector cells of the immune system, important in many biological responses including the control of viral infections and malignancies. The function of these cells is governed in part by the expression of a large number of both activatory and inhibitory cell surface receptors including the KIR family. Characterisation of the expression and function of these receptors is necessary to fully understand the role of NK cells in health and disease. Functional analysis of KIR and other NK cell receptors is hampered by genetic variation in these receptors within the population and further by the variation in receptor expression within an individual's NK cell pool. This study addresses the role of 3DL1 allelic polymorphism in NK cell function.

3.1.1 3DL1 genetic variability

A key feature of the KIR gene cluster is a high degree of variability. Much of this variability is due to differing numbers of KIR genes present – individuals can possess between 7 and 13 KIR genes. The 3DL1 gene is present in the vast majority of individuals (approximately 99.5%) and thus most of its variation within the population is due to allelic polymorphism (Gardiner et al, 2001). 3DL1 is a highly polymorphic with over 40 alleles reported to date. The nomenclature adopted for 3DL1 alleles (and other KIR genes) is analogous to that used for HLA alleles; after the gene name an asterisk is used as a separator before a numeric allele designation. Alleles that differ in sequence giving rise to an altered amino acid sequence are given a separate three digit code (*001, *002); the next two digits are used to distinguish alleles that differ by synonymous differences within the coding region (*00101, *00102); an additional two digits can be used to distinguish difference in noncoding regions of the gene.

3DS1 is an allele of 3DL1 with 98% sequence homology in the extracellular domain. Intracellularly 3DS1 resembles other short tailed KIR, lacking the inhibitory ITIM motifs and containing a charged amino acid in the transmembrane region, features associated with activatory KIR. There are five reported alleles of 3DS1 (designated 3DS1*010 to 014), although several Caucasian studies have reported only one (*013), suggesting that there is

one predominant allele in most Caucasian populations (Halfpenny et al, 2004; Martin et al, 2002).

The 3DL1 gene covers approximately 16 Kb on chromosome 19 with nine exons. The intron-exon structure of 3DL1 is similar to other KIR genes – exons 1 and 2 encode a leader sequence, each of the three Ig domains is encoded by a separate exon, the stem and transmembrane region are each encoded by a single exon and the remaining 2 exons encode the cytoplasmic domain (Wilson et al, 1997). The extracellular domain of 3DL1 contains 16 positions with defined amino acid polymorphisms (Table 3.1.1). Many of these polymorphisms occur in areas of the receptor that are predicted to interact with the HLA ligand, suggesting that this polymorphism may have functional consequences.

DOMAIN	D0						D1				D2						Cytoplasmic		
Amino acid no.	2	31	41	47	54	58	86	92	138	163	166	182	199	238	277	283	320	343	373
Consensus	M	R	R	V	I	S	S	V	G	P	L	P	P	G	R	W	I	C	E
*001	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*002	V	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*004	-	H	G	I	-	-	L	-	-	-	-	S	-	-	-	L	V	Y	Q
*005	n.d.	-	-	I	-	-	-	-	-	-	-	S	-	-	-	L	-	-	-
3DS1	-	-	-	-	-	G	-	M	W	S	R	-	L	-	-	-	DIVERGENT		
Predicted Protein Structure	Loop Loop Loop Loop Loop						S	Loop	S	Loop Loop	Loop Loop	Loop Loop	Loop Loop	Loop Loop	Loop Loop				

Table 3.1.1 Amino acid difference in 3DL1 alleles

A summary of the amino acid differences in the extracellular portion of 3DL1 alleles examined. 3DL1 alleles were aligned and amino acid differences relative to a consensus KIR sequence highlighted. Loop and stem (S) portions of the protein are indicated.

3.1.1.1 Methods of KIR genotyping

The presence and allelic polymorphism of KIR genes in the population has been assessed using a number of techniques including sequence-based typing (SBT), sequence specific conformational polymorphism (SSCP), sequence specific oligonucleotide probing PCR (SSOP-PCR) and sequence specific primers PCR (SSP-PCR). SBT is based on the sequencing of the PCR product of locus-specific amplification (Adams et al, 2001). SBT is the most comprehensive typing system, giving high-resolution sequence and allowing identification of new alleles. SSCP is a technique based on different electrophoretic mobility of DNA sequences and has been used in the identification of 2DL4 alleles (Witt et al, 2000). Small changes in the DNA sequences can give rise to altered secondary structure, which affects the mobility of the sequence and can identify single nucleotide changes.

SSOP-PCR has been used extensively in the identification of HLA polymorphism and the technique has been adapted for KIR genotyping (Crum et al, 2000). Group-specific primers are initially used to generate PCR products, which are then exposed to a panel of specific oligonucleotide probes. SSOP-PCR techniques have been used to identify KIR allele types for 2DL3, 2DL4, 3DL2, 2DS4 and 3DL1/S1. The 3DL1/S1 allele typing system is based on three separate PCR products (D0, D1D2 and the cytoplasmic tail of 3DS1), which are then applied to a charged nylon membrane and probed with a panel of 28 oligomers that are specific for polymorphic sequences in exon 3, 4, 5 and 9 of the 3DL1 gene (Halfpenny et al, 2004).

A method of KIR genotype based on the use of SSP-PCR that identified the presence of 11 KIR genes and distinguished between 3DL1 and 3DS1 was first described by Uhrberg *et al* in 1997. Primer pairs were designed that contained sequences to match polymorphic positions specific to each KIR gene - a PCR product is generated only in the presence of the specific KIR gene. This system of genotyping has been modified to allow allele typing of 3DL1/S1, based on the use of primer pairs that are specific for polymorphic positions that vary between 3DL1 alleles (Gardiner et al, 2001). SSP-PCR is the simplest and most convenient method of detecting the presence of known alleles but, unlike some of the other

methods, is not capable of identifying new alleles. This method was also adapted for RT-PCR identification of 3DL1 allele expression; different primer pairs were used for cDNA amplification (with the exception of 3DL1*001) to ensure that PCR product size was easily visualised (Gardiner et al, 2001). In this study, five of the most common 3DL1 alleles (3DL1*001, *002, *004, *005 and 3DS1) were identified in different donors using SSP-PCR on genomic DNA. Expression of these 3DL1 alleles was also assessed by SSP-PCR on cDNA generated from PBMCs, NK cells, sorted NK cell subsets or NK cell clones.

3.1.2 3DL1 expression

In addition to the allelic variability in 3DL1, there is considerable heterogeneity in the expression of the receptor. The anti-3DL1 antibody DX9 has been used in assessing the variability in 3DL1, showing large variation in the percentage of positive cells and in the intensity of staining (Gumperz et al, 1996). NK cells from individuals show four different patterns of DX9 staining intensity – unimodal high intensity binding; unimodal low intensity binding; bimodal high and low intensity binding and no binding. These differences in staining patterns can be attributed to 3DL1 polymorphism – with some alleles (*001, *002) giving rise to a high intensity peak, while others (*005) give a low intensity peak and some (*004 and 3DS1) show no staining (Gardiner et al, 2001). Allelic variation in levels of cell surface receptor expression rather than varying affinity for the antibody accounts for the different patterns of DX9 reactivity for the long tailed 3DL1 alleles. In the most extreme case, the allele 3DL1*004 is not expressed at the cell surface but is retained intracellularly due to amino acid substitutions in the D0 and D1 domain (Pando et al, 2003).

Individuals with specific 3DL1 alleles will only express this allele on a subset of their NK cells (and a minor subset of T cells). Both the levels of 3DL1 expression and percentage of 3DL1 expressing NK cells are stable over time. Early family studies found that the expression was genetically determined (with twins showing similar profiles), but not correlated with HLA expression (Gumperz et al, 1996). A more recent study reported that HLA can affect both the frequency and level of 3DL1 expression on NK cells (Yawata et

al, 2006). Yawata *et al* also found that the presence of the Bw4 ligand increased the percentage of 3DL1 positive cells in the donors with two copies of highly expressed 3DL1 alleles with a greater increase for Bw4/Bw6 heterozygotes than Bw4 homozygotes. The presence of additional cognate KIR-HLA ligand pairs (e.g. 2DL1 with HLA C2 allele) decreased the Bw4-dependent increase in the frequency of 3DL1 positive cells. In contrast, for individuals expressing a single highly expressed allele, the expression of HLA-Bw4 decreased the mean fluorescent intensity (MFI) of DX9 staining.

3.1.3 Non-KIR HLA-specific receptors

NK cells express a number of HLA-specific receptors in addition to the KIR family, including the CD94/NKG2 heterodimers and LILRB1. CD94/NKG2A (inhibitory) and CD94/NKG2C (activatory) both recognise HLA-E, which is expressed on target cells presenting the leader peptide derived from some HLA-A, -B, -C and -G alleles (Llano *et al*, 1998). Of the alleles of HLA-Bw4 examined in this study, it is predicted that some, but not all alleles, will support HLA-E expression and inhibit NK cell cytotoxicity via CD94/NKG2A. The LILRB1 inhibitory receptor, expressed on a subset of NK cells, recognises a broad range of HLA class I molecules.

3.1.4 NK cell proliferation

Expansion of NK cells is necessary to generate sufficient numbers of cells for functional experiments. NK cell proliferation can be supported by the provision of various cytokines and the addition of stimulated feeder cells, themselves a source of both cytokine and cell contact-dependent signals. The primary cytokines involved in NK cell proliferation are IL-2 and IL-15, but IL-12, IL-4 and IL-7 have also been reported to play a role.

IL-2 is capable of inducing NK cell proliferation in the absence of other stimuli, and mediates its effects by binding to a medium or high affinity IL-2 receptor (Trinchieri *et al*, 1984). The medium affinity IL-2 receptor (k_a approximately 10^9 M^{-1}) consists of the IL-2 R β chain and the γ -common chain and is constitutively expressed by the majority of peripheral blood NK cells (the CD56^{dim} subset) (Baume *et al*, 1992). The CD56^{bright} subset of NK cells additionally express the IL-2R α chain therefore having a high affinity IL-2 receptor (k_a approximately 10^{11} M^{-1}). These cells proliferate vigorously to picomolar

concentration of IL-2. In contrast, the CD56^{dim} cells respond to nanomolar concentrations of IL-2 to induce cytotoxicity but very poor proliferation.

IL-15 is a pleiotropic cytokine that acts to promote survival, proliferation and activation of NK and T cells. It was originally identified due to its ability to mimic IL-2 dependent T cell proliferation and despite a lack of sequence homology it is predicted to adopt a secondary structure similar to that of IL-2 (Grabstein et al, 1994). IL-15 shares the IL-2/15R β chain and the γ_c chain receptor signalling components with IL-2, but has a unique IL-15R α chain, that in conjunction with the IL-2/15R β and γ_c gives a high affinity IL-15 receptor. The IL-15R α chain, similar to the IL-2R α chain binds its ligand with high affinity but has no inherent signalling capacity. Due to the shared signalling machinery, IL-2 and IL-15 have similar (if not identical) signalling and biological properties, but both IL-15 and the IL-15R α have a much broader tissue distribution than IL-2 and the IL-2R α chain (Budagian et al, 2006).

IL-4, IL-7 and IL-12 are also capable of supporting NK cell proliferation, but to a much lesser extent than that seen with IL-2. Optimal concentrations of these cytokines produce less than one tenth of the expansion seen in the presence of optimal concentrations of IL-2. In addition, the concentration of both IL-4 and IL-7 needed to induce maximal proliferation is 100-1000 fold greater than that of IL-2 (Robertson et al, 1993). NK cells preactivated by IL-2 show an increased proliferative response to IL-12, but the degree of proliferation is inferior to that induced by IL-2 alone (Robertson et al, 1992). In contrast IL-12 inhibits the IL-2 induced proliferation of resting or preactivated NK cells. IL-4 is also capable of inhibiting IL-2 induced NK cell proliferation (Nagler et al, 1988).

3.1.5 Choice of experimental model system

In light of the complexity of the NK cell receptor expression, analysis of the role of individual proteins requires a careful choice of model system. One possibility is a reductionist approach involving the analysis of the receptor recognition in an isolated system such as that used in Chapter 4. Examination of 3DL1 function in primary NK cells is hampered by its large degree of variability (both in allelic polymorphism and in

expression), as well as by co-expression by NK cells of other HLA-specific receptors. NK cell cloning is a very attractive solution to this problem, allowing the generation of large number of cells for functional analysis that can be chosen based on the expression (or lack of expression) of specific receptors. To examine the function and ligand specificity of specific 3DL1 alleles using this approach, suitable individuals expressing these alleles must be identified before NK cells are isolated and cloned (Figure 3.1.1).

This study focuses on five 3DL1 alleles – 3DL1*001, *002, *004, *005 and 3DS1. These alleles were chosen as they are found at high frequency in Caucasian populations and contain polymorphisms that were thought likely to influence 3DL1 ligand recognition and function. These alleles also represent high expressed (*001, *002) and low expressed (*005) alleles of 3DL1 as well as the putative activatory allele 3DS1.

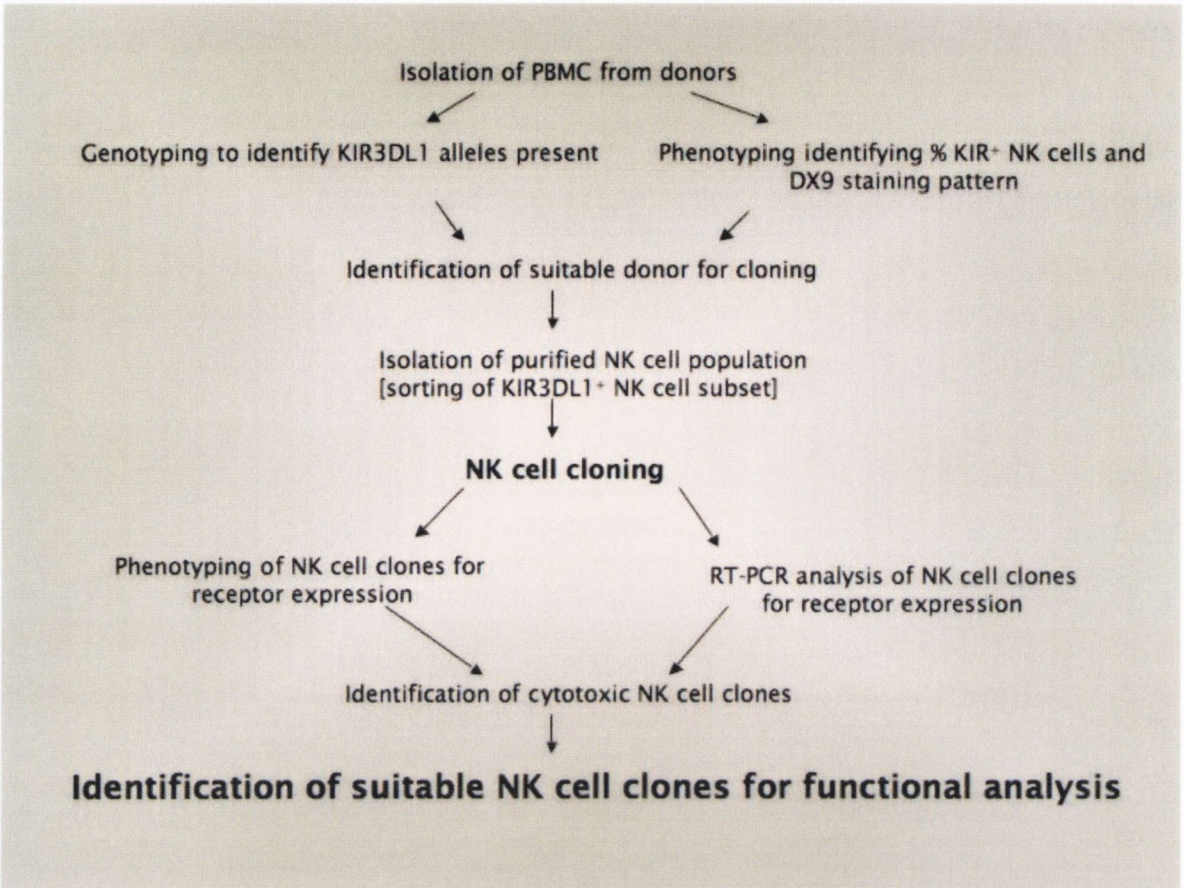


Figure 3.1.1 Generation of NK cell clones for analysis of 3DL1 functional polymorphism

Donors were characterised for the expression of 3DL1 alleles in order to choose suitable donors for NK cell cloning. NK cell clones were then analysed for the expression of 3DL1 and other HLA-specific receptors.

3.2 Aims

The aims of this chapter were

- To characterise 3DL1 allele and expression profile of a panel of donors
- To assess NK cell enrichment, purification and sorting protocols
- To establish NK cell cloning protocol – a comparison of three different protocols
- To characterise of a panel of NK cell clones

3.3 Results

3.3.1 Donor Profiling and Selection

To select donors expressing the required 3DL1 alleles protocols for allele typing and expression were optimised.

3.3.1.1 Validation of 3DL1 allele typing system

A previously reported 3DL1 allele typing system (Gardiner et al, 2001) was established in the laboratory using a panel of DNA samples from donors who had been previously sequenced to verify the accuracy of the system. Each DNA sample was amplified using primers specific for 3DL1 (recognising all alleles except 3DS1), 3DS1, 3DL1*001, 3DL1*002, 3DL1*004 and 3DL1*005. The quality of the DNA was assessed by amplification of the β -actin gene. Figure 3.1 shows a typical agarose gel profile with a positive band for each primer pair. Initial allele amplification identified a problem with non-specific *001 amplification in the presence of either the *004 or *005 allele. A separate PCR amplification program for this allele was then designed involving an increase in the primer annealing temperature (Table 2.4). This program eliminated the false positive results obtained using the less stringent conditions. Once these modifications had been applied the known 3DL1 allele types of each sequenced donor were successfully reproduced (Table 3.1).

3.3.1.2 3DL1 allele expression in a panel of donors

In total, 75 individual donors were typed for the presence of 3DL1 alleles (Table 3.2). The percentage of positive donors and the allele frequency was calculated for each allele based on the typing of 42 randomly chosen donors (Figure 3.2). The remaining 33 donors were excluded from this analysis as they were selected for typing based on a specific staining profile with the anti-3DL1 antibody Z27. Within the 42 randomly chosen donors, 3DL1*002 was the most common allele with 54% testing positive. The short-tailed 3DS1 was found in 37.2% of the donors analysed, 3DL1*005

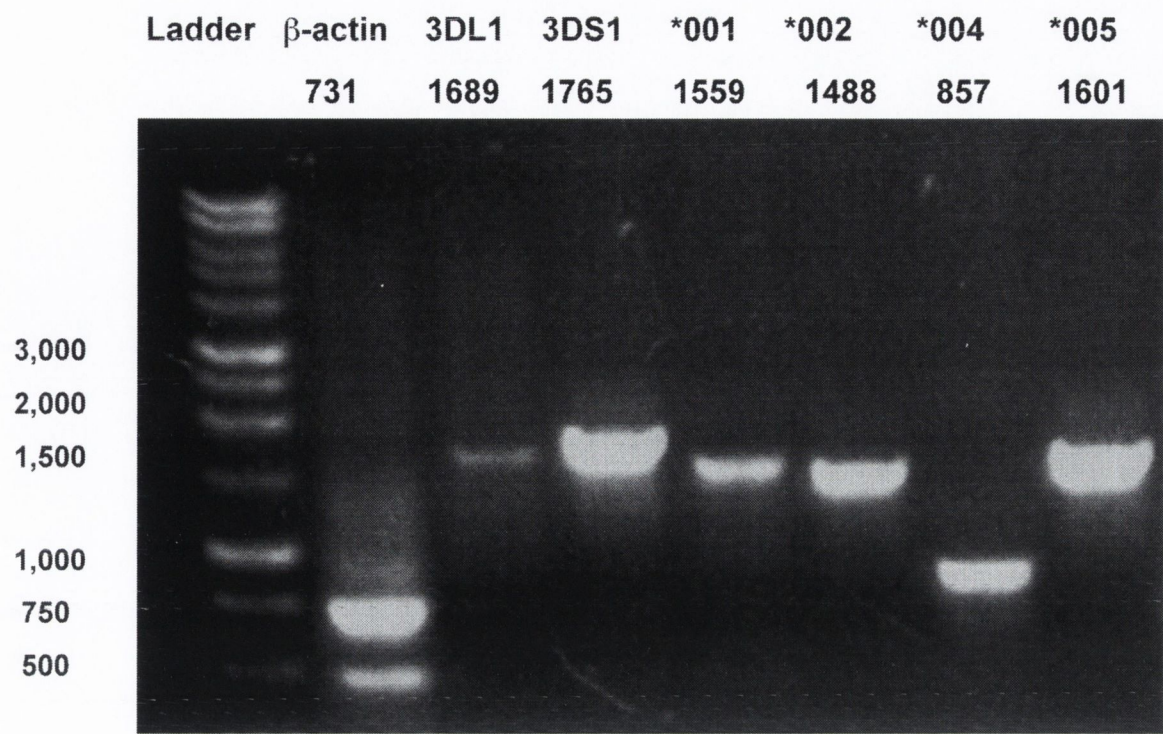


Figure 3. 1 3DL1 genomic DNA allele typing

PCR-SSP was carried on gDNA from sequenced donors (a gift from Peter Parham) for the amplification of 3DL1 alleles. Products were visualized using agarose gel electrophoresis. Primer sequences and PCR conditions are outlined in Table 2.3 and Table 2.4.

		3DL1	3DS1	*001	*002	*004	*005
Donor 1	sequencing	+	-	+	+	-	-
	typing	+	-	+	+	-	-
Donor 2	sequencing	+	-	-	+	-	+
	typing	+	-	-	+	-	+
Donor 3	sequencing	+	-	-	-	+	-
	typing	+	-	-	-	+	-
Donor 4	sequencing	+	-	-	+	-	-
	typing	+	-	-	+	-	-
Donor 5	sequencing	+	+	-	+	-	-
	typing	+	+	-	+	-	-

Table 3.1 Verification of 3DL1 genomic DNA allele typing

DNA from five sequenced donors was used to validate the 3DL1 allelic typing method. The table shows 3DL1 alleles for each of the five donors as determined by cDNA sequencing (top line) and by primer specific PCR (bottom line).

Donor	Allele 1	Allele 2	DX9 pattern	% DX9 ⁺ NK
1	*002	*005	bimodal	12.1, 3.04
2	*001		high	20.5
3	*002		high	17.9
4	3DS1		null	
5	*002	*005	bimodal	28.2, 10.9
6	*002	3DS1	high	9.0
7	*002	*004	high	8.2
8	*004	3DS1	null	
9	*002	3DS1	high	9.5
10	*001	*004	high	10.6
11	*002	*004	high	NA
12	*001	3DS1	high	18.2
13	*004	3DS1	null	
14	*001	*002	high	33.3
15	*002	*005	bimodal	4.0, 4.2
16	*001	*002	high	15.5
17	*004	3DS1	null	
18	*004	*005	low	14.9
19	*002		high	24.0
20	*005		low	14.1
21	3DS1		null	
22	*005	3DS1	null	
23	*002	*004	high	7.8
24	*005	3DS1	low	4.1
25	*001	3DS1	low	30.2
26	*005		low	11.4
27	*005	3DS1	low	6.7
28	*005	3DS1	low	NA
29	*002		high	6.3
30	*001	*002	high	20.2
31	*001	*002	high	25.6
32	*004	3DS1	null	
33	*004	3DS1	null	
34	*001	*004	high	17.9
35	*005	3DS1	low	12.2
36	*004	*005	low	9.4
37	*004		null	
38	*001	*002	high	31.7
39	*002		high	18.0
40	*002		high	2.7

(continued)

Donor	Allele 1	Allele 2	DX9 pattern	% DX9 ⁺ NK
41	*002	3DS1	high	14.5
42	*002	3DS1	high	12.5
43	*002		high	13.9
44	*002	*005	bimodal	9.9, 5.8
45	*002	3DS1	high	12.8
46	*004	3DS1	null	
47	*002	*004	high	11.3
48	*001	*002	high	23.7
49	*002		high	7.1
50	*001		high	15.2
51	*005		low	12.8
52	*002		high	15.2
53	*005	3DS1	low	11.7
54	*002		high	29.1
55	*001	*002	high	24.9
56	*004	3DS1	null	
57	*005		low	17.8
58	*002		high	22.1
59	*005	3DS1	low	10.0
60	3DS1		null	
61	*004	3DS1	null	
62	-	-	null	
63	*002		high	11.1
64	*002	3DS1	high	14.9
65	*002	3DS1	high	15.9
66	*002	3DS1	high	14.0
67	*005	3DS1	low	9.5
68	*005		low	35.8
69	*005	3DS1	low	10.1
70	*002	*004	high	15.7
71	*004		null	
72	3DS1		null	
73	*002	3DS1	high	26.8
74	*005	3DS1	low	5.2
75	*004	3DS1	null	

Table 3.2 Summary of donor 3DL1 allele typing and phenotyping

75 donors were typed by PCR-SSP to determine their 3DL1 allele type. Cells were stained with antibodies against CD56, CD3 and DX9; the DX9 staining pattern (null, low, high or bimodal binding) and the percentage of DX9⁺ NK cells was noted.

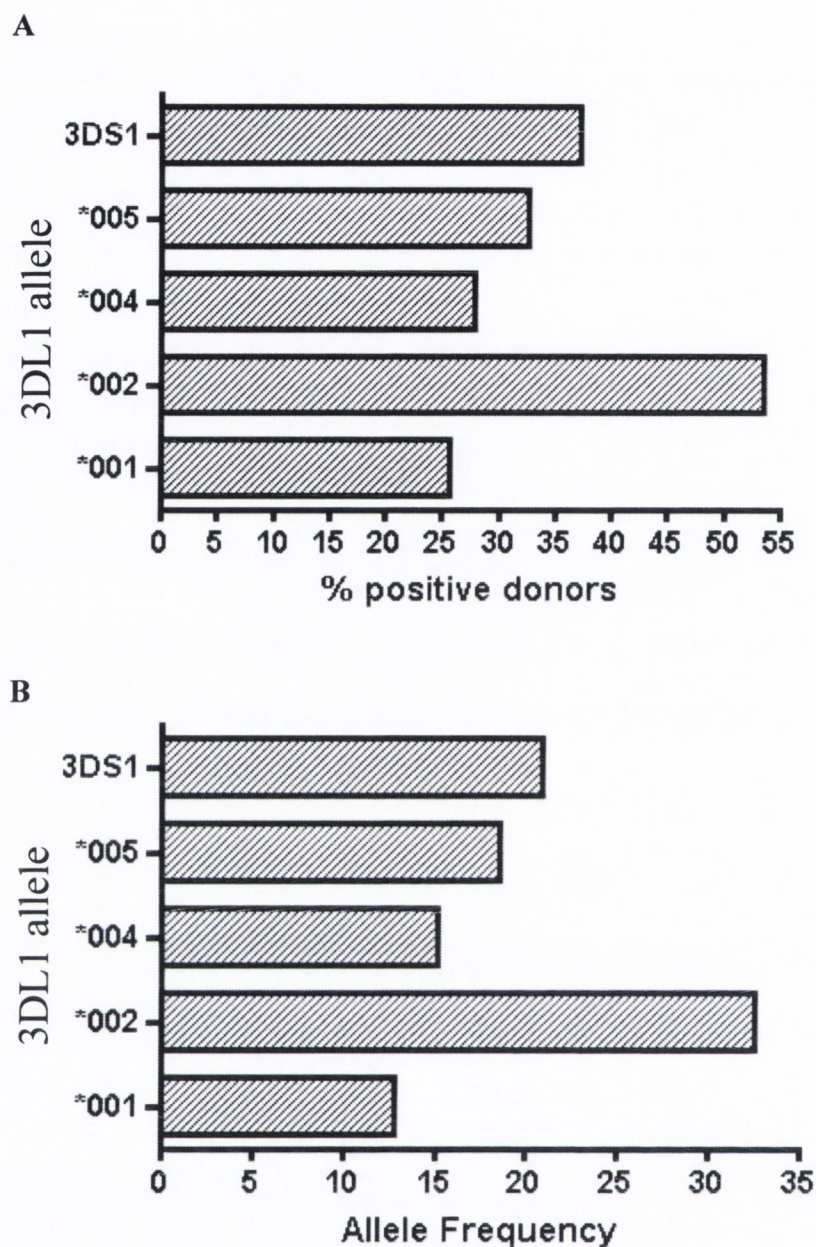


Figure 3.2 3DL1 allele frequencies in normal healthy donors

The percentage of positive donors (A) and the allele frequency (B) was determined for five 3DL1 alleles examined in a panel of 42 randomly chosen donors.

was found in 32.6%. 3DL1*001 and 3DL1*004 were present at comparable levels (25.6% and 27.9% of donors respectively).

3.3.1.3 3DL1 phenotyping

Cell surface expression of 3DL1 was examined in the panel of donors by flow cytometry. Cells were stained for CD56 and CD3 to identify NK cells (CD56⁺CD3⁻) and with the DX9 antibody to identify 3DL1 positive cells. We found four different staining patterns that correlated with the allele typing (Figure 3.3, Table 3.2). No binding was observed for 3DS1 or 3DL1*004; a low intensity peak was seen in the presence of 3DL1*005; 3DL1*001 and 3DL1*002 gave a high intensity peak and a combination of 3DL1*005 and either 3DL1*001 or 3DL1*002 gave a bimodal peak. These results are in line with previous reports of allelic differences in DX9 staining and different intensity of staining reflects varied levels of cell surface expression (Gardiner et al, 2001). The influence of 3DL1 alleles on the frequency of 3DL1⁺ NK cells was examined by analysing the percentage of 3DL1⁺ NK cells in donors where DX9 staining could be attributed to the presence of a single allele (Figure 3.4). Homozygous and heterozygous donors where DX9 staining for each allele could not be distinguished (e.g. *001/*002 heterozygotes) were excluded from the analysis. The percentage of positive NK cells was higher for alleles of 3DL1 that are highly expressed at the cell surface (*001, *002) than for *005 which is present at lower levels at the cell surface.

3.3.2 NK cell expansion using RPMI-8866 cell line

Obtaining an NK cell population of suitable purity and number is a prerequisite for functional experiments. Culturing of PBMCs with the irradiated RPMI-8866 B cell line leads to preferential expansion of the NK cell subset (Perussia et al, 1987). Three expansion experiments were carried out. In the first experiment with donor 1, on day 14, CD56⁺CD3⁻ comprised 51.8% of the lymphocytes, representing a greater than four fold increase in the percentage of NK cells (Figure 3.5); the number of NK cells present had increased

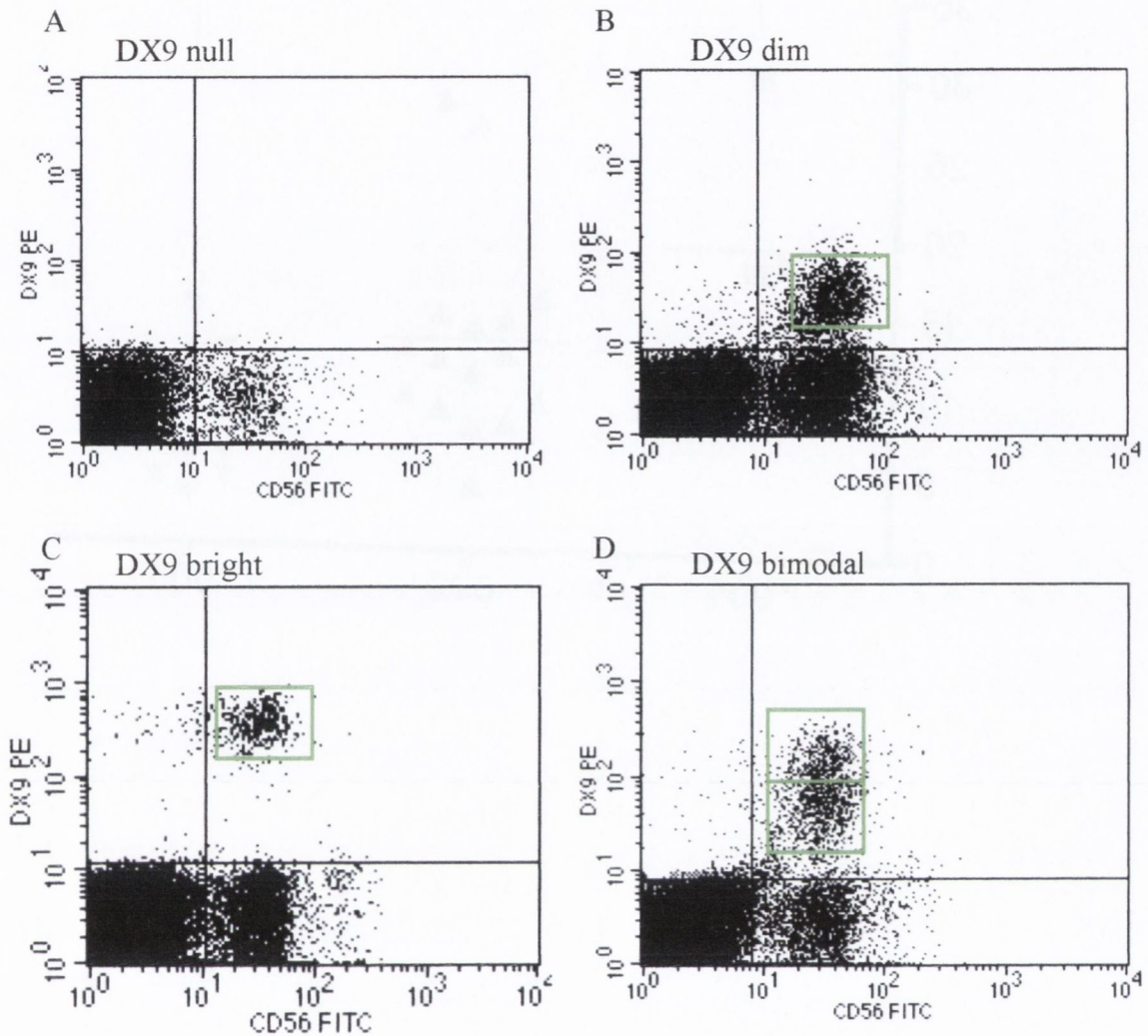


Figure 3.3 Allelic variants of 3DL1 are characterised by different DX9 staining patterns

PBMCs from 3DL1 allele-typed donors were stained with anti CD56-FITC, anti CD3-PerCP and DX9 PE and expression of DX9 on CD56⁺CD3⁻ cells was analysed by flow cytometry. Panel A shows a donor whose cells do not stain with the DX9 antibody. Panels B, C and D represent low, high and bimodal staining patterns.

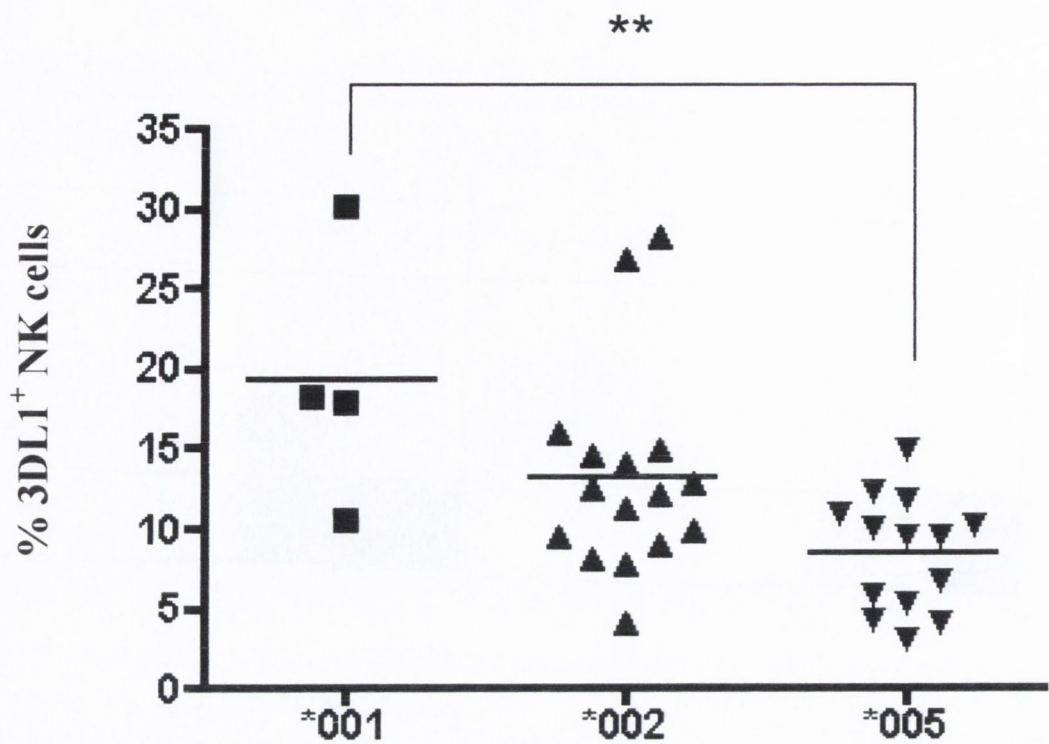


Figure 3.4 3DL1 alleles influence the frequency of expression on NK cells

NK cells from 3DL1 allele typed donors were analysed by flow cytometry, cells were stained with anti CD56, anti CD3 and DX9 to identify 3DL1⁺ NK cells. Homozygous donors and donors where DX9 staining for each allele could not be distinguished were excluded from the analysis. ** p< 0.01.

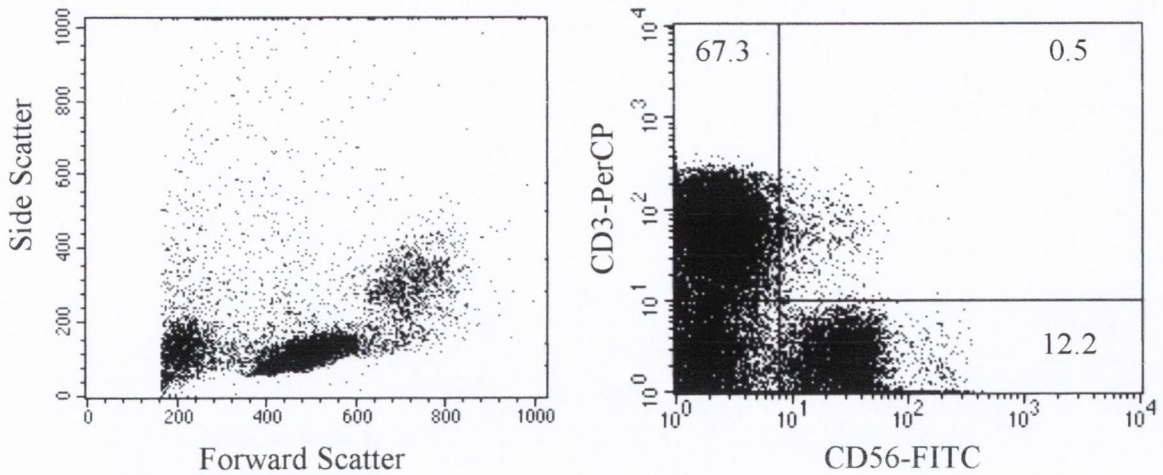
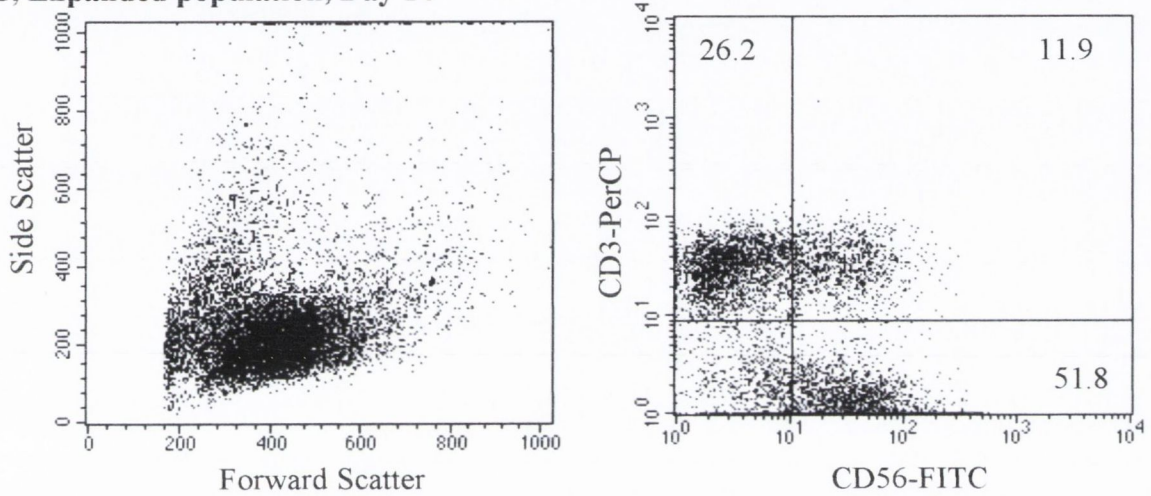
A, PBMC population, Day 0**B, Expanded population, Day 14**

Figure 3.5 Expansion of NK cells from PBMC culture using the RPMI-8866 cell line

Panels on left show the forward scatter/side scatter profile of stained cells. Right panels show dot plots of cells staining for CD56 and CD3, the numbers in each quadrant represent the percentage of lymphocytes. **A**, A typical profile of freshly isolated PBMCs. **B**, Isolated PBMCs were cultured with irradiated RPMI8866 cells in RPMI-1640 for 14 days to expand the NK cell population.

by a factor of 8.5. A second donor showed a similar level of NK cell expansion, but the third donor gave poorer results with a final NK cell population representing 31% of the lymphocyte population, only a 2.5 fold increase over the PBMC population. Due to the high degree of donor variation and the large number of contaminating CD3⁺ cells after expansion, this protocol was not found to be suitable for the generation of NK cell populations for functional experiments.

3.3.3 NK cell isolation

Considering the unsuitability of the expansion protocol, we decided to test various NK cell cloning protocols. These methods are based on single cell cloning, making it impossible to work with PBMCs as a starting population. We therefore started by standardising methods of NK cell isolation.

3.3.3.1 Magnetic bead isolation

Magnetic bead isolation of NK cells from PBMC can be based on either positive or negative selection. Initial experiments used the Miltenyi positive selection kit, which routinely gave purities of 70-80% CD56⁺CD3⁻ cells (Figure 3.6A, n=10). There was considerable donor-to-donor variation in the percentage of NK receptor positive T cells (NKR⁺ T cells, CD56⁺CD3⁺) cells in peripheral blood and these cells are present in the isolated NK cell fraction after positive selection. A second commercial isolation kit from Stem Cell Technologies, based on the same principle of positive selection, was also tested and gave comparable results (n=2). Given the high degree of contaminating cells present after positive selection, the Miltenyi negative selection protocol was tested and this routinely gave a purity of 85-95% CD56⁺CD3⁻ cells (Figure 3.6, n>50). CD3 cells are specifically depleted by this protocol and CD56⁺CD3⁺ cells represented less than 1% of the purified population. Similar results were obtained using a second negative isolation protocol from Stem Cell Technologies (n=2). The negative selection kit from Miltenyi was used in all further experiments.

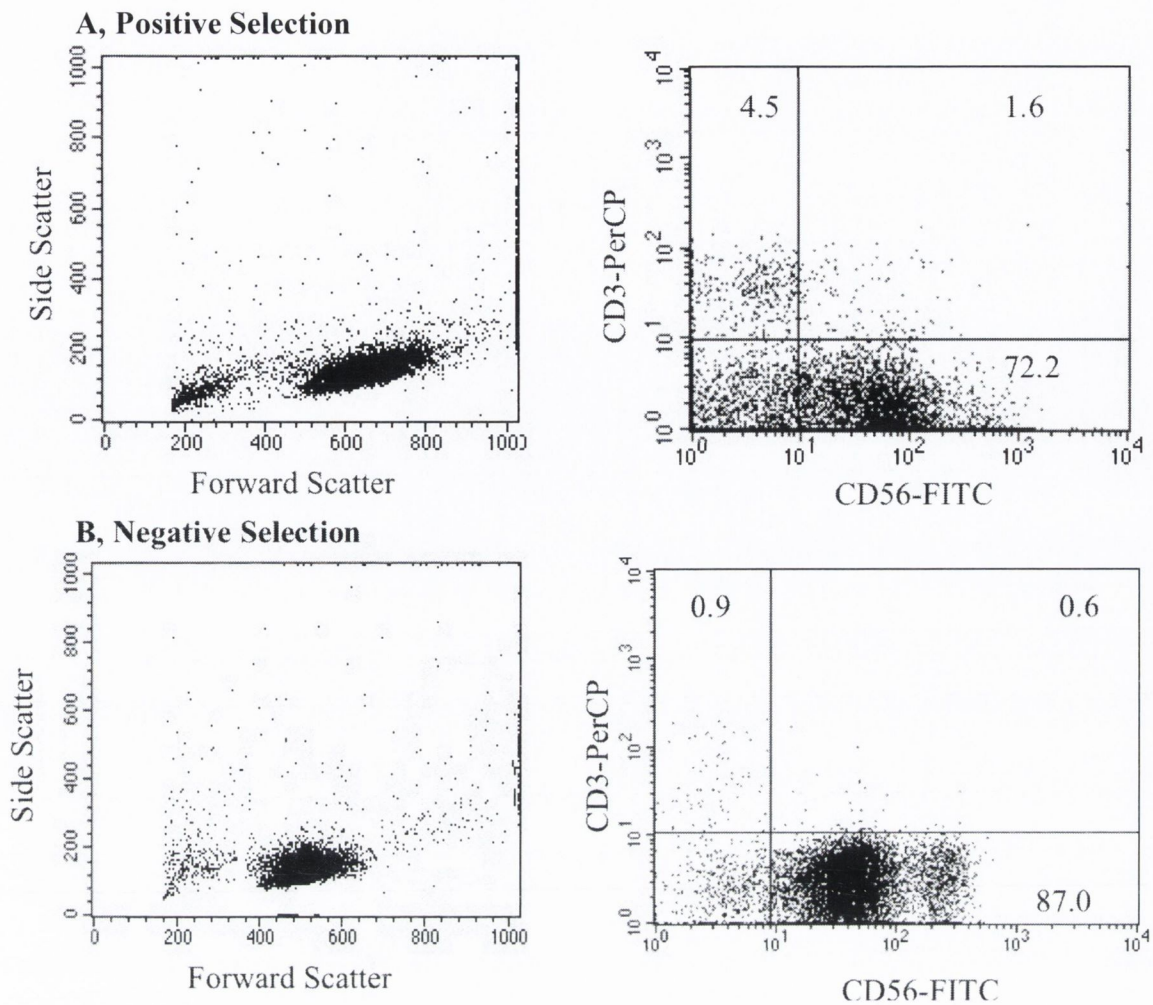


Figure 3.6 Flow cytometric analysis of isolated NK cells

Panels on left show forward scatter/side scatter profile of stained cells. Right panels show dot plots of cells stained for CD56 and CD3. **(A)** NK cells were isolated from freshly isolated PBMCs using the Miltenyi Positive NK cell isolation kit. **(B)** NK cells were isolated from freshly isolated PBMCs using the Miltenyi Negative NK cell isolation kit. Numbers represent percentage of cells in each quadrant. These are representative plots for a minimum of n=10 isolations and n=6 donors.

3.3.3.2 NK cell sorting

To obtain NK cell populations with greater purity than that achievable by magnetic bead isolation, a cell sorter was used. Initial trials used PBMCs as a starting point for NK cell isolation (n=4). Cells were stained with anti-CD56 and anti-CD3 antibodies and the CD56⁺CD3⁻ population was selected for sorting. The purity of the resulting isolated population was greater than 95% CD56⁺CD3⁻, but due to the high starting cell number, the flow rate of the cell sorting was low, leading to very long sorting times. In addition, the high number of negative cells caused a large number of positive cells to be rejected, reducing the yield of sorted cells. Due to these difficulties, bead purified NK cells (85-95% CD56⁺CD3⁻) were used as a starting population to sort NK cells. Sorted NK cell populations obtained in this manner were consistently greater than 99% CD56⁺CD3⁻ (Figure 3.7). Cell yields were typically 80-90% of the theoretical cell yield. Additional modifications to the protocol that increased purity and viability of the cells included filtering the cells before sorting, exclusion of doublet cells, addition of HEPES (10mM) to both the sheath fluid and the collection medium (as a buffering agent), addition of FCS (20%) to the collection medium and the coating of the collection vial with FCS.

Cell sorting also allowed the isolation of specific subsets of the NK cell population. 3DL1⁺ NK cells were initially identified as DX9⁺CD56⁺CD3⁻ lymphocytes and sorted. The purity of the sorted population was typically 92-96% DX9⁺CD56⁺CD3⁻ (Figure 3.8). We found that the greater the separation in fluorescence intensity between the positive and negative populations, the greater the purity of the sorted population. A second anti-3DL1 antibody (clone Z27) was found to give a higher intensity of staining and was subsequently used to identify 3DL1⁺ cells for cell sorting.

3.3.4 NK cell cloning

Three different protocols were tested for NK cell cloning, differing from each other primarily in the composition of the cloning medium. Limiting dilution of bead purified or cell sorted NK cells was used to generate single cell clones in all cases.

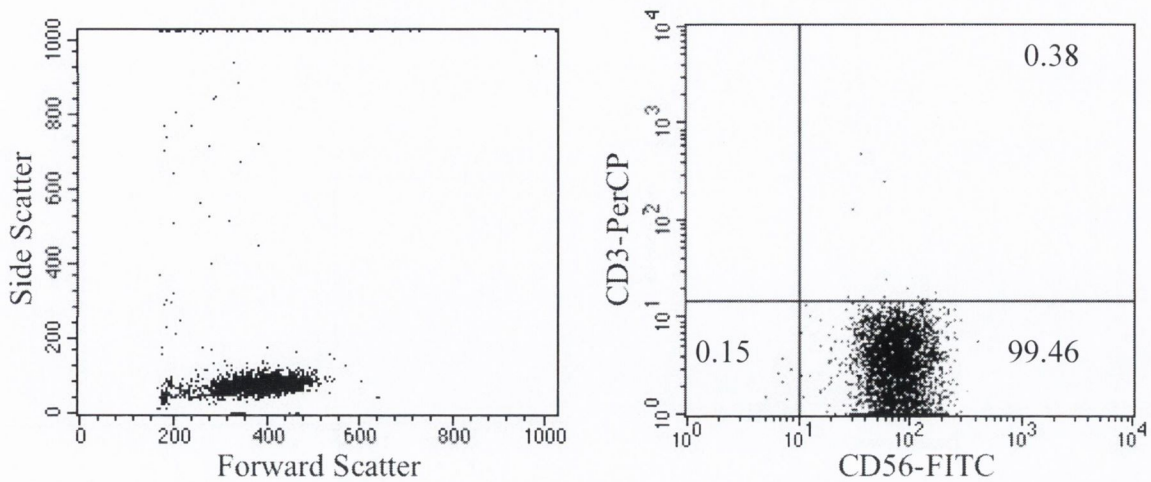


Figure 3.7 Purity of sorted NK cells

Magnetic bead isolated NK cells (typically 85-95% CD56⁺) were further purified by cell sorting on a BD FACS Aria cell sorter. Cells were stained with anti CD56-FITC and anti CD3-PerCP. CD56⁺/CD3⁻ cells account for 99.46% of the purified population with 0.38% CD56⁺/CD3⁺ cells and 0.15% CD56⁻/CD3⁻ cells. Graphs are representative of n=10 cloning experiments.

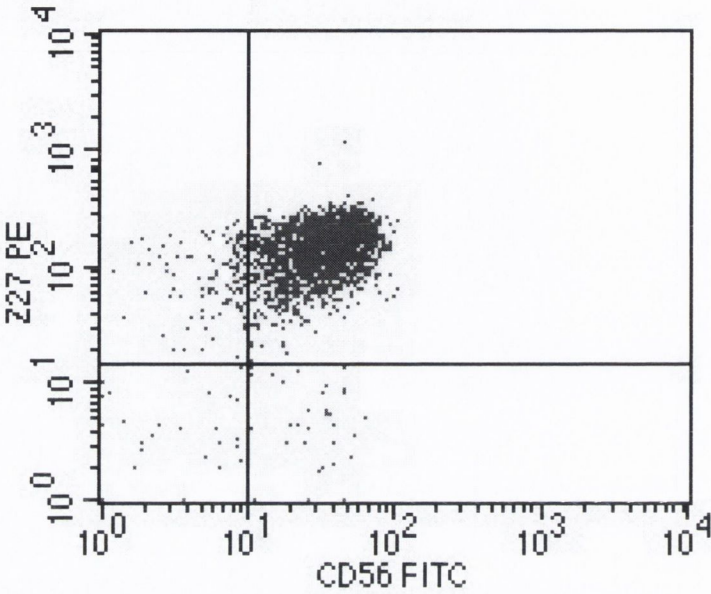


Figure 3.8 Cell sorting resulted in pure populations of 3DL1⁺ NK cells

3DL1⁺ NK cells were isolated from magnetic bead isolated NK cells (typically 85-95% CD56⁺) by cell sorting on a BD FACS Aria cell sorter. Cells were stained with anti CD56-FITC, Z27-PE and anti CD3-PerCP. CD56⁺/Z27⁺/CD3⁻ lymphocytes were selected, sorted and account for 93.05% of the final population.

3.3.4.1 RPMI-8866-based cloning protocol

In this protocol the cloning medium consisted of RPMI-1640 supplemented with irradiated RPMI-8866 cells (see 2.7.2). While this medium was capable of supporting a parallel polyclonal NK cell culture, no clonal cultures showed any growth (n=4 donors).

3.3.4.2 Yssel's medium based protocol

Initial attempts to clone NK cells using this protocol used Yssel's medium prepared by the addition of fatty acids, BSA, ethanolamine, transferrin, insulin, PHA, L-glutamine, FCS, human AB serum and antibiotics to Iscove's modified Dulbecco's medium (see 2.7.1). In later trials this was replaced by a commercially available Yssel's medium, which proved to be more successful in the generation of NK cell clones. When limiting dilution cloning in commercial Yssel's medium gave some clone growth, a number of parameters were modified to optimise clone growth and number. Yssel's medium is supplemented with irradiated feeder cells to encourage NK cell clone growth. These feeder cells consisted of the EBV-transformed B cell line JY and a mixture of three allogenic PBMC preparations. Irradiation of the cells was found to cause substantial immediate cell death (20-30%) which could be minimised (<10%) if cells were irradiated in the absence of phenol-red containing medium and in the presence of 10% FCS.

The influence of the well shape on the initial expansion from the single NK cell was examined using a number of different tissue culture plates. Standard U- bottomed 96 well plates (capacity 200 μ l), V- bottomed 96 well plates (capacity 200 μ l) as well as 60 well high profile tissue typing plates (capacity 40 μ l) and 384 well plates (capacity 120 μ l) were evaluated. 200 purified NK cells were cloned by limiting dilution in Yssel's medium in 20 μ l volume in each culture plate. On day 7 an additional 20 μ l of Yssel's medium, supplemented with 500 IU hrIL-2/ml, was added to each well and on day 14 clone growth was assessed based on pH changes of the culture medium (resulting in a colour change). The most successful clone growth was seen in the 60 well tissue-typing plates, with 9% of wells showing some degree of growth (Figure 3.9). V-bottomed 96 well plates showed the next highest growth level; U-bottomed 96 well and 384 well plates showed poor results, especially for donor 2 (Figure 3.9). Due to the small culture volume in each well,

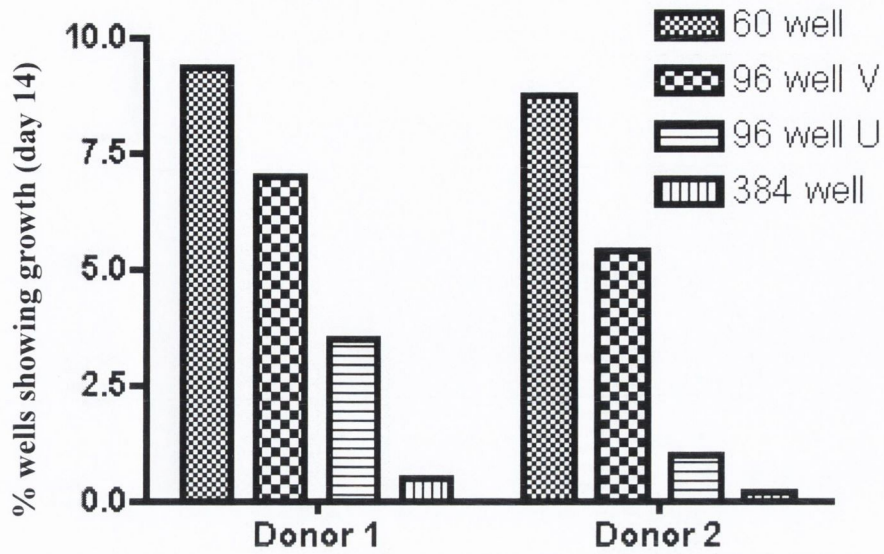


Figure 3.9 Clone growth is influenced by tissue culture well shape

NK cells were cloned by limiting dilution in a volume of 20 μ l in 60 well Terasaki tissue typing plates, 96 well V-shaped, 96 well U-shaped and 384 well tissue culture plates. Clones were fed with Yssel's medium containing IL-2 on day 7 and the growth of clones on day 14 was assessed.

evaporation of medium proved to be a problem. Breathable plate sealers and Parafilm® sealing reduced this problem, while maintaining gaseous exchange. In addition, the outer row of wells along each of the four sides was not used for clone growth and the humidity in the incubator was increased by the addition of sterile water in open containers.

The standard Yssel's medium protocol involves the addition of IL-2 to the clonal cultures on day 7. Addition of IL-2 to the cloning medium from the start of the culture period was found to have no effect on clone growth (data not shown). The effect of IL-15 addition on clone growth was assessed. IL-2 (500 IU/ml), IL-15 (10 ng/ml) or both were added to the cells on day 7 and clone growth was assessed on day 21. There was no observable improvement in clone growth in the presence of IL-15 either alone or in combination with IL-2 (Figure 3.10); less positive wells were seen for donor 2 in the presence of IL-15.

3.3.4.2.1 Characterisation of NK cell clones generated using Yssel's medium

Cloning wells that showed cell growth were expanded and fed on a weekly basis. After approximately 25-27 days a cell sample was taken from positive wells and analysed by flow cytometry. The forward scatter/ side scatter profile of unstained cells was examined and compared to that of a "feeder cells only" control sample (Figure 3.11). Expansion of lymphocyte cells was detected by the presence of a cell population with a characteristic lymphocyte FACS profile that was absent in the control cultures. Cells were then stained with anti-CD56 and anti-CD3 antibodies to identify the presence of NK cell clones (Figure 3.12). Initial cloning experiments using 85-95% pure NK cells (n=5) generated a large number of non-NK cell clones that were identified at this stage. CD3⁺ and CD56⁺CD3⁺ cells appear to be preferentially expanded by this protocol as they represented a disproportionately large fraction of the growing cells. In one experiment with a starting purity of 90% CD56⁺CD3⁻, over 50% of the clones generated were CD3⁺.

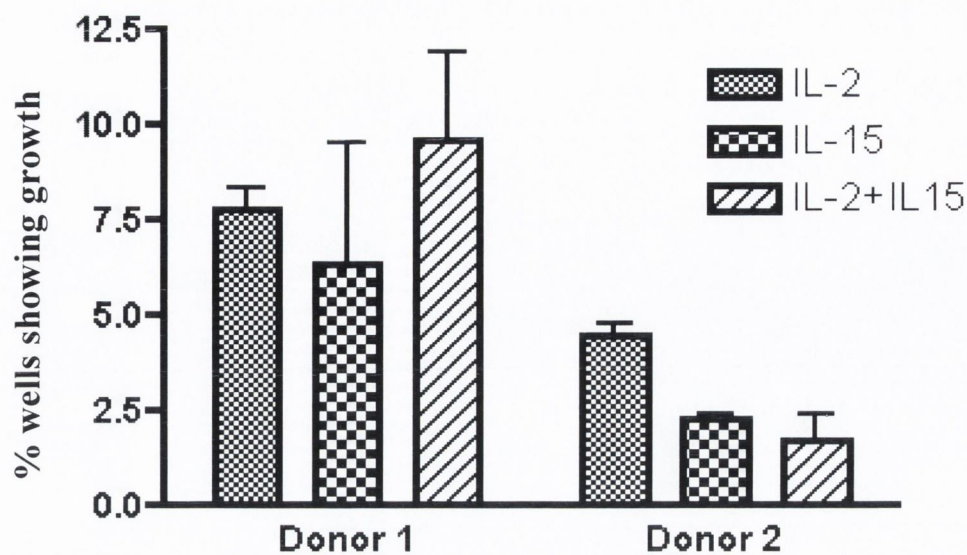


Figure 3.10 Influence of IL-15 in NK cell clone growth

NK cells were cloned by limiting dilution in medium containing feeder cells but without supplementing cytokines. Clones were fed with Yssel’s medium containing IL-2 (500 IU/ml), IL-15 (10 ng/ml) or both on day 7. Growth of clones was assessed on day 21. Error bars represent standard deviation.

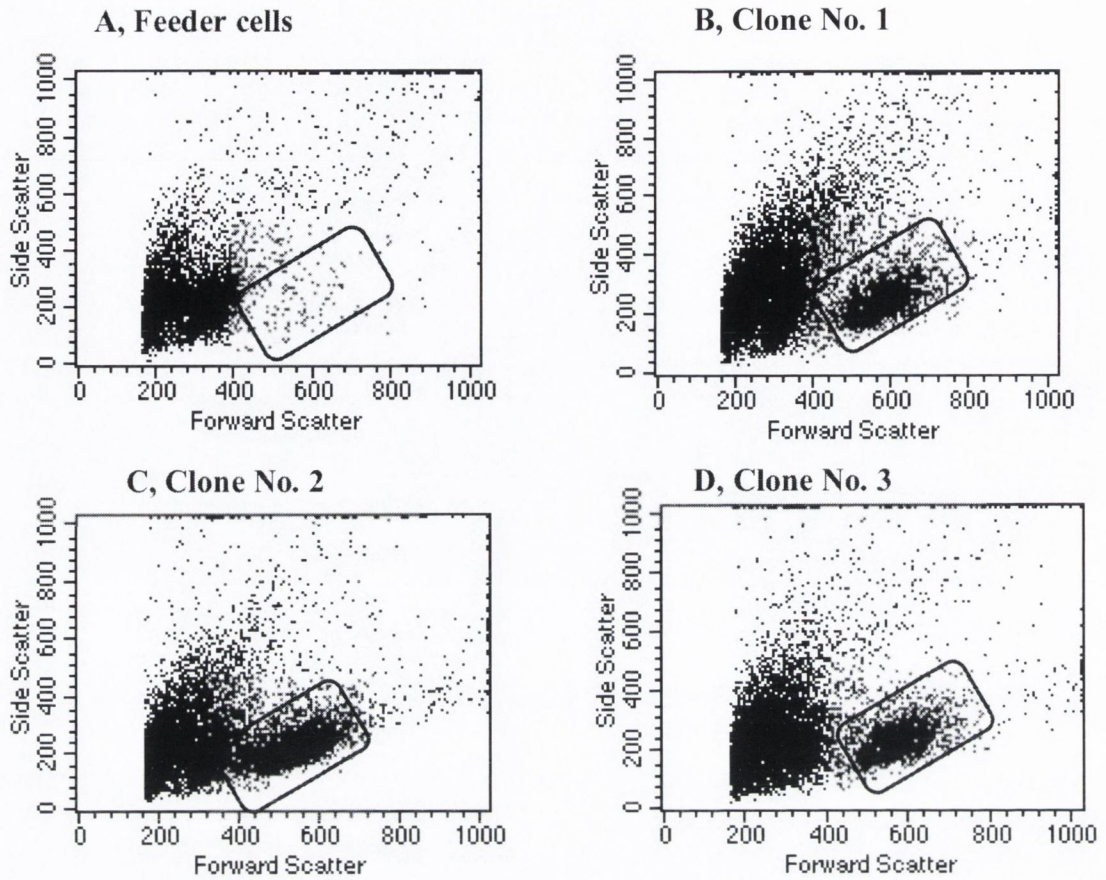


Figure 3.11 Clone growth was identified by the presence of a lymphocyte population

Cells from wells showing initial signs of growth were analysed by flow cytometry. The forward scatter/side scatter profile of unstained cells was examined. Panels B-D show representative plots of positive clone proliferation. Samples from control “feeder cells only” wells were also analysed (panel A).

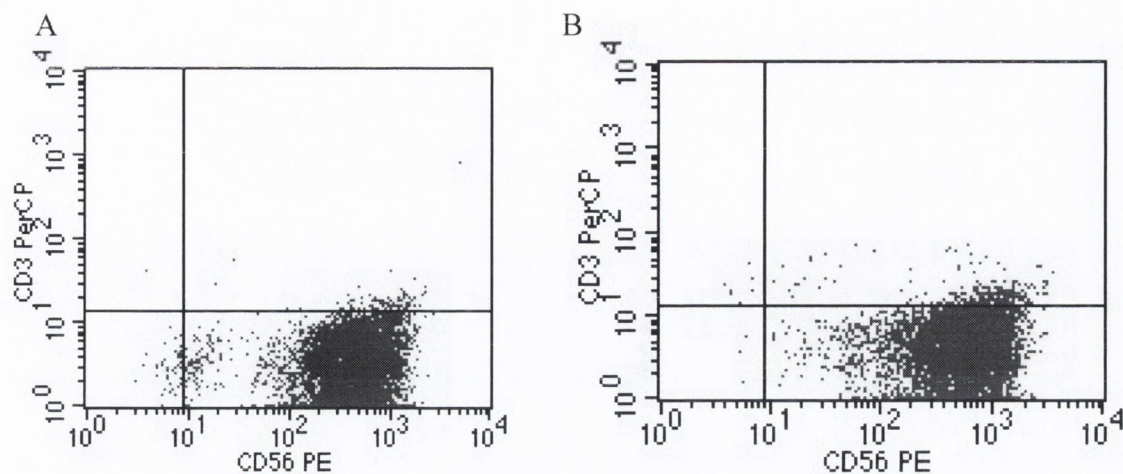


Figure 3.12 Clones exhibited an NK cell phenotype

Cloned cells were stained with anti CD56-PE and anti CD3-PerCP and analysed by flow cytometry. Cells were stained 6 days after the addition of feeder cells and gated on the lymphocyte gate to exclude feeder cells from the analysis. Panels A and B show representative staining of CD56⁺ CD3⁻ clones.

NK clone cytotoxicity

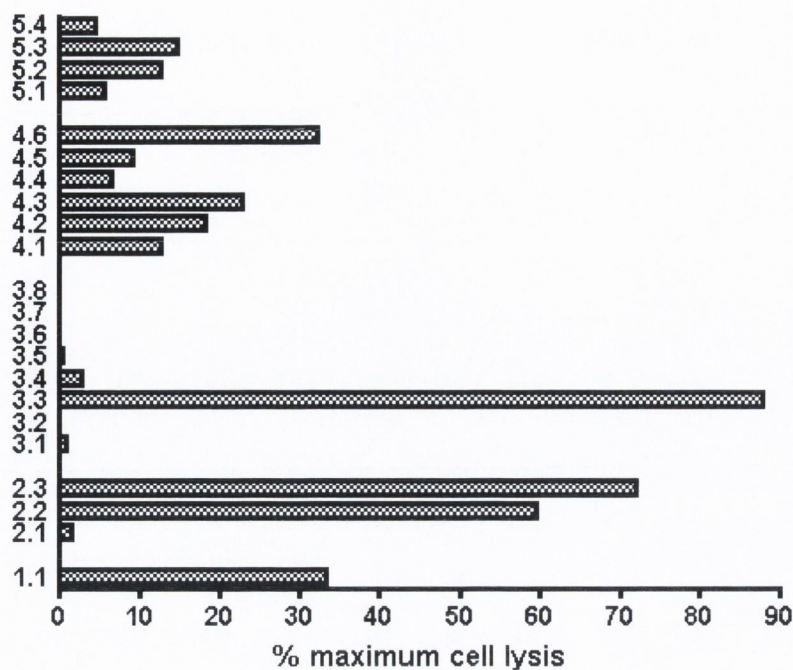
The cytotoxic potential of the NK cell clones was measured in a standard four-hour ⁵¹Cr release assay to assess the suitability of the clones for use in a killing assay. A standard volume (50 µl) was taken from wells containing growing NK cell clones and used as effector cells against the K562 cell line. The cell number was not normalised for this preliminary assay, but on average approximately 5×10^4 NK cells were used, giving an effector to target ratio (E: T) of 5:1. Approximately one half of the NK cell clones showed cytotoxic potential against the K562 cell line (Figure 3.13 A). Some clones showed only low levels of killing that would be unsuitable for use in functional assays, a proportion however lysed the target cells efficiently, with 5 of the 22 clones shown in Figure 3.13 giving 30 – 85 % of maximum cell lysis.

The E: T against the K562 cell line of cytotoxic NK cell clones identified in this assay was then titrated. NK cell clones were counted using ethidium bromide/acridine orange staining, as it proved difficult to quantify the clones using trypan blue staining due to the presence of dead and dying feeder cells. The titration of E: T of two NK clones is shown in Figure 3.13 B. Two NK cell clones were used as effector cells at an effector to target ratio (E: T) of 6:1 and 4:1 and killing of the K562 cell line was expressed as a percentage of maximum cell lysis. Both clones gave high levels of cytotoxicity at an E: T of 6:1 and showed a slight reduction at an E: T of 4:1. A feeder control was included to exclude any killing due to the presence of these cells in the clonal population by taking an equivalent volume of cells from “feeder only” wells. These controls wells showed no cytotoxicity against the K562 cells.

3DL1⁺ NK cell clones

The percentage of 3DL1⁺ NK cells varied between donors (Table 3.2) and those expressing a large 3DL1⁺ NK cell population were preferentially chosen for cloning to maximise the potential for 3DL1⁺ NK cell clones. Initial cloning experiments provided very few if any 3DL1⁺ NK cell clones and FACS analysis of clones generated showed that very few clones expressed any of the KIR examined (3DL1, 2DL1, 2DL2, 2DL3, 2DS1, 2DS2). From a donor expressing 3DL1 on 25% of the peripheral blood NK cells, 19 clones were generated

A



B

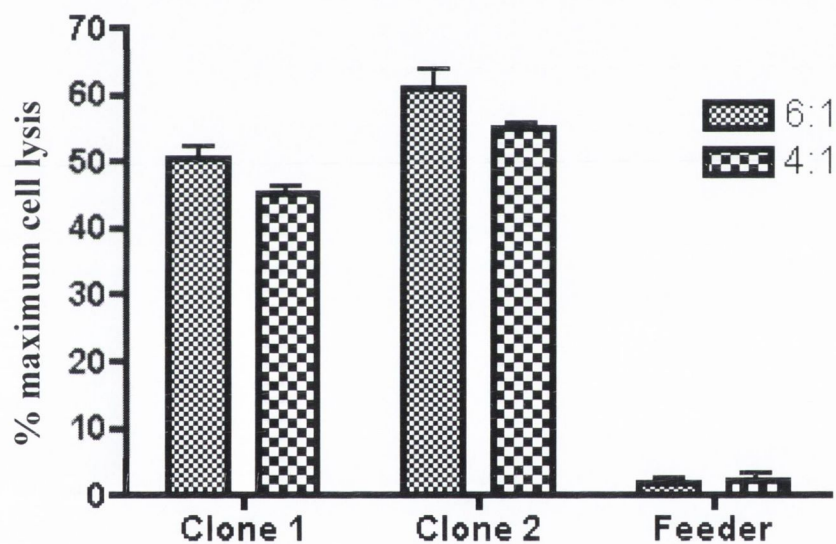


Figure 3.13 NK cell clones show cytotoxicity against the K562 cell line

(A) A selection of clones was used as effector cells against the K562 cell line in a standard 4-hour ⁵¹Cr release cytotoxicity assay. (B) Two NK cell clones were tested at two different effector:target ratios (6:1 and 4:1). A corresponding volume of control feeder cells was used as a negative control. Lysis is expressed as a percentage of maximum cell lysis.

of which all were 3DL1 negative, suggesting that KIR⁻ NK cells were preferentially expanded (Figure 3.14A). For this reason, sorted 3DL1⁺ NK cells were isolated and cloned. This approach proved successful in generating 3DL1⁺ NK cell clones (Figure 3.14). Identification of the allele of 3DL1 expressed on NK cell clones was primarily dependent on the genomic KIR allele typing of the donors in combination with the pattern of DX9 staining intensity. cDNA was also generated from the NK cell clones to confirm expression of 3DL1 alleles by the cDNA typing system. This proved useful in some cases but was hampered by false positives generated by cDNA from feeder cells.

NK cell clone phenotyping

To characterise the expression of a number of relevant receptors NK cell clones were stained with various antibodies and analysed by flow cytometry. Expression of CD94, NKG2A and LILRB1 receptors were examined. Figure 3.15 shows a representative positive staining for each antibody. During the analysis of NK cell clones, occasionally a very broad or double peak was observed (Figure 3.16), suggesting the presence of two or more cell populations. These cells were not considered to be clones and were not used in any further analysis.

3.3.4.3 Stem Cell Growth Medium (SCGM) clones

A third protocol for NK cell cloning (see 2.7.3), using the commercially available stem cell growth medium described by Morris *et al* was also examined (n=8 cloning experiments with 6 different donors). Comparison showed that NK cell cloning efficiency achieved using the SCGM protocol was greater than that found with the Yssel's medium protocol; although there was a high degree of donor-to-donor variation for both (Figure 3.17). Initial growth of the SCGM cultures could be as high as 40% but was typically around 20%. Many of these NK cell clones failed to continue to grow and the number of clones actively growing after 4 weeks was approximately 10%. Yssel's medium cloning efficiencies varied from < 1% to 7%.

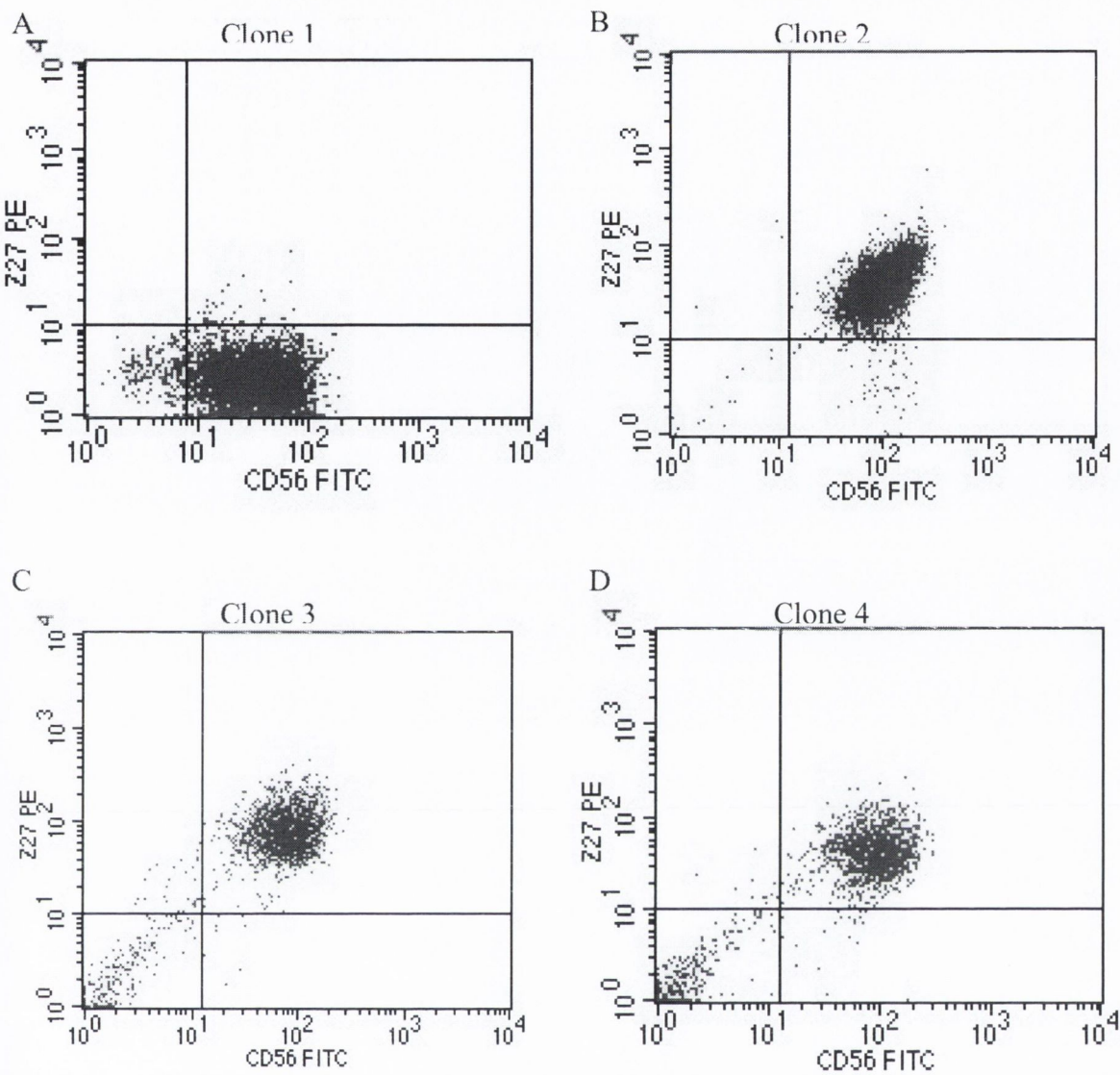


Figure 3.14 3DL1 expression by NK cell clones

Cells were stained with anti CD56-FITC and anti 3DL1 (Z27)-PE and analysed by flow cytometry. Cells were stained 6 days after the addition of feeder cells and gated on the lymphocyte gate to exclude feeder cells from the analysis. Panel A shows a representative DX9 negative clone. Panels B-D show three representative DX9 positive clones.

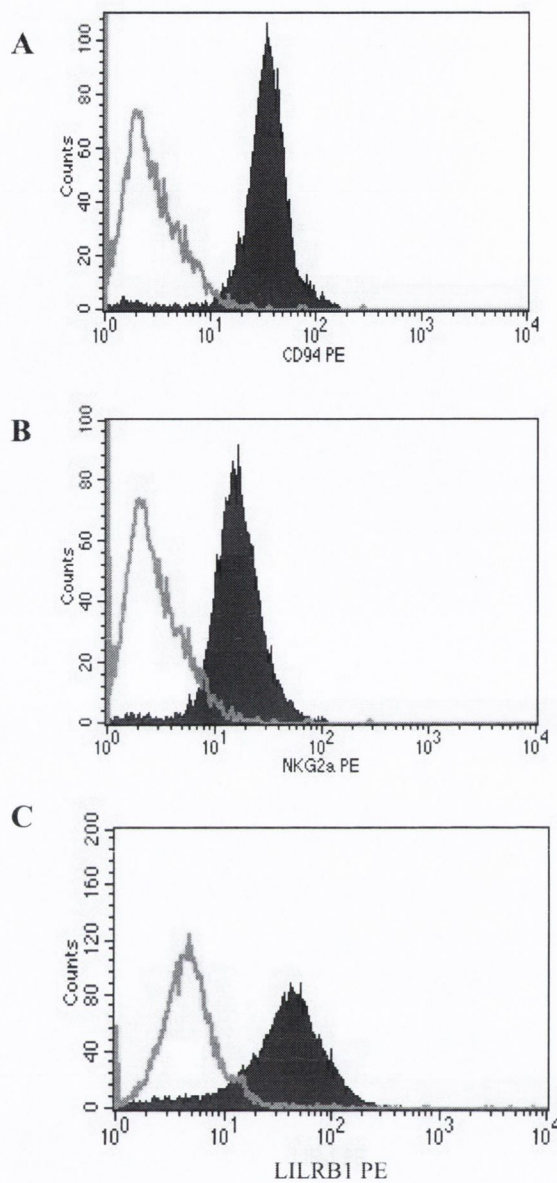


Figure 3.15 NK cell clones express additional inhibitory receptors for HLA

Expression of HLA-specific inhibitory receptors by NK cell clones was examined. Cells were stained with anti CD94-PE(A), anti NKG2a-PE(B) and anti LILRB1-PE(C) and analysed by flow cytometry. Cells were stained 6 days after the addition of feeder cells and gated on the lymphocyte gate to exclude feeder cells from the analysis. A representative histogram from a positive staining is shown for each antibody. Open grey histogram represents staining with control isotype antibody.

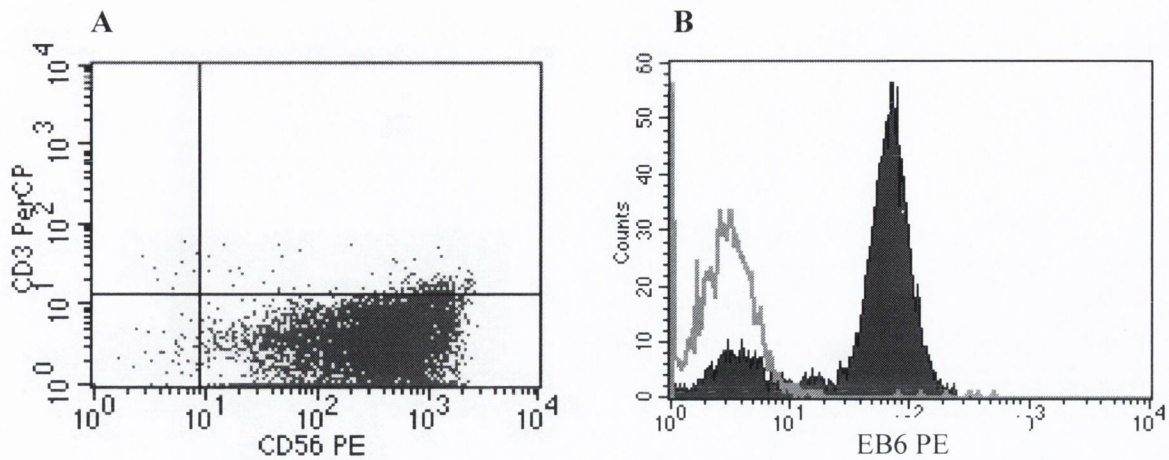


Figure 3.16 Identification of non-clonal NK cell populations

Cells were stained with a panel of antibodies to characterise the cell surface phenotype. Staining was carried out 6 days after the addition of feeder cells and gated on the lymphocyte gate to exclude feeder cells from the analysis. **A**, A non-typical CD56/CD3 dot plot showing a very broad profile. **B**, EB6 staining (anti 2DL1/S1) showing the presence of both an EB6⁺ and an EB6⁻ population.

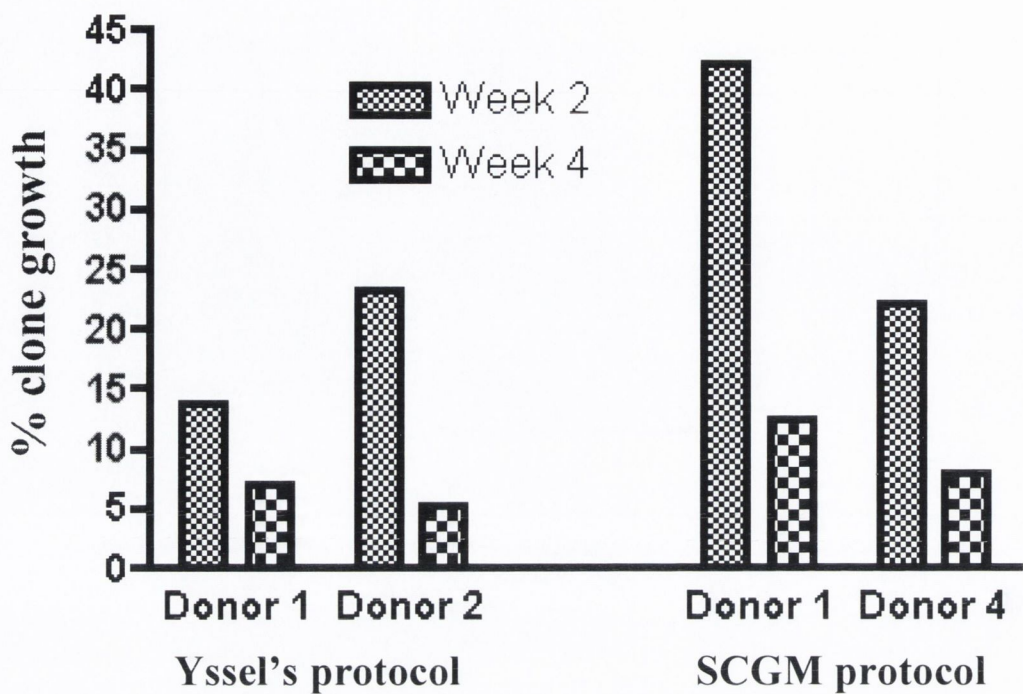


Figure 3.17 A comparison of NK cell cloning efficiencies

NK cells were cloned from donors using either the Yssel's medium or SCGM protocol. The growth of clones was measured on day 14 and again on day 28 and the percentage of clones that had grown was calculated.

The SCGM protocol used only allogenic PBMCs as feeder cells and at half the concentration of those used for the Yssel's medium protocol. As a result of this, NK cell clonal populations derived from this method displayed a much cleaner forward scatter/ side scatter profile by flow cytometry (Figure 3.18). Clones generated by this method also expanded to a greater degree, routinely giving 4×10^6 cells and up to 6×10^6 cells/clone compared to a typical maximum of 4×10^6 cells using the Yssel's protocol.

The cytotoxic potential of SCGM medium clones was assayed as for the Yssel's medium clones. The majority of SCGM clones showed some level of killing of the K562 cell line (31/37 clones in Figure 3.19); of these cells more than half (64%) were suitable for use in cytotoxicity assays, giving greater than 30% of maximum cell lysis. This protocol was then used for all later cloning experiments.

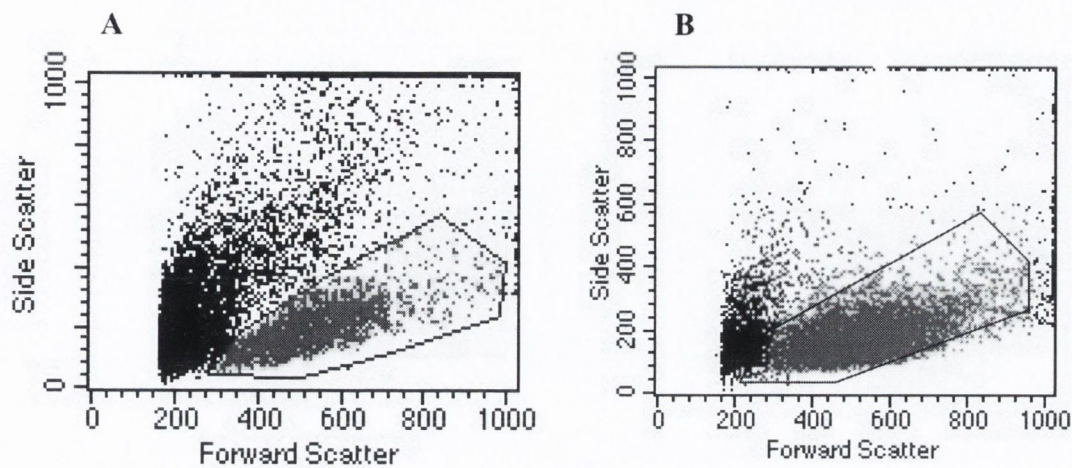


Figure 3.18 StemCell Medium generated clones contain less feeder cells

A comparison of the forward scatter/side scatter profile of Yssel's medium clone (A) and StemCell medium clones (B). Unstained cells were analysed by flow cytometry 6 days after the addition of feeder cells. The same number of cells (10,000) was acquired for each NK cell clone.

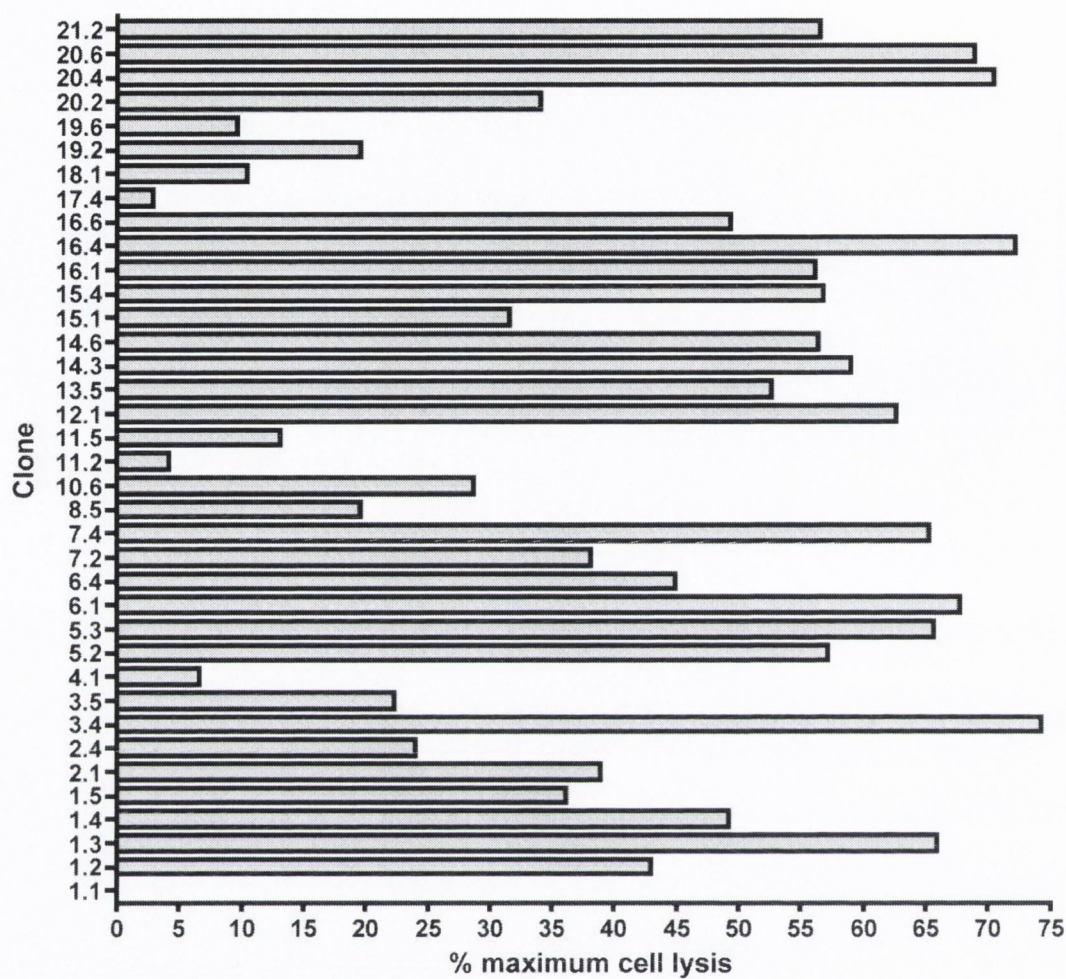


Figure 3.19 A high proportion of StemCell medium generated clones display cytotoxic potential

A panel of Stem Cell Growth medium generated clones was used as effector cells against the K562 cell line in a standard 4-hour ⁵¹Cr release cytotoxicity assay. Lysis is expressed as a percentage of maximum cell lysis.

3.4 Discussion

The aim of the work described in this chapter was to establish the techniques necessary to generate the NK cell populations needed to address the functional role of 3DL1 polymorphism. To this end, it was necessary to characterise the 3DL1 allele type and expression pattern of a panel of donors, as well as to establish an efficient NK cell isolation and cloning protocol for the generation of 3DL1⁺ NK cell clones.

3DL1 allele type was examined using SSP-PCR, giving allele information on a range of individuals. 3DL1 was found in all but one of the 75 donors, in line with previous reports of high prevalence of this gene. 3DS1 was found in 37% of individuals, in close agreement with its reported frequency in the Northern Ireland (NI) cohort (34%), which investigated the 3DL1 allele type of 100 unrelated individuals using SSOP-PCR (Halfpenny et al, 2004). The primers used in our typing system recognise all reported alleles of 3DS1 with the exception of 3DS1*014 (which has only been reported in non-Caucasian populations; Paul Normal, personal communication). The SSOP approach used with the NI cohort was capable of detecting and differentiating between all five described alleles of 3DS1, but only 3DS1*013 was found in this population.

The frequency of 3DL1*004 and *005 positive individuals also closely matched those reported by the NI study, while *001 was less frequent in this study. Over one half of the donors examined here tested positive for the *002 allele, double the frequency seen in the NI cohort. This reflects a lack of specificity in the primers used in this study which recognise, in addition to *002, the less frequent alleles *006, *007, *008, *015, *017, *018 and *020. To further characterise the 3DL1 allele type of donors who tested positive for the *002 allele, gDNA samples were analysed by SSOP-PCR using a system capable of greater discrimination between alleles. The allele type of donors used in functional experiments was confirmed by this method. This work was carried out by Rodat Cunningham of the Northern Ireland Regional Histocompatibility and Immunogenetics Laboratory, City Hospital, Belfast, Northern Ireland.

The allele pattern of DX9 reactivity seen in this study is consistent with that previously described - *001 and *002 are both expressed on the cell surface at a higher level than *005, while *004 is not detected at the cell surface. There are several lines of evidence that suggest that DX9 staining intensity reflects levels of allele cell surface expression rather than allotype affinity for the antibody. The rates of dissociation of DX9 F(ab')₂ and Fab fragment from NK cell clones expressing 3DL1 alleles that stain with different intensity are similar. The divalent F(ab')₂ fragment would be expected to dissociate with slower kinetics than the Fab fragment from highly expressed alleles if there were differences in antibody affinity among 3DL1 alleles (Gardiner et al, 2001). The kinetics and titration of DX9 antibody binding is similar for different alleles, varying only in the final levels of binding (Yawata et al, 2006).

We observed a large range of 3DL1 expression frequency, with the percentage of 3DL1⁺ NK cells varying among donors from 3% to 36%. While the level of cell surface expression is readily explained by allelic variability, the factors determining the frequency of 3DL1 expression on NK cells are less clear. Early studies showed that HLA ligands had no discernable effect on the percentage of 3DL1⁺ NK cells with HLA-Bw4 homozygous donors showing a similar distribution to HLA-Bw4/Bw6 heterozygotes and HLA-Bw6 homozygous donors (Gumperz et al, 1996). Similarly the presence of specific HLA-Bw4 sequence motifs did not correlate with the degree of 3DL1 expression in the NK cell population. While monozygotic twins have similar levels of 3DL1⁺ NK cells, genetic factors controlling expression levels are unclear, with children showing levels intermediate between those of the parents or higher than either parent (Gumperz et al, 1996). A more recent study found an association with 3DL1 allele type and the frequency of NK cells expressing that allele, with alleles associated with high cell surface expression having a high expression frequency within the NK cell population (Yawata et al, 2006). Our results here support that finding, with the percentage of NK cells expressing 3DL1*005 (an allele with low cell surface expression) tending to be lower (average of 5% of NK cells) than the percentage expressing 3DL1*001 (19%) or 3DL1*002 (13%) - both highly expressed at the cell surface.

An efficient method of isolating NK cells from peripheral blood is a prerequisite for the functional analysis of NK cell receptors. Density gradient centrifugation can isolate a PBMC population from blood, consisting of NK cells, T and B cells, and monocytes with some level of contaminating red blood cells, granulocytes and neutrophils. A comparison of the available magnetic isolation protocols for NK cells proved that isolation of highly purified NK cells required negative selection, based on the specific depletion of all unwanted cells types. Positive selection, based on the expression of the CD56 surface antigen, copurified the CD56⁺CD3⁺ population of T cells, which could account for up to 30% of the recovered population in some individuals. An additional advantage of the negative isolation is that the recovered cells have not been bound by antibody, reducing the possibility of stressing or activating these cells. Cells isolated by negative selection have been found to be less activated with respect to expression of the activation marker CD69 relative to those isolated by positive selection (Oren et al, 2005).

An enriched NK cell population can also be obtained by the preferential expansion of NK cells from PBMCs by coculture with the RPMI-8866 cell line (Perussia et al, 1987). We found that the purity of this expanded population is highly variable among donors and can contain up to 40% T cells and as such was unsuitable for generating the NK cell populations desired. Perussia *et al* originally described this protocol for the expansion of NK cells from PBMC population in 1987, involving the culture of PBMCs with an irradiated B lymphoblastoid cell line. The presence of CD4⁺ T cells in the PBMC culture was essential for NK cell proliferation. These cells are believed to produce IL-2 upon interaction with the B cell line and depletion of the CD4⁺ cells or anti-IL-2 antiserum prevents NK cell proliferation. Activated B lymphoblastoid cells are also a source of IL-12, which can increase the proliferation of IL-2 activated NK cells (Robertson et al, 1992).

Our study compared three different methods of generating NK cell clones based on different cloning media – an RMPI-1640-based method, Yssel's method and Stem Cell Growth Medium (SCGM) method as described by Morris *et al* (Cella and Colonna, 2000; Morris et al, 2005; Spits and Yssel, 1996; Yssel et al, 1984). These protocols differ in the composition of the medium and the nature of the feeder cells used to support NK cell

growth. Both Yssel's method and the use of SCGM were found to be vastly superior to the use of RPMI medium with RPMI-8866 B cells and allogeneic PBMCs feeder cells as described by Cella and Colonna. NK cell clones could be generated in sufficient numbers for functional experiments using both Yssel's medium and SCGM protocols. SCGM had a number of advantages over the Yssel's protocol, generating a (marginally) greater number of clones, giving a clear clonal population (fewer feeder cells) and a larger fraction of cytotoxic NK cell clones.

Factors influencing NK cell clonal growth include the composition of the culture medium used, the addition of specific cytokine and the nature of the feeder cells added (a source of both cell contact and soluble factors). IL-2 is the only exogenously added cytokine present in the NK cell clonal culture in each of the protocols examined (after a delay of 7 days for the Yssel's protocol). This cytokine is a powerful inducer of NK cell proliferation (and activation). IL-2 induced expansion of bulk NK cells has been attributed to the expansion of the CD56^{bright} NK cell subset (see below). Other cytokines, themselves incapable of inducing proliferation, may co-stimulate NK cell proliferation in the presence of IL-2. The effect of addition of IL-15 in place of, or in addition to, IL-2 to Yssel's medium cultures was examined. Based on the results obtained, there is no advantage to cloning by the addition of IL-15. IL-15 has been found to induce proliferation of CD56^{bright} NK cells to a similar extent as IL-2, but it does so with a much steeper dose-response curve than IL-2, which can cause significant NK proliferation at concentration that only partially saturate the IL-2R (Carson et al, 1994).

Cytokine-induced NK cell proliferation has been found to result in the preferential expansion of the CD56^{bright} subset of NK cells (Baume et al, 1992; Robertson et al, 1993). The SCGM protocol has also been reported to result in preferential selection of the CD94^{bright} subset of NK cells, which is typical of CD56^{bright} NK cells (Voss et al, 1998). This NK cell subset is characterised by poor natural cytotoxicity and ADCC but greater levels of cytokine production in response to monokine stimulation (Carson et al, 1994; Cooper et al, 2001a; Cooper et al, 2001b; Sedlmayr et al, 1996). The cell surface expression of many receptors, including KIR, LILRB1, CD16 and CD94 also differs across the two

NK cell subsets. KIR expression, with the exception of the atypical 2DL4, is almost exclusively confined to the CD56^{dim} subset of NK cells. In this study, we found a bias towards the generation of 3DL1⁺ NK cell clones which we believe is (at least in part) a reflection of the preferential expansion of the CD56^{bright} (KIR⁻) subset.

The preferential expansion of CD56^{bright} cells may be due to their constitutive expression of the high affinity IL-2 receptor, which allows them to respond to low (picomolar) concentrations of IL-2. However, stimulation with IL-2 induces the expression of the IL-2R α chain on CD56^{dim} cells, but even with the expression of a high affinity IL-2R, these cells do not show significant proliferation (Baume 1992). Expansion of CD56^{bright} NK cells therefore appears to be due to an inherent greater proliferative capacity of this subset. CD56^{bright} NK cells are believed to be less differentiated than the CD56^{dim} subset based on the phenotypical similarities between CD56^{bright} cells and immature NK cells generated *in vitro* and increased telomere length in CD56^{bright} cells relative to CD56^{dim} cells (Mrozek et al, 1996; Ouyang et al, 2007). Thus, CD56^{dim} cells may be more resistant to expansion as they represent a terminally differentiated cell subset. To ensure maximum efficiency when KIR⁺ NK cell clones are desired, isolated NK cells were sorted based on 3DL1 expression prior to cloning.

While cytokine stimulation leads to a preferential expansion of CD56^{bright} subset, CD56^{dim} NK cells can be successfully expanded in clonal cultures, but this requires costimulation of the cells, provided by the presence of feeder cells (Robertson et al, 1993). NK cell cloning protocols involve the addition of irradiated feeder cells that provide both soluble and cell contact mediated support for NK cell proliferation. The strategies used here involve the use of allogenic PBMC from multiple donors and lymphoblastoid B cell line JY or RPMI 8866. Feeder cells are activated both through additions to the medium (PHA in the Yssel's protocol, anti-CD3 antibody in the SCGM protocol) and because of mixed lymphocyte reaction (MLR) caused by the presence of cells from three different donors. A number of cytokines are believed to be produced by the feeder cells, including IL-12 (from activated B cells) (Valiante et al, 1992), IL-1 and TNF- α (both from activated monocytes and lymphocytes). Although these cytokines on their own are not capable of inducing

proliferation, they cause an increase in the IL-2 induced proliferation of CD56^{dim} NK cells (Naume et al, 1991; Robertson et al, 1996).

The SCGM protocol proved to be more effective at generating NK cell clones than Yssel's medium protocol. This is despite a much lower amount of feeder cells (half the number of allogenic PBMCs and no addition of the JY B cell line) in this protocol. In place of the polyclonal activating lectin PHA used in the Yssel's protocol, the feeder PBMCs here are stimulated by the use of the anti-CD3 antibody OKT3. The medium is a commercially available medium that has been formulated for the expansion of human stem cells. The exact composition of this medium is not available, but the manufacturer's information states that it contains only human derived or recombinant human proteins and does not contain any growth factors. From the results obtained here, it appears that the SCGM contents can substitute for the factors provided by larger numbers of feeder cells in the Yssel's protocol, and that these unidentified factors provide a better source of stimulation for NK cell proliferation.

In addition to the production of soluble mediators, the presence of feeder cells in NK cell clonal cultures also provide direct cell-cell contact that may influence NK cell proliferation. Our results here support the important role played by cell-cell contact in supporting NK cell proliferation, as we see a profound effect of cell shape on NK clone growth. Well shape that encourages intimate cell contact produced more cell growth in the initial stage of culture. Culturing of NK cells in the presence of some susceptible target cell lines, activated T cells or PBMCs but not NK-resistant cell lines has been shown to increase cytokine-induced NK cell proliferation in a cell contact dependent manner (Pierson et al, 1996; Robertson et al, 1996). The exact nature of the signal is not known although it does not appear to be mediated by any of the common T cell costimulatory receptors including CD2, CD27, CD28, CD29 or LFA-1 (Robertson et al, 1996).

To optimise the generation of NK cell clones the cell culture medium used contains a number of additions to the basal medium used. Yssel's medium (based on Iscove's modified Dulbecco's medium (IMDM)) is enriched with a number of factors including

insulin, transferrin, bovine serum albumin (BSA) and fatty acids, as well as FCS and human serum. Many of these growth-promoting factors have been described as additions to basal medium for use in serum-free conditions (Barnes and Sato, 1980). Transferrin is the major iron transport protein, and is important for cellular proliferation, due to the increased requirement for iron during DNA synthesis. The receptor for transferrin is up-regulated on activated NK cells (Seligman et al, 1992) and it has been reported that transferrin increases NK cell cytotoxicity (Shau et al, 1986). Fatty acids and insulin also act as promoters of cellular proliferation by interactions with the thyroid hormone receptors (Jump and Clarke, 1999) and the induction of DNA synthesis (Cheatham and Kahn, 1995) respectively. BSA acts as binding protein for the free fatty acids reducing the toxicity of free lipid in solution (Guilbert and Iscove, 1976).

Conclusion

The 3DL1 allelic genotype and expression profile of over 70 donors was successfully established during this study. This enabled the selection of suitable blood donors for the generation of NK cell clones. An optimal protocol involving the isolation of highly purified NK cells, cell sorting of NK cell subsets and cloning using both the Yssel's and SCGM protocols allowed for the generation of 3DL1⁺ NK cell clones.

Chapter Four

3DL1 polymorphism is functional

4.1 Introduction

The ligand for 3DL1 has been identified as the Bw4 epitope expressed by a subgroup of HLA-B molecules. Variation within the sequence of Bw4 allotypes, the peptide presented by the HLA-Bw4 molecule and polymorphism of 3DL1 all have the potential to modulate the interaction between 3DL1⁺ NK cells and HLA expressed on target cells. These differences may in turn influence the NK cell response.

4.1.1 Variation within HLA-Bw4

HLA-B allotypes can be divided into two subclasses – Bw4 and Bw6 - depending on the amino acid sequence at position 77-83 of the HLA α 1 domain (Barnstable et al, 1979, Figure 4.1.1). 3DL1 recognises HLA molecules that contain the Bw4 public epitope (Gumperz et al, 1995). Bw6 is present on about two thirds of known HLA-B allotypes, while the Bw4 motif is found on the remaining third. Some HLA-A allotypes, including A*23, A*24, A*25 and A*32 members also contain the Bw4 epitope. HLA-Bw6 allotypes are relatively homogenous at the Bw6 epitope, while there is a greater degree of variability within Bw4 allotypes, with at least five different sequence motifs at position 77-83 (Muller et al, 1989). A sequence alignment of the Bw4/Bw6 epitope of HLA allotypes is shown in Table 4.1.1.

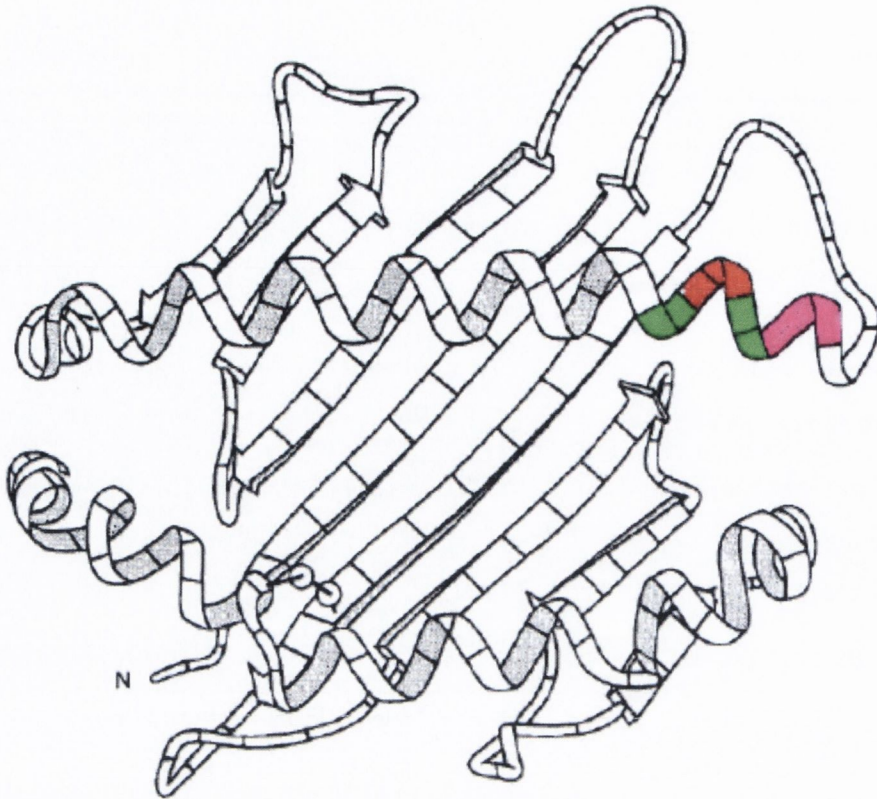


Figure 4.1.1 HLA-B allotypes possess a Bw4 or Bw6 epitope at position 77-88

HLA-B allotypes are characterised as Bw4 or Bw6 based on the amino acid sequence at position 77-88 (highlighted). Residues conserved across all class I allotypes are in red; purple residues are common to all Bw4 allotypes, while the amino acid positions in green vary. Diagram adapted from (Parham, 1992).

	Class I position							Allotype
	77	78	79	80	81	82	83	
HLA-Bw6	Ser	Leu	Arg	Asn	Leu	Arg	Gly	B*0702
	Gly	Leu	Arg	Asn	Leu	Arg	Gly	
HLA-Bw4								B*1513,
								B*3801,
								B*5101,
								B*5801
	Asn	Leu	Arg	Ile	Ala	Leu	Arg	
	Ser	Leu	Arg	Thr	Leu	Leu	Arg	
	Asp	Leu	Arg	Thr	Leu	Leu	Arg	B*2705
	Ser	Leu	Arg	Ile	Ala	Leu	Arg	
	Asn	Leu	Arg	Thr	Ala	Leu	Arg	

Table 4.1.1 Amino acid sequence at the Bw4/Bw6 motif

The amino acid sequence motifs at position 77-88 found in Bw4 and Bw6 HLA-B allotypes are shown. The sequence present at this position in the HLA-B allotypes used in this study is indicated.

Identification of the Bw4 epitope as the ligand for 3DL1 was made after comparison of the recognition of two HLA-B allotypes, B*1502 (Bw6) and B*1513 (Bw4). These allotypes differ only at residues 77-83 which define the Bw4 epitope (Gumperz et al, 1995). Expression of B*1513, but not B*1502, on target cells inhibited killing of these cells by 3DL1⁺ NK cells. Residues outside the Bw4 epitope were also implicated in recognition by 3DL1 as HLA-A allotypes with the Bw4 epitope but which differ substantially in other areas of the protein were not recognized by 3DL1. Within the Bw4 motif, 2 residues (78 and 79) are conserved across HLA-A, -B and -C allotype; residues 82 (leucine) and 83 (arginine) are present in all Bw4 allotypes and positions 77, 80 and 81 are polymorphic among Bw4 allotypes. Due to the high level of conservation, residues 78 and 79 are unlikely to play a role in 3DL1 recognition of Bw4 proteins. Mutation of Leu82 and Arg83 in B*1513 showed that these residues are involved in the interaction, but are not essential, implicating one or more of the three polymorphic Bw4 residues in the interaction between 3DL1 and HLA-Bw4 (Gumperz et al, 1997). The role played by these variable residues in the interaction between 3DL1 and HLA-Bw4 suggests that Bw4 allotypes with different amino acids at these positions may act as stronger or weaker ligands for 3DL1 and influence the level of inhibition generated.

4.1.2 The role of peptide

Polymorphism of HLA molecules affects their peptide specificity and thus the profile of presented peptides. Comparison of two Bw4⁺ HLA-B allotypes (B*1513, B*1516) that serve as ligands for 3DL1 revealed that they have distinct and non-overlapping peptide requirements (Barber et al, 1997). In contrast, a closely related Bw6 allotype (B*1502), has a similar peptide binding motif to B*1513 but does not act as a ligand for 3DL1 (Gumperz et al, 1997). Although this suggested that there is no correlation between the profile of presented peptides and recognition by 3DL1, individual peptides bound by HLA can influence 3DL1 recognition. Many of the studies of the role of peptide have been done using the B*2705 protein (Malnati et al, 1995; Peruzzi et al, 1996a; Peruzzi et al, 1996b). TAP (transporter associated with antigen presentation) deficient cells expressing B*2705 were exogenously loaded with a range of peptides and used as target cells for 3DL1⁺ NK

cell clones. While certain peptides did prevent recognition by 3DL1 this was not a truly peptide-specific recognition as 3DL1 could recognize that same HLA molecule presenting very different peptides (Malnati et al, 1995). Single amino acid substitutions in both a protective and a non-protective peptide presented by HLA-B*2705 revealed the importance of residues 7 and 8 for recognition by 3DL1 (Peruzzi et al, 1996a). Based on inferences made from the crystal structure of 2D KIR in association with their ligand, these peptide residues are predicted to interact with 3DL1.

4.1.3 3DL1 polymorphism

At a genetic level, 3DL1 shows extensive polymorphism with more than 40 alleles described to date. This genetic polymorphism is believed to give rise to functionally distinct proteins – this hypothesis is supported by several lines of evidence including the pattern of polymorphic amino acid residues, the expression profile of different alleles and anecdotal evidence from early functional work on ligand specificity.

Analysis of the amino acid variation among 3DL1 allotypes reveals that the majority of the polymorphic residues occur in parts of the protein that are predicted to be involved in loops of the Ig-like domains, rather than the β -pleated sheet structures. Based on our knowledge of HLA/KIR interaction these sites are more likely to come in contact with the HLA-Bw4 ligand and thus variation here may lead to alterations in the interaction. Amino acid substitutions distal from the direct contact site may also play a role due to their influence on protein conformation or ligand affinity. The finding that different 3DL1 allotypes give distinct patterns of staining with the monoclonal antibody DX9 also supports the hypothesis that the polymorphism of the 3DL1 gene is functional (Gardiner et al, 2001). Allelic polymorphism influences the level of cell surface expression and thus the intensity of antibody staining (see Chapter 3). In one example, the common allotype *004, is not present at the cell surface but is retained intracellularly (Pando et al, 2003). This difference in cellular localisation has been mapped to a unique substitution in the D0 domain of *004 and an additional substitution in the D1 domain. This substitution in the D1 domain (serine in *004) is also present in the *005 where it is believed to contribute to the low cell surface expression of this allotype.

Initial functional analysis of 3DL1 was done before the polymorphism present at the 3DL1 gene was fully appreciated and as such, some of the initial experiments were done using different allotypes (Gumperez et al, 1995; Cella et al, 1994; Luque et al, 1996). DX9 or Z27 antibodies, which recognise a range of 3DL1 allotypes, were used to identify 3DL1⁺ cells. Much of the initial work characterising 3DL1 was carried out by Gumperz *et al* working on what was later determined to be the *002 allotype of 3DL1. Data generated using this allotype provided evidence that the Bw4 epitope, irrespective of the amino acid sequence present, is the ligand for 3DL1 (Gumperz et al, 1995). Independently, Cella *et al* found Bw4 allotypes with isoleucine at this position (Ile80) were associated with strong inhibition while threonine (Thr80) was associated with weaker levels (Cella et al, 1994). In contrast, Luque *et al* found that four B*27 allotypes with a Thr80, but not the B*2702 allotype which contains isoleucine at position 80, provided protection from NK cell lysis (Luque et al, 1996). Site-directed mutagenesis of Thr80 to Ile80 in B*2705 lead to the abrogation of its protective effect. The conflicting reports of 3DL1 specificity may be attributable to the polymorphism of 3DL1 and the differences seen may reflect the use of alternative forms of 3DL1 that have different ligand specificities. Functional polymorphism of 3DL1 is also supported by the report of heterogeneity between B*2705-specific clones in the recognition of three B*2705 mutants containing single amino acids changes in the peptide binding groove. Within a panel of five clones generated from two different donors the pattern of protection from lysis differed (Malnati et al, 1995).

In the present chapter, we address the impact of both 3DL1 and HLA-B polymorphism on their mutual interaction. We used the common 3DL1 allotypes *001, *002 and *005 and a panel of HLA-B molecules (including B*1513, B*2705, B*3801, B*5101 and B*5801). These HLA-B allotypes include members of a number of HLA-B families, including a number that have been implicated in the progression of certain disease conditions.

4.2 Aims

The aims of this chapter were

- To characterise the influence of variation within Bw4⁺ allotypes on recognition by 3DL1
- To assess the role of allelic polymorphism of 3DL1 on inhibition by HLA-Bw4 allotypes

4.3 Results

The interaction between HLA-B and 3DL1 allotypes was examined using a panel of Jurkat cell lines stably transfected with chimeric constructs bearing the extracellular domain of 3DL1 fused to the signalling CD3 zeta chain (see Figure 4.1, generated by Clair Gardiner). These cell lines were stimulated with a panel of 721.221 single HLA transfectants. 721.221 cells are an HLA class I negative cell line; transfection of this cell line with a plasmid encoding a single HLA allele has resulted in the generation of a series of daughter cell lines that express a single, defined HLA allotype.

4.3.1 Transfection of the Jurkat T cell line

Detection of KIR/HLA interaction was dependent on transient transfection of the Jurkat cell lines with an NFAT-sensitive reporter plasmid. Reports in the literature have used electroporation to introduce equivalent reporter plasmids to Jurkat cells (Khakoo et al, 2002). However due to advantages such as reduced cytotoxicity to the cells and the smaller amount of plasmid DNA required, we investigated if transient transfection of Jurkat cells was possible using commercially available polyamine based transfection reagents (GeneJuice and Lipofectamine 2000). For convenience in measuring transfection efficiency a GFP plasmid was used during standardisation, allowing quantification of transfection by flow cytometry. Various conditions for transfection including amount of DNA, cell density and amount of transfection reagent were tested according to the manufacturer's instructions (see Table 4.1). Cells were analysed for GFP expression by flow cytometry 24 and 48 hours after transfection. No transfection of the Jurkat cell line was seen with any of the conditions used (Figure 4.2).

Transfection of the Jurkat cell line was then attempted using electroporation. Two protocols were used; the first using a 4 mm gap cuvette with a single pulse at 250 V and 950 μ F and the second using a 2 mm gap cuvette with two pulses, each at 240 V and 100 μ F, 30 seconds apart. Both transfection protocols were successful at introducing the GFP plasmid into Jurkat cells (Figure 4.3). A greater level of transfection (10% versus 6.7%)

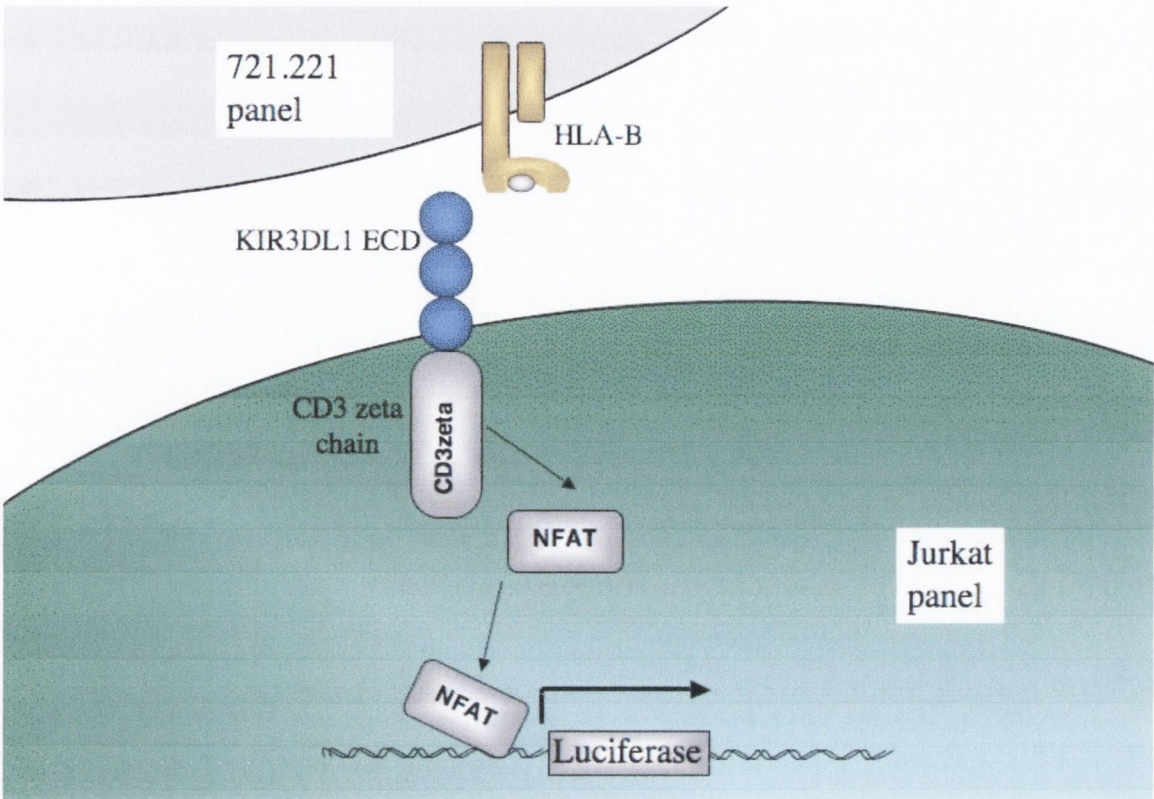


Figure 4.1 3DL1/HLA-Bw4 interaction was measured using a system of chimeric receptors

Jurkat cell lines stably expressing a chimeric construct consisting of the extracellular domain (ECD) of a 3DL1 allotype (shown in blue) and the signalling CD3 zeta chain were used to investigate the influence of allelic variation in the interaction between 3DL1 and HLA-B. These cells were stimulated using a panel of 721.221 cell lines that express a single HLA-B allotype at the cell surface. Interaction between HLA-B and the ECD of 3DL1 lead to signalling through the CD3 zeta chain and consequent NFAT activation. The level of 3DL1/Bw4 interaction was detected using an NFAT sensitive promoter plasmid in the Jurkat cell line.

DNA concentration	Cell density	GeneJuice/DNA ratio
1µg/ml	1 x 10 ⁶ /ml	6µl/µg DNA
1µg/ml	1 x 10 ⁶ /ml	3µl/µg DNA
1µg/ml	1 x 10 ⁶ /ml	2µl/µg DNA
1µg/ml	1 x 10 ⁵ /ml	6µl/µg DNA
1µg/ml	1 x 10 ⁵ /ml	3µl/µg DNA
1µg/ml	1 x 10 ⁵ /ml	2µl/µg DNA

Table 4.1 Conditions for GeneJuice® Transfection

A GFP plasmid was used to establish conditions for the transfection of the Jurkat cell line. A GeneJuice®/Serum-free medium mixture was added to the plasmid DNA in a 96-well V-bottomed plate and incubated for 10 minutes. This mixture was then added to the cells, which were analysed for GFP expression 24 and 48 hours later. Cell concentration and GeneJuice ® /DNA ratio was varied as outlined as above.

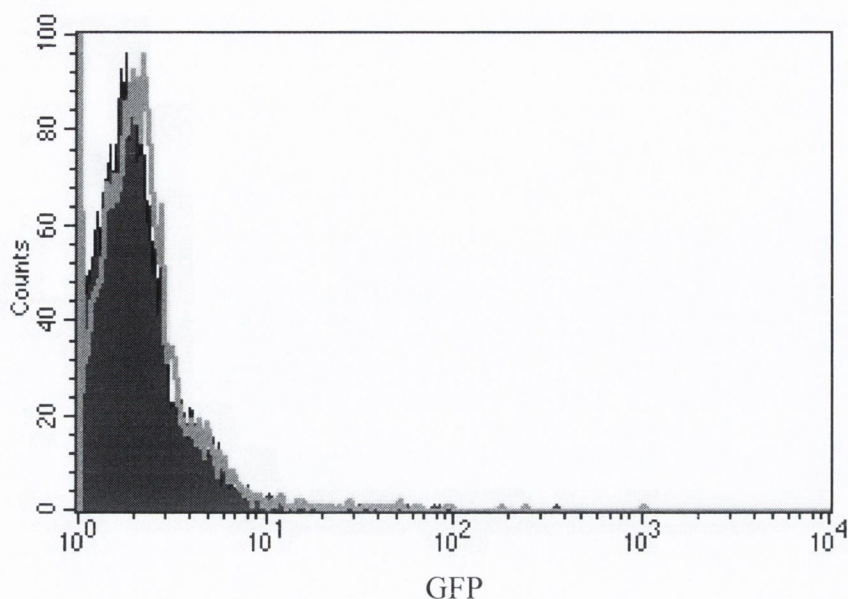


Figure 4.2 The polyamine based GeneJuice® transfection reagent does not achieve transfection of the Jurkat cell line.

GeneJuice® reagent was used to transfect Jurkat cells with a GFP plasmid. Cells were treated according to the manufacturer's instructions (see Table 4.1) and GFP expression was monitored by flow cytometry. A representative histogram is shown based on transfection using 1×10^5 cells/ml; 1 μ g/ml DNA and 6 μ l GeneJuice/ μ g DNA, 48 hours after transfection. The filled histogram represents cells transfected with the GFP plasmid, the open histogram represents mock-transfected controls.

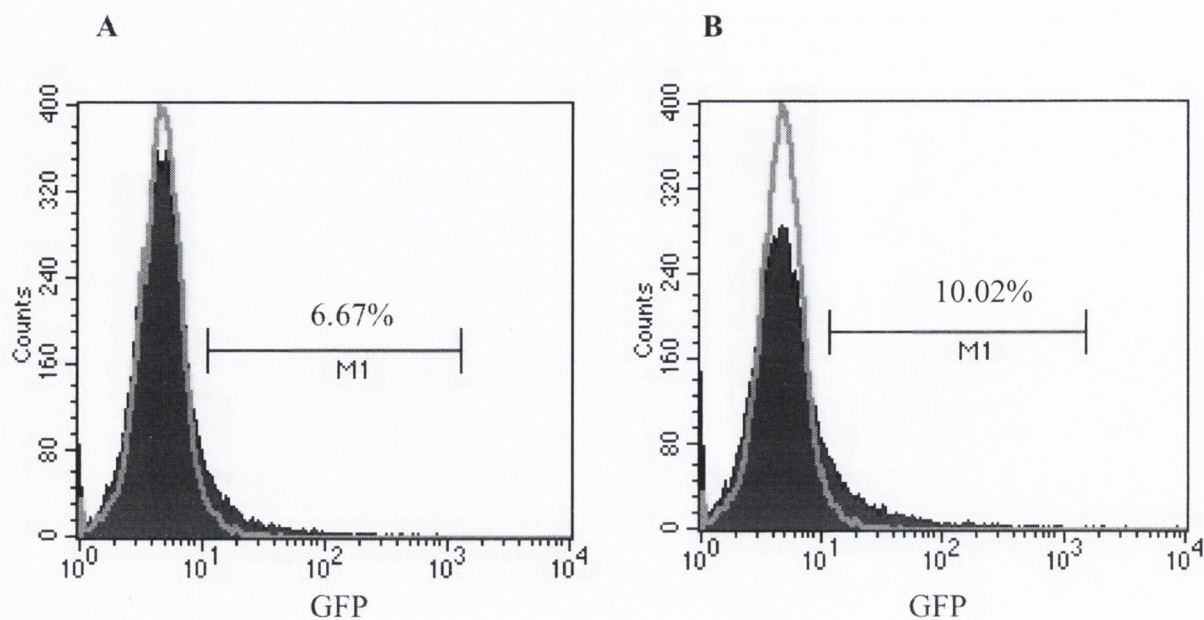


Figure 4.3 Transfection of the Jurkat cell line by electroporation

1×10^7 Jurkat cells were electroporated in a 2mm (A) or 4mm (B) gap cuvette according to the conditions outlined in 2.9.3. GFP expression was analysed 24 hours after transfection. Filled histogram represents cells transfected with the GFP plasmid, the open histogram represents mock transfected controls.

was seen using the 4 mm gap cuvette protocol. Electroporation caused significant cell death; this was minimised by using a specialised buffer (cytomix buffer) designed to mimic the intracellular environment. In the presence of this buffer, the cell viability following electroporation was routinely higher than 70% (compared to 40-60% in its absence, n=2)

4.3.2 Monitoring of stably-transfected cell lines

The expression of the transfected receptors on different cell lines needed to be comparable if any difference in the level of interaction could be attributed to biological differences. Therefore the level of expression of HLA on the 721.221 cell line transfectants and KIR expression on the Jurkat cell lines were monitored. The 3DL1 allele construct contains a GFP sequence, which allows for expression levels to be monitored by flow cytometry. 3DL1*001 and 3DL1*002 transfectants were monitored routinely for GFP expression (Figure 4.4). Staining of the cell lines with an anti-3DL1 antibody gave a similar pattern of reactivity. In some cases, a proportion of the cells were found to have reduced (or no) expression of the transfected construct; in these cases, the cells were sorted to ensure a homogenous population of expressing cells. HLA expression by the 721.221 transfectants was monitored with the use of a pan anti-HLA class I antibody (clone w6/32). Expression of the HLA antigens was found to be less stable than the expression of the KIR constructs and the 721.221 cell lines were frequently monitored and routinely sorted before their use in experiments to ensure equivalent levels of expression (Figure 4.5).

4.3.3 Optimisation of stimulation conditions

To optimise the detection of any interaction between the 3DL1 allotypes and the HLA transfectants, various ratios of stimulator cells (721.221 HLA transfectants) to responder cells (3DL1 Jurkat cells) were tested. An HLA-Bw4 allotype (B*2705) and an HLA-A allotype (A*0101) transfectant cell line were used as a positive and negative control respectively for the interaction with 3DL1. CD3/CD28 microbeads were added as a positive control for the detection of NFAT signalling.

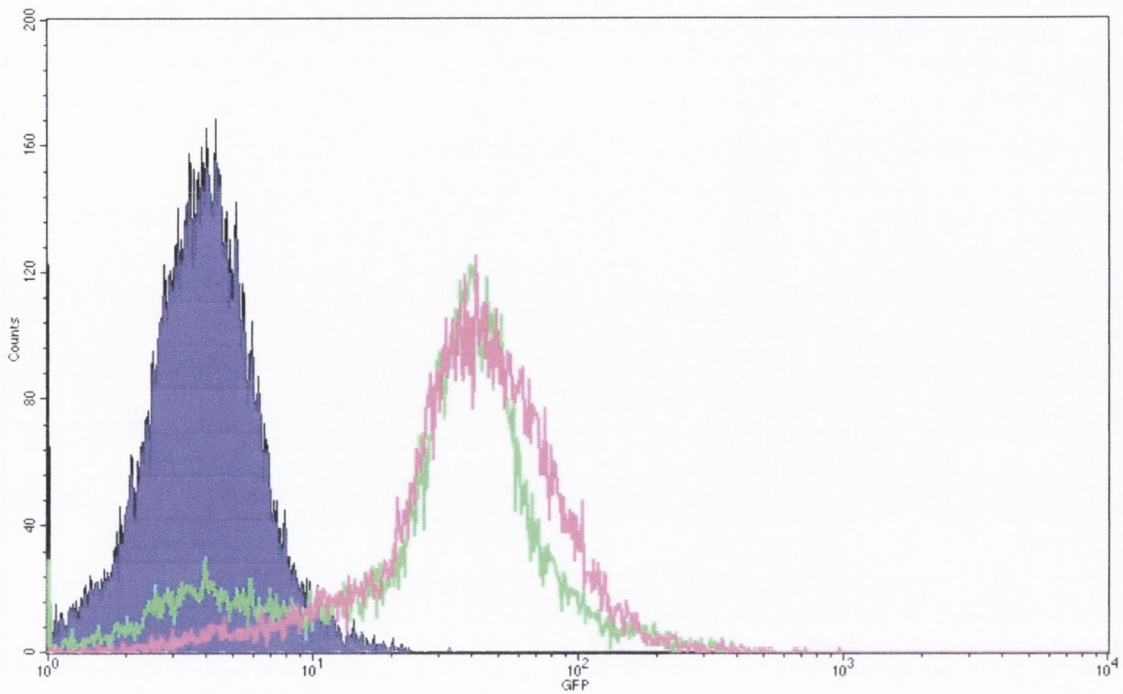


Figure 4.4 3DL1*001 and 3DL1*002 are expressed at comparable levels

Levels of expression of the 3DL1*001ECD and 3DL1*002ECD by transfected Jurkat cell lines were monitored through expression of the GFP tag. Pink histogram represents 3DL1*001 staining, green histogram represents 3DL1*002 staining and purple filled histogram represents control parental Jurkat cells.

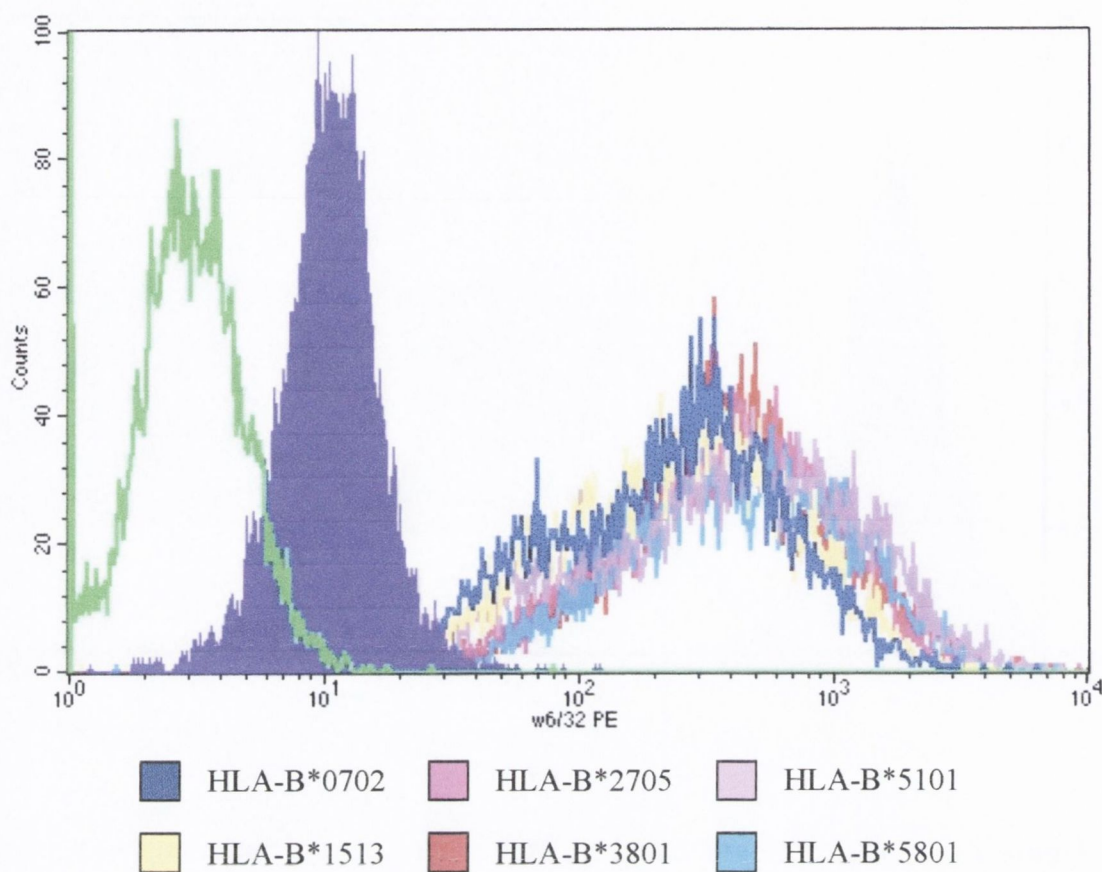


Figure 4.5 721.221 cell line transfectants express HLA at comparable levels

The panel of 721.221 transfectant cell lines was analysed for HLA class I expression using anti-HLA w6/32 antibody. Purple filled histogram represents staining by the parental 721.221 cell line and green histogram represents isotype-matched control staining of the parental cell line.

Stimulation conditions with a greater amount of stimulator cells (721.221) than responder cells (Jurkat) were not effective for the detection of KIR/HLA interactions (Figure 4.6A). Stimulation with 0.2X or 0.4X amount of stimulator cells gave an NFAT signal for B*2705 stimulation, but not A*0101 stimulation (Figure 4.6B). The strongest signal was detected with the use of 0.2X stimulator cells and this was used for all further experiments. CD3/CD28 microbeads gave a clear positive signal, although the level of stimulation was found to vary. Cells were initially stimulated for 18 hours before harvesting and analysis for the level of the reporter protein. Harvesting of the cells at 6, 10, 12 and 24 hours was also examined. The level of luciferase activity was found to increase up to 12 hours after which the levels remained constant (data not shown). Cells were harvested at 18 hours for all future experiments.

The experiments described above used an NFAT promoter linked to the luciferase reported gene. A second NFAT reporter plasmid, using secreted alkaline phosphatase (SEAP) as a reporter protein was tested to examine if it had any advantage over the use of the luciferase reporter. A similar pattern and strength of response was seen using this reporter system (Figure 4.7). Detection of luciferase activity was found to be less time consuming and so the NFAT-luciferase reporter plasmid was used in all future experiments.

4.3.4 3DL1*001 interacts with HLA-B allotypes

Interaction between 3DL1*001 ECD transfected Jurkat cells and a panel of 721.221 HLA transfectants was assayed using the conditions optimised above. Jurkat cells were transiently transfected with the NFAT-luciferase reporter plasmid and stimulated with 0.2X 721.221 cells for 18 hours. Cells were stimulated with medium alone and with CD3/CD28 microbeads as negative and positive controls respectively. Luciferase activity in the presence of HLA was measured relative to the unstimulated control. A strong interaction was seen between HLA-B*3801, B*5101 and B*5801 and 3DL1*001 (Figure 4.8A). HLA-B*2705 gave a weak (but reproducible) level of stimulation, while B*1513 failed to give any stimulation higher than that seen with the Bw6 allotype B*0702. As the level of stimulation seen with CD3/CD28 activation varied between experiments, the data

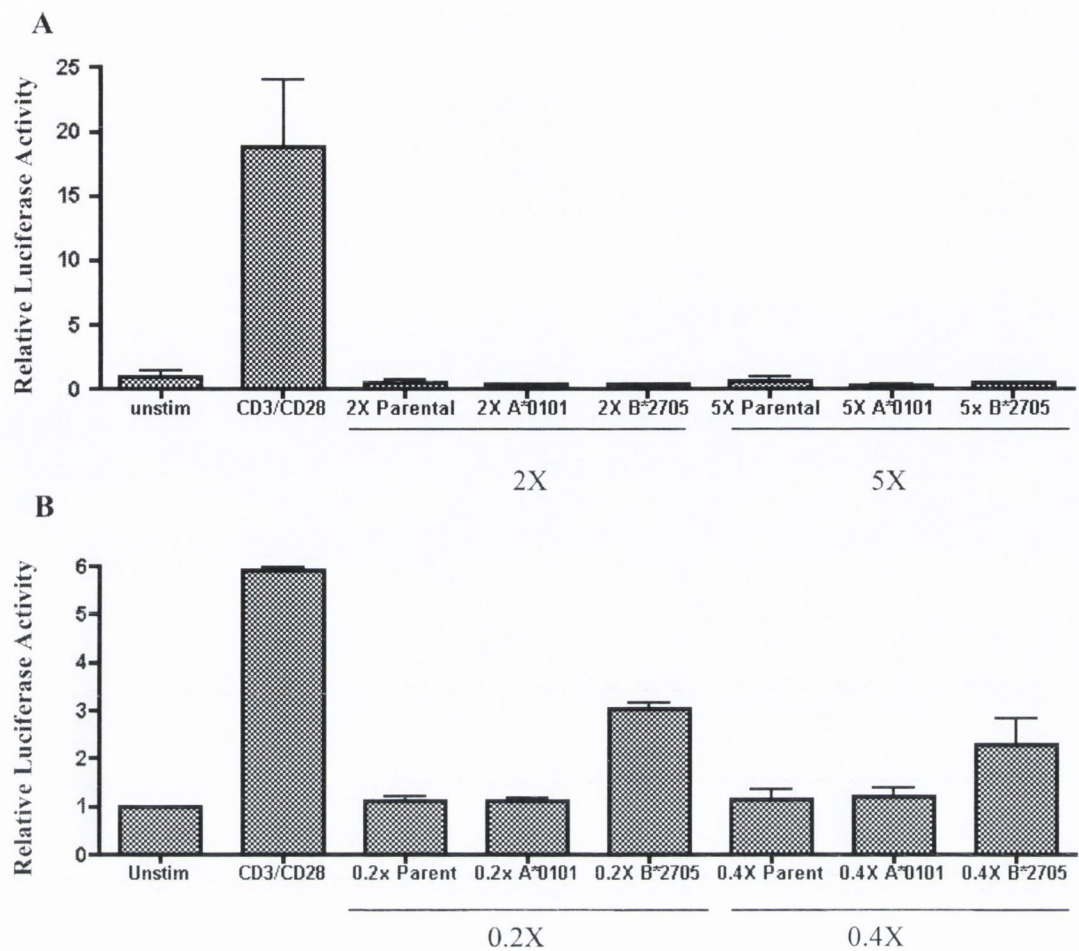


Figure 4.6 Optimisation of stimulation conditions

3DL1 Jurkat cells were plated at a concentration of 1×10^6 cells/ml and stimulated with the 721.221 parental cell line or a stable transfectant expressing HLA-A*0101 (negative control) or HLA-B*2705 (Bw4 epitope). The ratio of 721.221 cells used to stimulate the Jurkat cell line varied from 2X and 5X (top panel) to 0.2X and 0.4X (lower panel). Luciferase activity is expressed relative to an unstimulated control. CD3/CD28 microbeads (3 beads/cell) were used as a positive control. Data shown is representative of two independent experiments and error bars represent standard deviation.

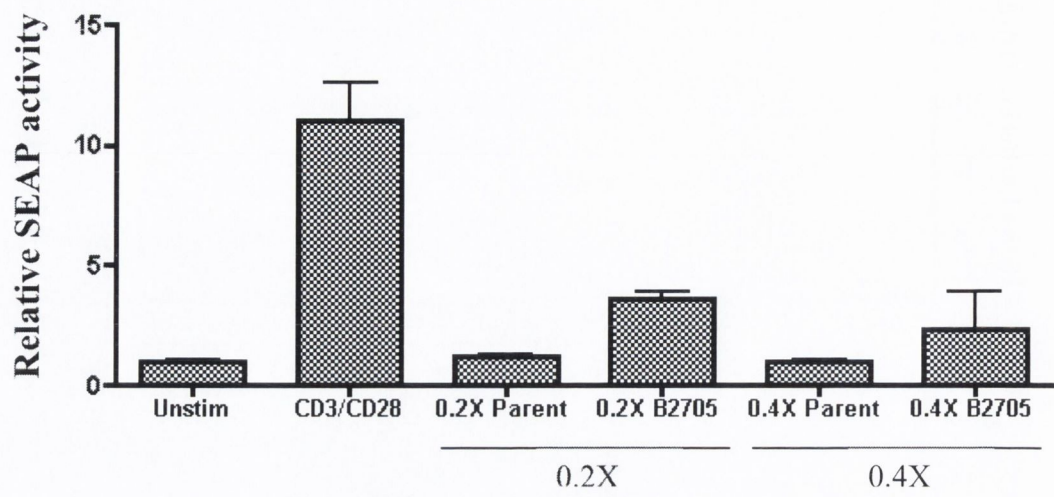


Figure 4.7 Evaluation of SEAP activity as reporter readout

3DL1 Jurkat cells were plated at a concentration of 1×10^6 cells/ml and stimulated with the 721.221 parental cell line or a stable transfectant expressing HLA-B*2705. The ratio of 721.221 used to stimulate the Jurkat cell line varied from 0.2X to 0.4X. SEAP activity is expressed relative to an unstimulated control. CD3/CD28 microbeads (3 beads/cell) were used as a positive control. Data shown is representative of two independent experiments and error bars represent standard deviation.

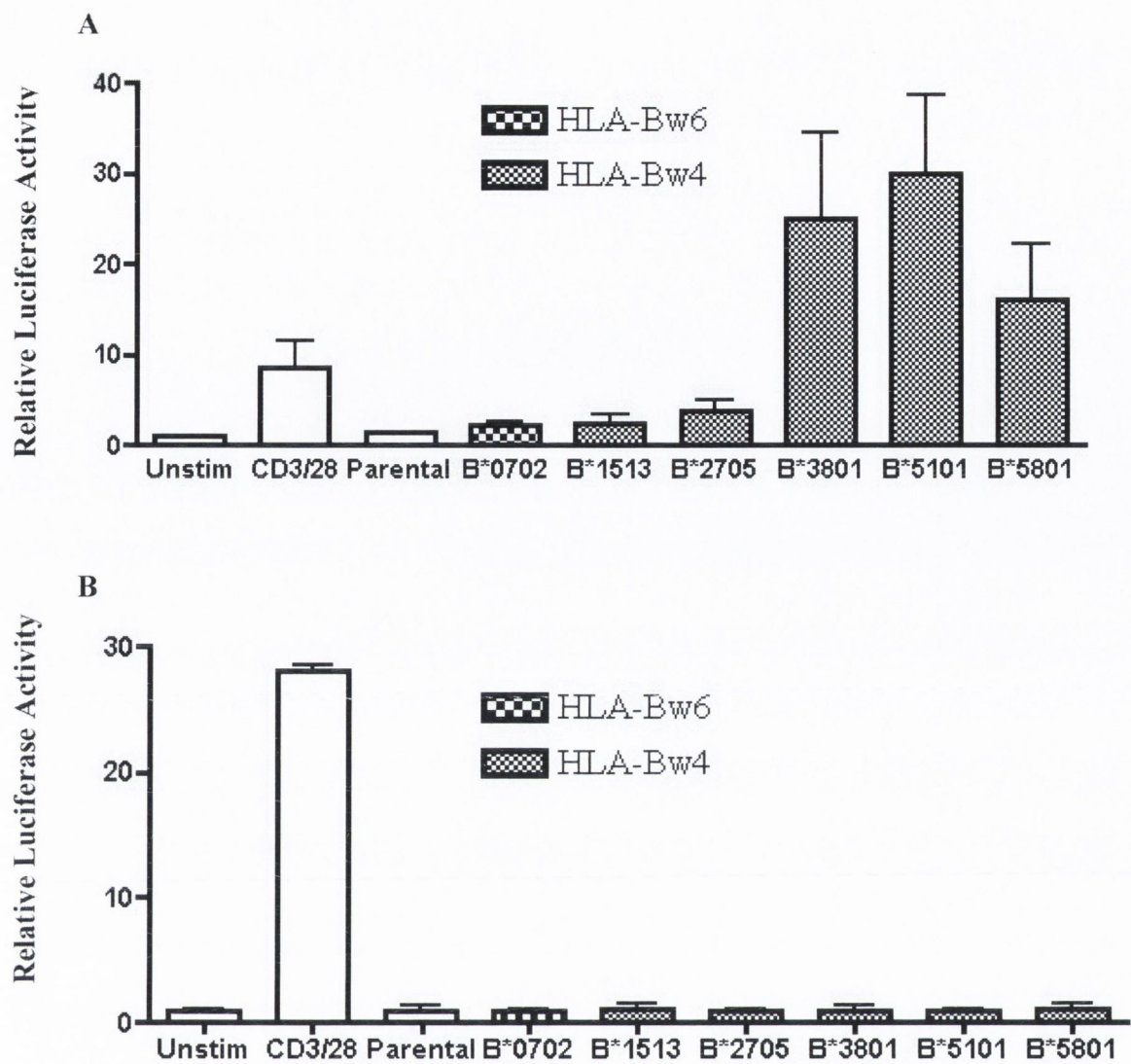


Figure 4.8 3DL1*001 interacts with HLA-B allotypes

A, 3DL1*001 Jurkat cells were transfected with NFAT-luciferase reporter plasmid and stimulated with a panel of 721.221 transfectants (0.2X) for 18 hours. Cells were then lysed and assayed for luciferase activity. Panel B shows stimulation of the control empty vector transfected Jurkat cell line. Relative luciferase activity is expressed relative to an unstimulated control. CD3/CD28 microbeads (3 beads/cell) were used as a positive control. HLA-B Bw4⁺ allotypes are shown with hashed bars. Data shown is the average of four independent experiments; error bars show standard error of the mean.

was also expressed as a percentage of CD3/CD28 stimulation – this gave the same pattern of reactivity (data not shown). The hierarchy of interaction between 3DL1*001 and the HLA-Bw4 allotypes detected in this system is

$$B^*5101 > B^*3801 > B^*5801 > B^*2705 > B^*1513$$

Jurkat cells transfected with empty pcDEFIII vector were also stimulated with the panel of 721.221 HLA transfectants as a negative control. As expected no stimulation was seen with any of the HLA allotypes tested (Figure 4.8B).

4.3.5 3DL1*002 interacts with HLA-B allotypes

Interaction between 3DL1*002 ECD transfected Jurkat cells and a panel of 721.221 HLA transfectants was assayed as above (4.3.4). A strong interaction was seen between HLA-B*2705, B*3801 and B*5101 and 3DL1*002 (Figure 4.9). HLA-B*5801 gave a lower level of stimulation, while B*1513 failed to give any stimulation higher than that seen with the Bw6 allotype B*0702. As above, the data was also expressed as a percentage of CD3/CD28 stimulation – this gave the same pattern of reactivity (data not shown). The hierarchy of interaction between 3DL1*002 and the HLA-Bw4 allotypes detected in this system is

$$B^*5101 > B^*3801 > B^*2705 > B^*5801 > B^*1513$$

4.3.6 The role of CD94 in the recognition of HLA panel

CD94 (in combination with NKG2 receptors) recognizes HLA-E, which is expressed on the surface of 721.221 cells when stabilized by the presence of the leader sequence from some but not all HLA allotypes. To determine if the CD94/NKG2 – HLA-E interaction had any effect on the lysis of the 721.221 transfectants, the experiment was carried out in the presence or absence of a CD94 blocking antibody. Polyclonal NK cells maintained in IL-2 were used as effector cells at an E: T of 6:1. As expected, the killing of the parental 721.221 and HLA-B*1513, B*2705, and B*5801 transfectants was unaffected by blocking CD94 ((Llano et al, 1998; Phillips et al, 1996), Figure 4.10). The lysis of B*5101 was also unaffected by blocking of CD94 – although the leader sequence of this HLA allotype has been reported to bind HLA-E, evidence suggests that this complex does

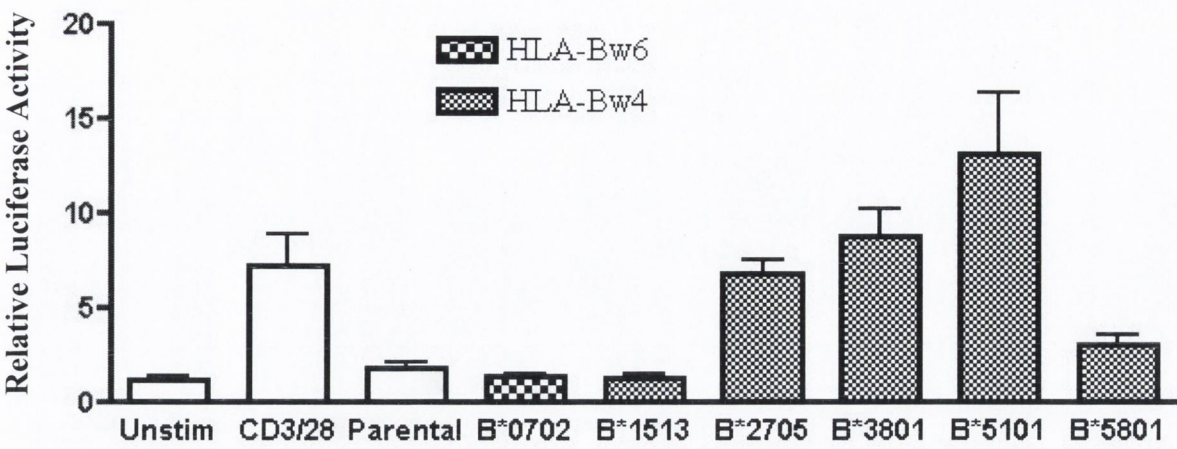


Figure 4.9 3DL1*002 interacts with HLA-B allotypes

3DL1*002 Jurkat cells were transfected with NFAT-luciferase reporter plasmid and stimulated with a panel of 721.221 transfectants (0.2X) for 18 hours. Cells were then lysed and assayed for luciferase activity, which is expressed relative to an unstimulated control. CD3/CD28 microbeads (3 beads/cell) were used as a positive control. HLA-B Bw4⁺ allotypes are shown with hashed bars. Data shown is the average of four independent experiments; error bars show standard error of the mean.

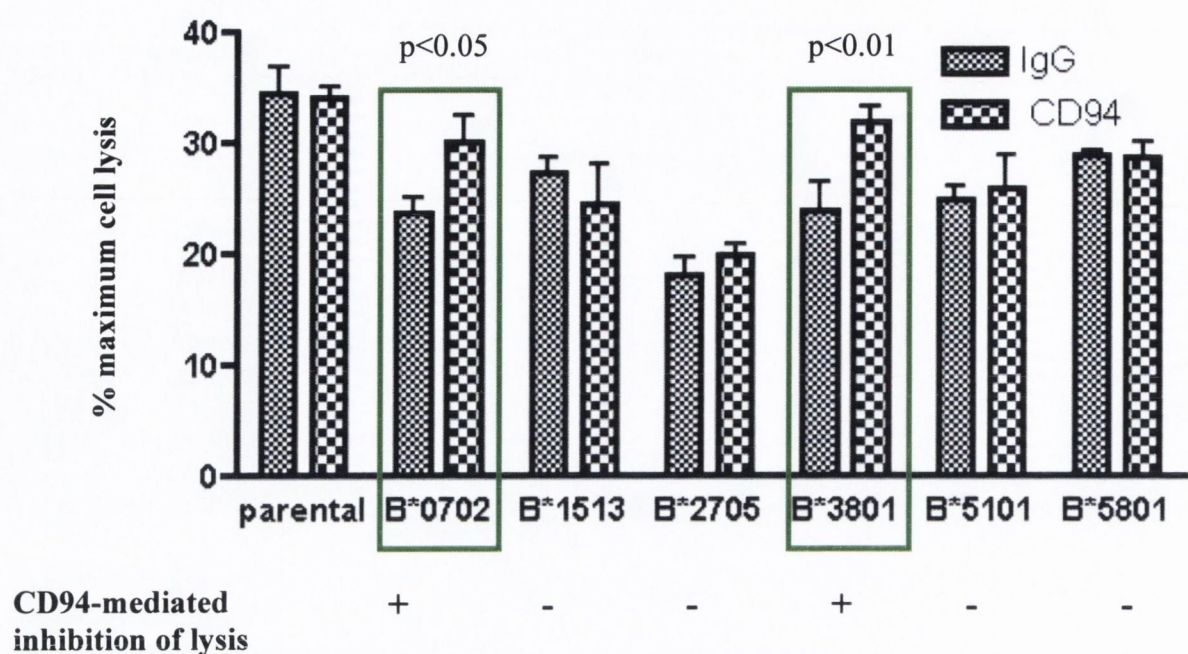


Figure 4.10 The role of CD94 in the recognition of HLA panel

Polyclonal NK cells were used as effector cells against a panel of 721.221 transfectants in a standard 4-hour ⁵¹Cr release assay at an effector to target ratio of 6:1. Cytotoxicity is expressed as a percentage of maximum cell lysis and was measured in the presence of a control IgG antibody (10 µg/ml, open bars) or anti-CD94 (10 µg/ml, hatched bars). Data was compared using a Student's t-test. HLA allotypes where killing was statistically increased in the presence of anti-CD94 are highlighted in green boxes. Data shown is a representative of six independent experiments and error bars show standard deviation.

not provide inhibition from killing (Phillips et al, 1996). Levels of cytotoxicity against HLA-B*0702 and B*3801 were greater in the presence of the CD94 blocking antibody, but there was a considerable degree of killing (approximately 25% maximum cell lysis or 70% of parental cell lysis) in the absence of the blocking antibody. As the HLA-B*3801 transfectant decreases lysis via interaction with CD94, later experiments with this transfectant were carried out in the presence of anti-CD94 antibody (10 µg/ml).

4.3.7 3DL1*001 specificity in a clonal cytotoxicity assay

The generation of NK cells clones is described in chapter 3. In brief, 3DL1⁺ NK cells from a 3DL1*001 genotyped donor were sorted and cloned by limiting dilution using either Yssel's medium or Stem Cell Growth Medium. Clones were positive for 3DL1*001 as measured by DX9 (and Z27) staining and RT-PCR analysis. Cytotoxicity of the clones was measured as percentage of maximum cell lysis against the K562 cell line.

3DL1*001⁺ NK cells clones were used as effector cells against the panel of 721.221 transfectants at an E: T of 6:1. Killing was measured in the presence and absence of the blocking anti-3DL1 antibody DX9 to demonstrate the specificity of the interaction. Killing of all HLA-Bw4⁺ transfectants was reduced relative to the killing of the 721.221 parental cell line and the Bw6⁺ B*0702 transfectant (Figure 4.11A). The presence of the blocking DX9 antibody reversed this inhibition of killing. The degree of inhibition of lysis was greater for some allotypes than for other; the hierarchy of inhibition in this assay is

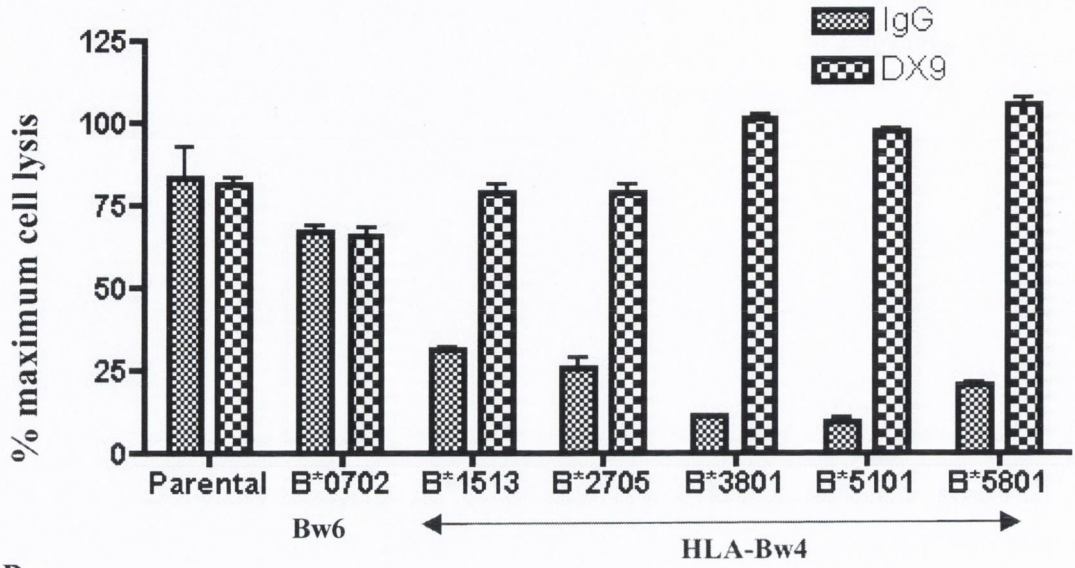
B*5101>B*3801>B*5801>B*2705>B*1513

The data are also expressed as the percentage of killing in the presence of the DX9 antibody, giving the same pattern of reactivity (Figure 4.11B).

4.3.8 3DL1*002 specificity in a clonal cytotoxicity assay

Using a similar approach to that described above for 3DL1*001 (4.3.7) 3DL1*002⁺ NK cell clones were generated. These clones were used as effector cells against the panel of

A



B

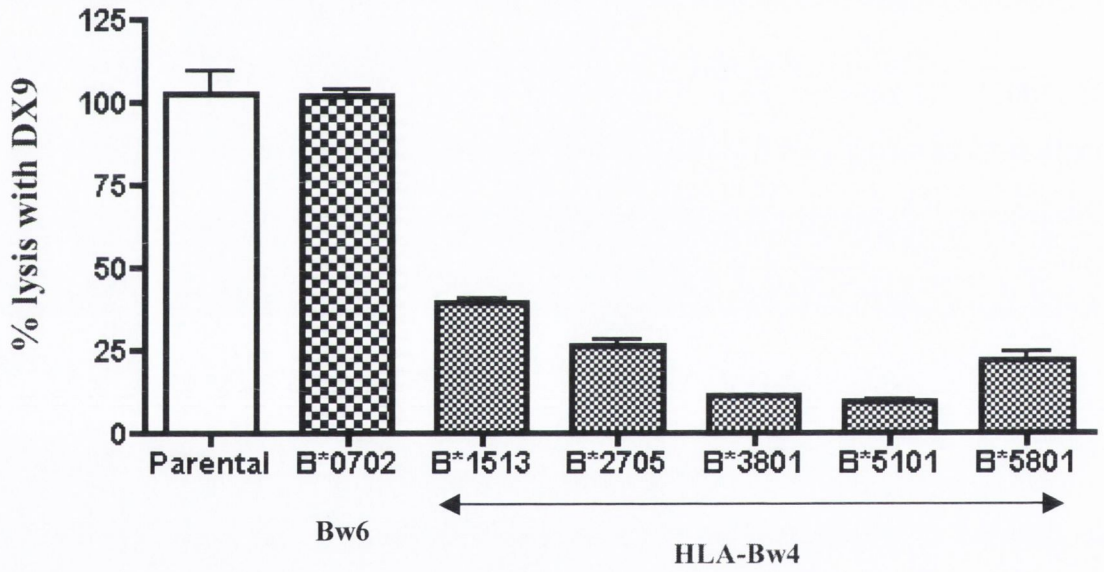


Figure 4.11 3DL1*001 specificity in clonal cytotoxicity assay

(A) 3DL1*001⁺ NK cell clones were used as effector cells against a panel of 721.221 transfectants in a standard 4 hour ⁵¹Cr release assay (6:1 effector to target ratio). Cytotoxicity is expressed as a percentage of maximum cell lysis and was measured in the presence of a control IgG antibody (10 µg/ml, hatched bars) or DX9 (10µg/ml, checked bars). Data shown is a representative of two independent experiments and error bars show standard deviation. Killing of the B*3801 cell line was assayed in the presence of 10 µg/ml anti-CD94 antibody. (B) The data from (A) is expressed as a percentage of the killing in the presence of the blocking DX9 antibody for each of the cell lines. HLA-B Bw4⁺ allotypes are shown with hashed bars.

721.221 transfectants at an E: T of 6:1. Killing was measured in the presence and absence of the blocking anti-3DL1 antibody DX9 to demonstrate the specificity of the interaction. Killing of all HLA-Bw4⁺ transfectants was reduced relative to the killing of 721.221 parental cell line and the Bw6⁺ B*0702 transfectant (Figure 4.12A). The presence of the blocking DX9 antibody reversed this inhibition of killing. The degree of inhibition of lysis was greater for some allotypes than for others; the hierarchy of inhibition in this assay is

$$B^*5101 > B^*3801 > B^*2705 > B^*5801 > B^*1513$$

The data are also expressed as the percentage of killing in the presence of the DX9 antibody, giving the same pattern of reactivity (Figure 4.12B).

4.3.9 3DL1*005 specificity in a clonal cytotoxicity assay

3DL1*005⁺ NK cells clones were generated as described above (4.3.7) and used as effector cells against the panel of 721.221 transfectants at an E: T of 6:1. Killing was measured in the presence and absence of the blocking anti-3DL1 antibody DX9. Killing of all HLA-Bw4⁺ transfectants was reduced relative to the killing of 721.221 parental cell line and the Bw6⁺ B*0702 transfectant (Figure 4.13A). The presence of the blocking DX9 antibody reversed this inhibition of killing. The degree of inhibition of lysis was greater for some allotypes than for others; the hierarchy of inhibition in this assay is

$$B^*5101 > B^*5801 > B^*1513 > B^*2705 > B^*3801$$

The data are also expressed as the percentage of killing in the presence of the DX9 antibody, giving the same pattern of reactivity (Figure 4.13B).

4.3.10 3DL1*005 specificity in a polyclonal cytotoxicity assay

A polyclonal population of sorted 3DL1*005⁺ NK cells were used as effector cells against the panel of 721.221 transfectants at an E: T of 6:1. Killing was measured in the presence and absence of the blocking anti-3DL1 antibody DX9. Killing of all HLA-Bw4⁺ transfectants was reduced relative to the killing of 721.221 parental cell line (Figure 4.14A). The presence of the blocking DX9 antibody reversed this inhibition of killing.

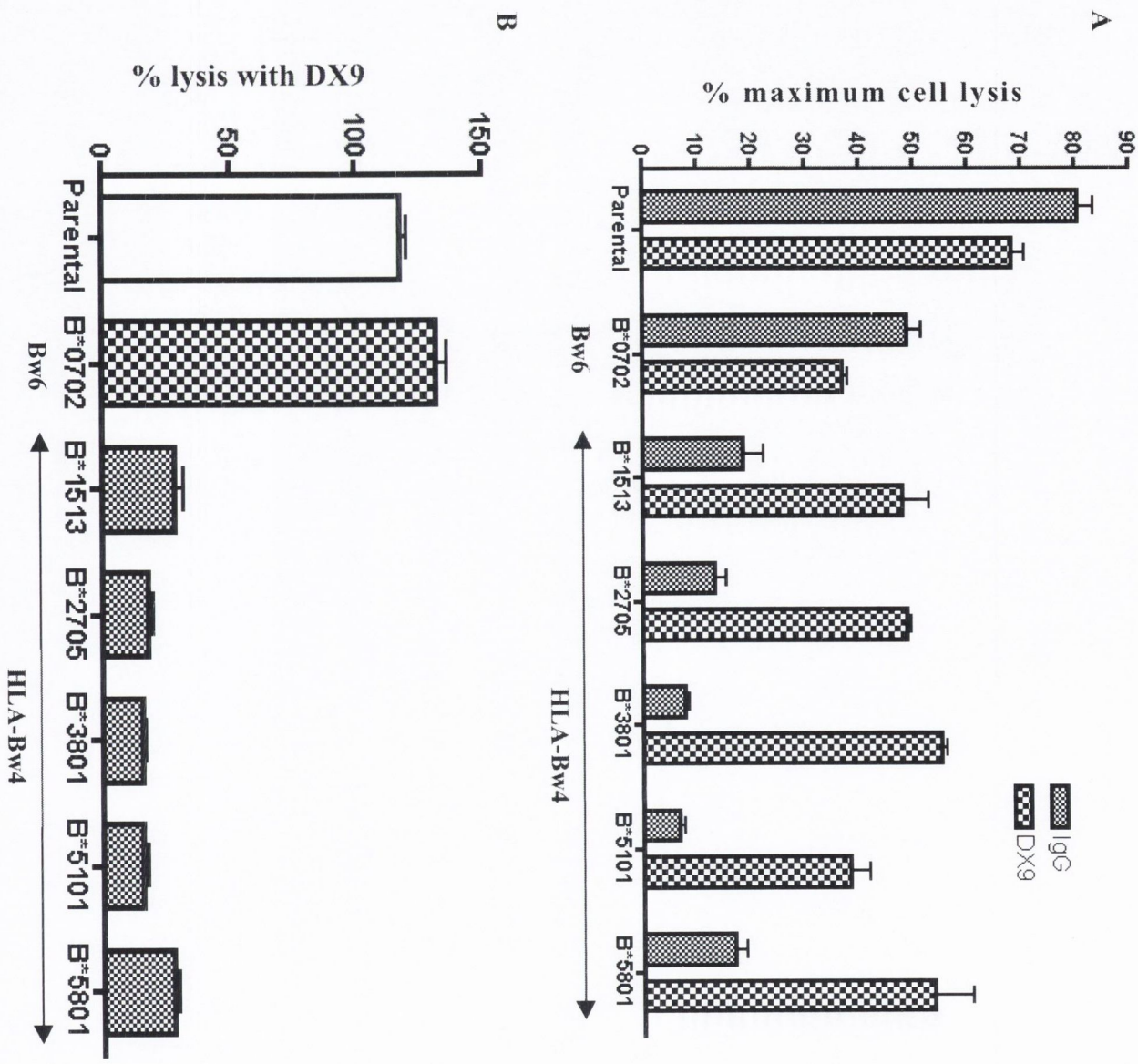


Figure 4.12 3DL1*002 specificity in clonal cytotoxicity assay

3DL1*002⁺ NK cell clones were used as effector cells against a panel of 721.221 transfectants in a standard 4 hour ⁵¹Cr release assay (6:1 effector to target ratio). Cytotoxicity is expressed as a percentage of maximum cell lysis and was measured in the presence of a control IgG antibody (10 µg/ml, hatched bars) or DX9 (10µg/ml, checked bars). Data shown is representative of four independent experiments showing a consistent pattern and error bars show standard deviation. Data is also expressed at the percentage of lysis in the presence of the DX9 antibody for each cell line. Killixng of the B*3801 cell line was assayed in the presence of 10 µg/ml anti-CD94 antibody.

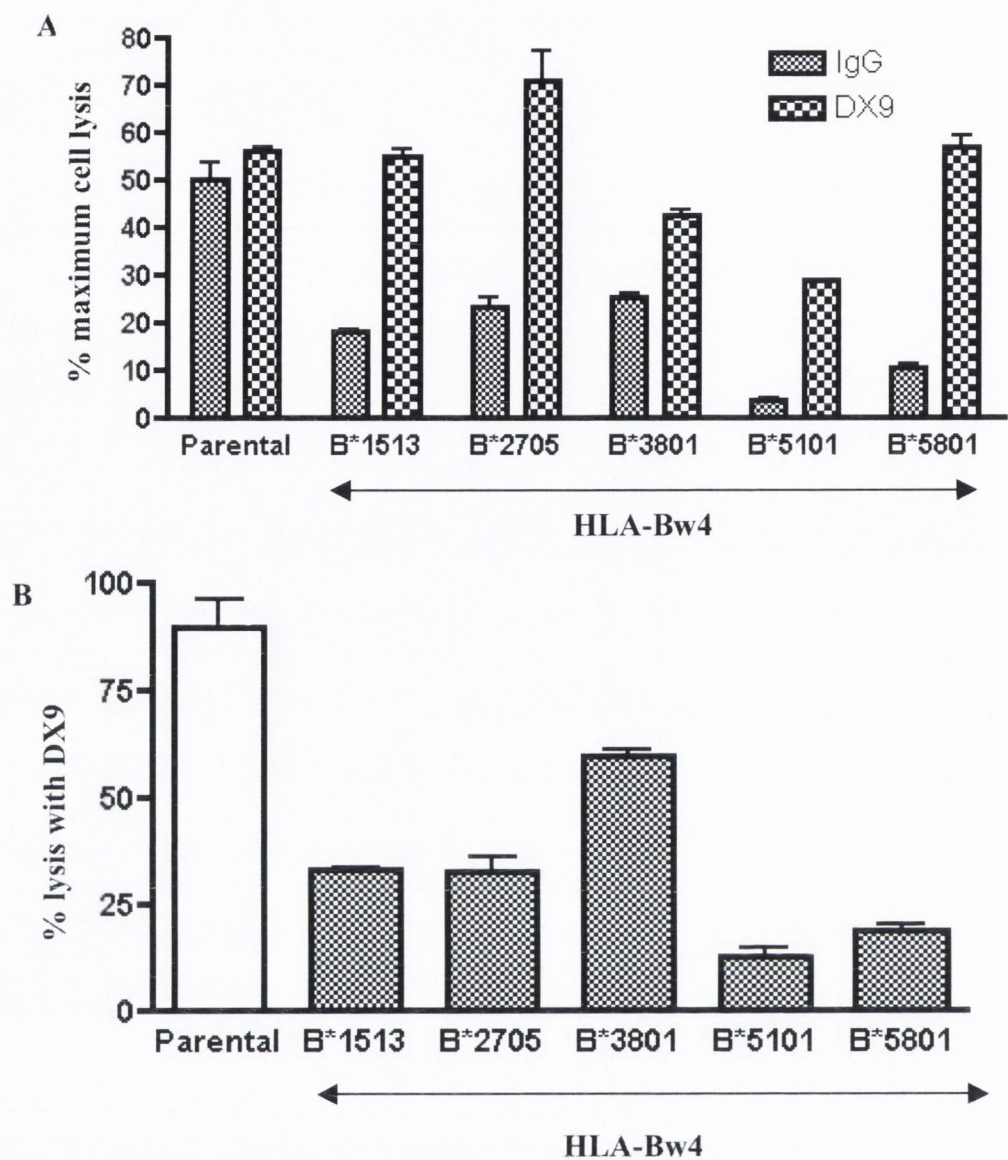


Figure 4. 13 3DL1*005 specificity in clonal cytotoxicity assay

(A) 3DL1*005⁺ NK cell clones were used as effector cells against a panel of 721.221 transfectants in a standard 4 hour ⁵¹Cr release assay (6:1 effector to target ratio). Cytotoxicity is expressed as a percentage of maximum cell lysis and was measured in the presence of a control IgG antibody (10 µg/ml, hatched bars) or DX9 (10µg/ml, checked bars). Data shown is a representative of two independent experiments and error bars show standard deviation. Killing of the B*3801 cell line was assayed in the presence of 10 µg/ml anti-CD94 antibody. (B) The data from (A) is expressed as a percentage of the killing in the presence of the blocking DX9 antibody for each of the cell lines.

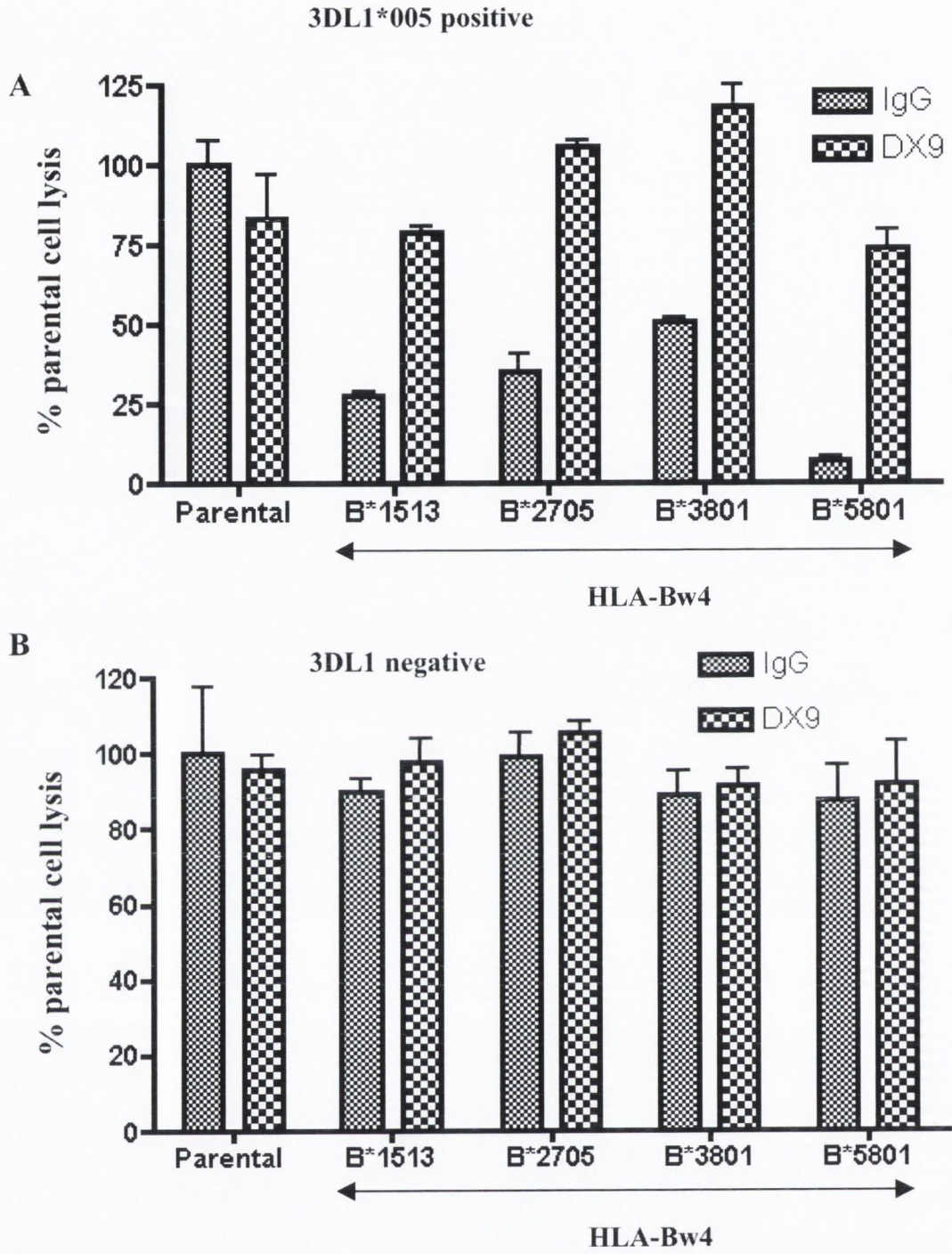


Figure 4.14 3DL1*005 specificity in polyclonal cytotoxicity assay

(A) 3DL1*005⁺ NK cells were sorted, expanded and used as effector cells against a panel of 721.221 transfectants in a standard 4 hour ⁵¹Cr release assay (6:1 effector to target ratio). Cytotoxicity is expressed as a percentage of parental cell lysis and was measured in the presence of a control IgG antibody (10 µg/ml, hatched bars) or DX9 (10µg/ml, checked bars). Data shown is a representative of two independent experiments and error bars show standard deviation. Killing of the B*3801 cell line was assayed in the presence of 10 µg/ml anti-CD94 antibody. (B) 3DL1 negative cells from the same donor as A were sorted and expanded and used as effector cells as above.

The degree of inhibition of lysis was greater for some allotypes than for other; the hierarchy of inhibition in this assay is

$$B^*5801 > B^*1513 > B^*2705 > B^*3801$$

The data are also expressed as the percentage of killing in the presence of the DX9 antibody, giving the same pattern of reactivity (data not shown). This pattern of reactivity with polyclonal cells mirrored that seen with the clonal NK cells, but does not include the B*5101 allotype. Polyclonal cultures of 3DL1⁻ NK cells were also used as effector cells against the panel of 721.221 transfectants. All transfectants were lysed to a comparable degree irrespective of the presence of DX9 blocking antibody (Figure 4.14B)

4.3.11 Activation of NK cells by 721.221 cell lines is inhibited by HLA-B*5801

The interaction of NK cells with susceptible target cells such as the class I negative 721.221 cell line activates NK cell cytotoxicity, which can be measured by the conventional chromium release assay. This assay provides a measure of the death of the target cells but the degree of NK cell activation is also of interest. Expression of activation markers such as CD69 and CD25, the degranulation marker CD107a, and IFN- γ production can be assessed by flow cytometry. We investigated if activation of NK cells in response to a susceptible target cell could be measured using these markers and if this activation could be inhibited when 721.221 cell transfected with HLA-B allotypes were used. Co-staining of samples with the Z27 antibody was used to identify 3DL1⁺ and 3DL1⁻ subsets.

Freshly isolated NK cells from two donors (Donor 1 *001 homozygous, Donor 2 *002 + *004) were stimulated with parental 721.221 cells for 18 hours. The baseline levels of CD69 expression varied between the donors, but in both cases stimulation increased the level of CD69 expression on both 3DL1⁺ and 3DL1⁻ NK cell subsets (Figure 4.15A). Stimulation with HLA-B*5801 transfected 721.221 induced a lower level of CD69 expression in the 3DL1⁺ NK cells on both donors (although this inhibition does not reach baseline levels for donor 1). The level of inhibition of 3DL1⁻ cells for both donors is less

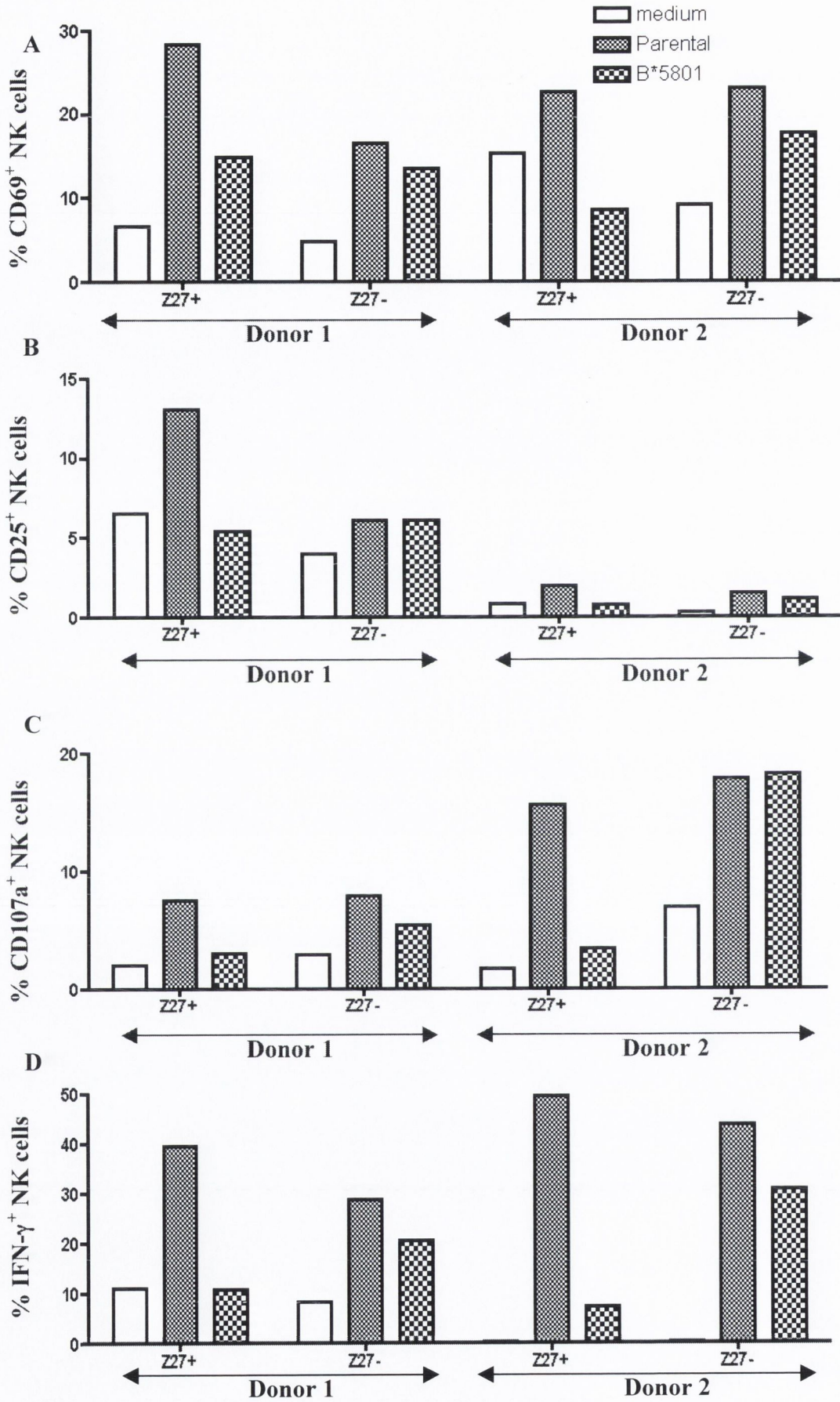


Figure 4.15 Activation of NK cells by 721.221 cell lines is inhibited by HLA

Purified NK cells from two donors (D1 and D2) were stimulated with 721.221 parental cell line and the HLA-B*5801 transfectant and analysed for the expression of CD69, CD25, IFN- γ and CD107a. Cells were stained with Z27, anti-CD56 and anti-CD3 to identify 3DL1⁺ NK cells. (A) Cells were stimulated for 18 hours and stained with anti-CD69 FITC. (B) Cells were stimulated for 18 hours in the presence of hrIL-2 (500 IU/ml) and stained with anti-CD25. (C) Cells were stimulated in the presence of anti-CD107a FITC (20 μ l/ml). Monensin (6 μ g/ml) was added 1 hour post stimulation and the cells were incubated for a further 5 hours. (D) Cells were stimulated for 18 hours in the presence of hrIL-2 (500 IU/ml). BD GolgiStopTM (1 μ g/ml) was added for the final 6 hours of stimulation. Cells were then fixed and permeabilised and stained intracellularly for IFN- γ . Open bars represent medium-only control, hatched bars represent stimulation with parental 721.221 and checked boxes represent stimulation with the 721.221 HLA-B*5801 cell line.

that seen for 3DL1⁺ cells, although there is a slight reduction for both donors. This may reflect inhibition of CD94/NKG2A⁺ or LILRB1⁺ cells within this population. Similar to the percentage of CD69⁺ cells, the intensity of CD69 staining (mean fluorescence intensity, MFI) was found to increase on NK cells upon stimulation with the 721.221 parental cell line. This increase was also inhibited by the expression of HLA-B*5801 (data not shown).

Stimulation of NK cells with 721.221 parental cells did not cause any appreciable increase in CD25 expression in the absence of IL-2 costimulation (data not shown). In the presence of 500 IU/ml hrIL-2 for 18 hours, costimulation with the 721.221 cell line caused an increase in CD25 expression for both donors (although the levels of positive cells seen is much higher for donor 1 than donor 2) (Figure 4.15B). Expression of HLA-B*5801 by the 721.221 cell line inhibited this increase in CD25 expression for the 3DL1⁺ but not the 3DL1⁻ populations of both donors.

Stimulation of NK cells with 721.221 parental cells, in the presence of an anti-CD107a antibody and monensin (a Golgi apparatus inhibitor), caused an increase in CD107a expression in both 3DL1 positive and negative populations (Figure 4.15C). Expression of HLA-B*5801 by the 721.221 cell line inhibited this increase in CD107a expression for the 3DL1⁺ cells of both donors. The 3DL1⁻ cells of donor 2 were not inhibited by HLA-B*5801 expression, while the 3DL1⁻ cells of donor 1 show a small degree of inhibition.

Stimulation of NK cells with 721.221 parental cells, in the presence (for the last 4 hours of stimulation) of GolgiPlug®, caused an increase in the intracellular expression of IFN-γ (Figure 4.15D). Expression of HLA-B*5801 by the 721.221 cell line inhibited this increase in IFN-γ expression for the 3DL1⁺ cells of both donors. The 3DL1⁻ cells of both donors show a much smaller level of inhibition by HLA-B*5801.

4.3.12 3DL1*001 specificity is maintained in primary NK cells

Differences in 3DL1*001 interaction with HLA-B allotypes were assessed using CD69, CD107a and CD25 as described above. NK cells were purified from an *001 donor where the Z27 staining patterns allowed for the identification of the *001⁺ population (*001

homozygous (n=1) or *001/*004 heterozygous (n=1) or *001/*005 heterozygous (n=1; *001⁺ cells were distinguished from *005⁺ cells by the intensity of staining)). CD69 and CD107a expression were measured on cells stimulated in the absence of IL-2. The pattern of CD69 up-regulation and its inhibition by HLA-B allotypes agreed with the hierarchy of recognition as seen in previous assays

$$B^*5101 > B^*3801 > B^*5801 > B^*2705 > B^*1513$$

Stimulation with B*1513 and B*2705 transfected cells gave a similar and very modest degree of inhibition relative to parental cells. B*5101 provided the greatest level of inhibition, with intermediate levels generated by both B*3801 and B*5801 (Figure 4.16A).

The pattern of CD107a externalisation, and the inhibition of this by HLA-B allotypes gave a similar hierarchy of recognition (Figure 4.16B). B*5101 provided the greatest level of inhibition, with lower levels generated by both B*3801 and B*5801. In two of the three donors, B*1513 and B*2705 both gave a significant level of inhibition relative to stimulation with parental cells, while the third donor did not show significant levels of inhibition with B*1513.

Isolated NK cells were stimulated with 721.221 cells in the presence of 500 IU/ml hrIL-2 to assay CD25 up-regulation. Expression of CD25 on 3DL1⁺ cells was inhibited to some degree by all HLA-B allotypes tested (Figure 4.17). However, no consistent pattern was detected among the three donors (or within repetitions of a single donor). The representative donor shows greater inhibition with B*5101/B*3801 than with B*1513/B*2705 consistent with the pattern seen for other markers, but this was not a reproducible result.

Measurement of the inhibition of IFN- γ induction was not used to examine the hierarchy of HLA-B allotype affinity by *001 as the fixing of the NK cells altered the pattern of Z27 (and DX9) staining and identification of low and high intensity binding was not possible.

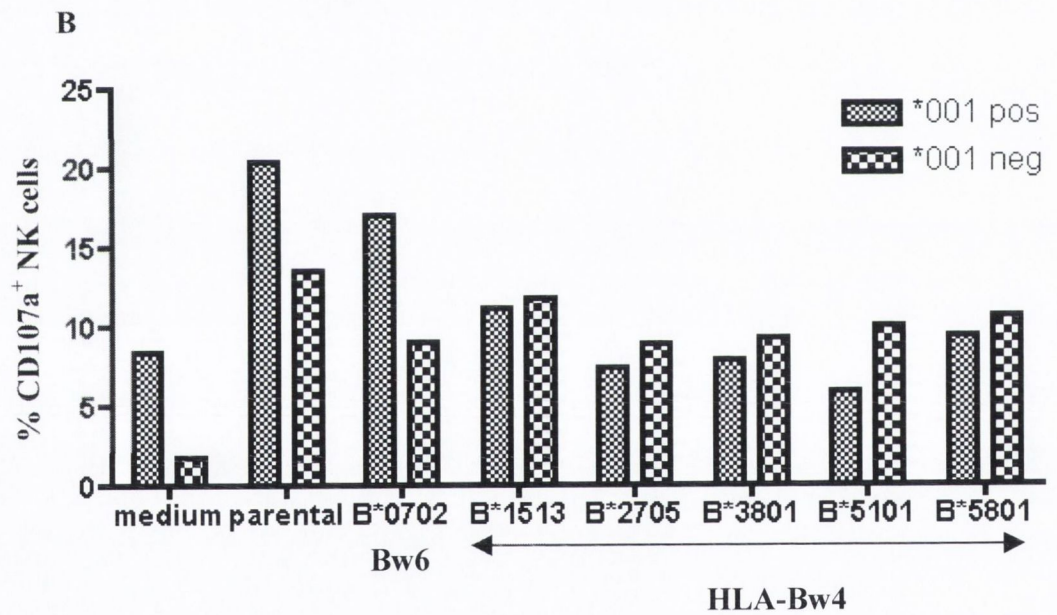
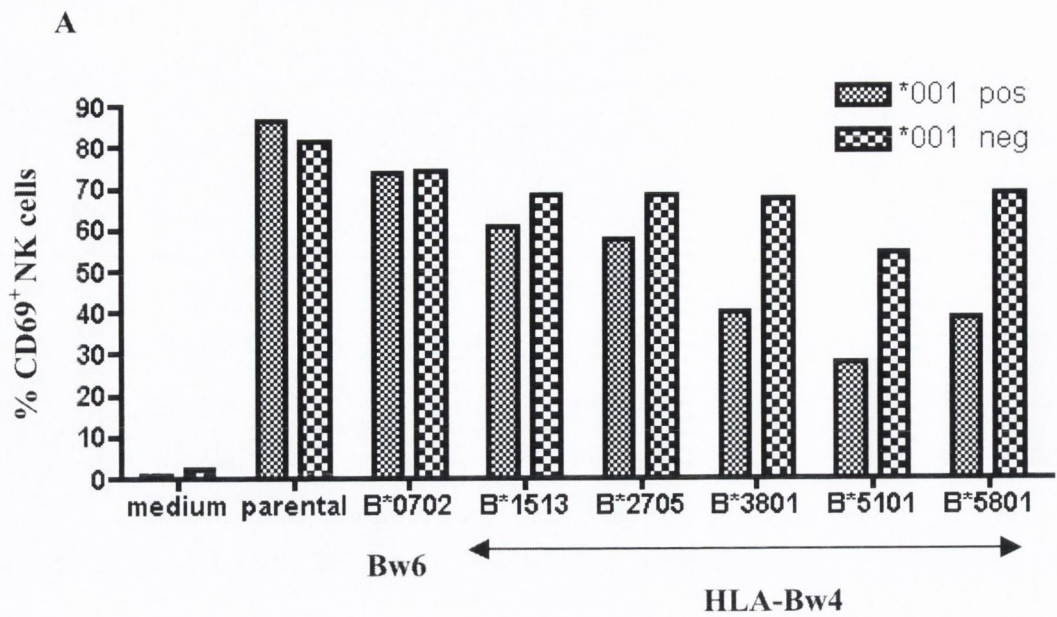


Figure 4.16 3DL1*001 specificity is maintained in primary NK cells

Purified NK cells from 3DL1*001 positive donors were stimulated with 721.221 parental cell line and a panel of its transfectants. (A) 3DL1*001 positive and negative cells were analysed for the expression of CD69 after 18 hour stimulation with the 721.221 cell line. (B) 3DL1*001 positive and negative cells were analysed for the expression of CD107a. Cells were stimulated in the presence of anti-CD107a FITC (20 μ l/ml). Monensin (6 μ g/ml) was added 1-hour post stimulation and the cells were incubated for a further 5 hours. Hatched bars represent 3DL1*001 positive cells, checked boxes represent 3DL1*001 negative cells. Data shown were generated from one donor, with a consistent pattern also seen in another 3DL1*001 positive individual.

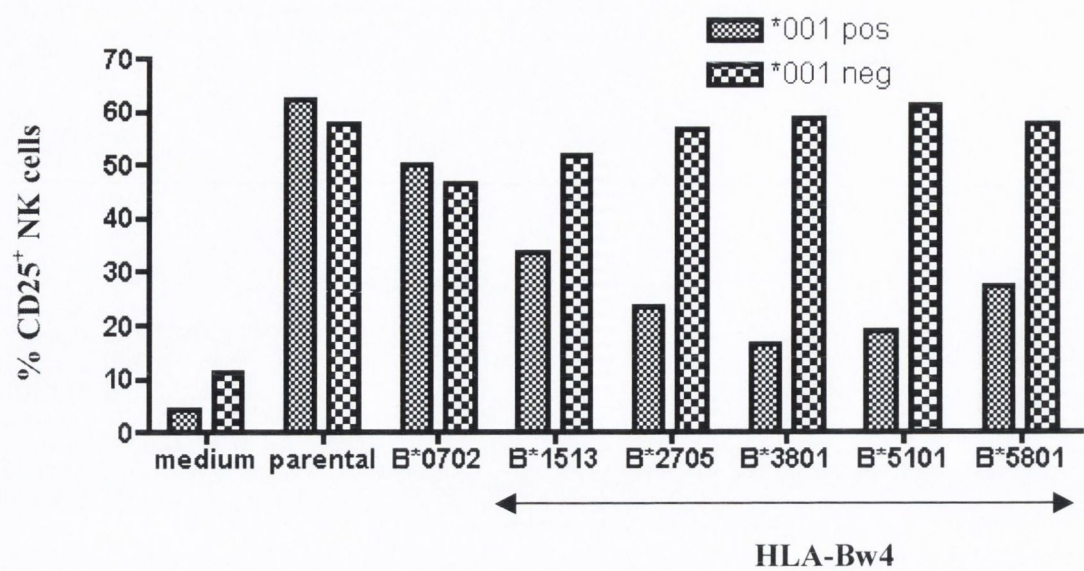


Figure 4.17 3DL1*001 specificity in short term IL-2 stimulated cultures

Purified NK cells from a 3DL1*001 positive donor were stimulated with 721.221 parental cell line and a panel of its transfectants. 3DL1*001 positive and negative cells were analysed for the expression of CD25 after 18 hour stimulation with the 721.221 cell line in the presence of hrIL-2 (500IU/ml). Hatched bars represent 3DL1*001 positive cells, checked boxes represent 3DL1*001 negative cells. Data shown were generated from a single donor.

4.4 Discussion

A direct and systematic comparison of the interaction between allelic variants of 3DL1 and HLA-Bw4 was investigated using a number of experimental approaches. The data generated gave a consistent hierarchy of reactivity that demonstrates that polymorphism of both HLA-B and 3DL1 influences their interaction.

The inhibitory nature of 3DL1, coupled with its clonal expression and the co-expression of additional HLA-specific receptors complicates functional analysis. One approach used to simplify the system was the use of a transfected cell line. The stable expression of a panel of 3DL1 allotypes in the KIR negative Jurkat cell line allows for the dissection of the role of 3DL1 and its allotypes in a controlled homogenous background. The use of chimeric constructs, where binding to 3DL1 results in activation of CD3 zeta chain, allows for a positive output signal that can be easily measured as the production of a reporter protein. In a more biologically relevant setting, NK cell clones expressing 3DL1 alleles were used as effector cells in the lysis of target cells expressing HLA-B alleles. Interaction between 3DL1 and HLA-B was measured as the inhibition of lysis of HLA-B transfected cells relative to the HLA Class I negative parental cell line. Polyclonal NK cell cultures were used in a similar fashion. These cultures were homogenous in the expression of a single 3DL1 allele but co-expressed other HLA-specific receptors on subpopulations of the NK cells. In contrast to the clonal and polyclonal cultures that were maintained in IL-2 containing medium for several weeks, stimulation of freshly isolated NK cells allowed investigation of the interaction of resting primary NK cells. As cells of the innate immune system, NK cells are expected to act early in infection and short term cultures (here with and without cytokine) may be a more accurate reflection of *in vivo* interactions. Identification of 3DL1⁺ cells and assessment of the activation status by flow cytometry also removed the need to sort the cells or generate specific NK cell clones, but did not eliminate the presence of additional HLA-specific receptors present on subsets of 3DL1⁺ cells.

The complementary approaches used all confirmed the reported specificity of 3DL1 for HLA-Bw4, while providing evidence that variation within the HLA-Bw4 epitope and

between 3DL1 allotypes influence this interaction. For the *001 3DL1 allotype B*5101, B*3801 and B*5801 gave a strong signal in the Jurkat reporter assay, while B*1513 and B*2705 gave a very weak signal. For the same 3DL1 allotype inhibition of NK cell clone cytotoxicity gave a similar pattern with B*5101, B*3801 and B*5801 giving the greatest protection. B*1513 and B*2705 gave less protection in this assay, but in contrast to the Jurkat assay, both of these allotypes showed significantly more interaction than the Bw6 allotype B*0702. Similarly, while this pattern was also seen in the stimulation of primary cells, inhibition of both CD107a externalization and (to a lesser extent) induction of CD69 expression by B*1513 and B*2705 was greater than that seen with the Bw6 allotype. This disparity in the results between the transfection based assay and the NK cell clone and primary cells suggest that the interactions detected in the NK cell setting may be more informative. It is possible that additional receptor/ligand interactions in these cells promote or enhance binding between 3DL1 and B*1513 and B*2705.

3DL1 polymorphism also impacts on Bw4 recognition, with a different pattern of reactivity seen with 3DL1*002 in the Jurkat transfectant and clonal assays to that seen with 3DL1*001. 3DL1*005 was also found to have altered recognition as assessed by clonal and polyclonal inhibition assays. B*5101 proved to be a strong inhibitory ligand for all the allotypes tested, but the interaction with other HLA-Bw4 allotypes differed. A comparison of B*2705 and B*5801 interaction with *001 and *002 Jurkat transfectants suggests that B*5801 is a stronger ligand for *001 while B*2705 interacts to a greater extent with *002. This preference is also seen (less dramatically) in the clonal inhibition assay. *005 was not available as a chimeric receptor but clonal and polyclonal assay repeatedly identified B*3801 as a relatively weak ligand for this allotype.

Of the five HLA-Bw4 tested, four had identical sequences at the Bw4 epitope while the fifth (B*2705) was different. Among the four allotypes with the same Bw4 epitope, there was considerable variation in the interaction with 3DL1, suggesting the amino acids outside of the Bw4 epitope influence the interaction. This may be due to amino acid changes that cause conformational changes in the HLA protein. The importance of sites other than the Bw4 epitope had also been suggested by the report that an HLA-A allotype containing an

identical Bw4 sequence to B*1513 did not serve as a ligand for 3DL1, while B*1513 did (Gumperz et al, 1997). Previous reports had implicated the amino acid at position 80 as having particular importance – with reports of a preference for threonine or isoleucine made by separate groups (Cella et al, 1994; Luque et al, 1996). B*2705 contains threonine at this position while all other Bw4 types tested contain isoleucine, and this factor may contribute to the trend towards less protection seen by expression of this allotype in particular with 3DL1*001 and *005.

In addition to the altered amino acid sequences of HLA-Bw4 allotypes causing a direct influence on the interaction with 3DL1, the repertoire of presented peptides is also sensitive to polymorphism of HLA, especially within the α 1 helix (Barber et al, 1997). Altered profile of peptide may be responsible for the differing interaction as the area of contact between 3DL1 and HLA is predicted to involve the peptide binding groove. This interaction has been shown to be sensitive to peptide changes, especially at position P7 and P8 (Peruzzi et al, 1996a).

Much of the variation between *001 and *002 lie in the D0 domain of 3DL1. By comparison with 2D KIR that lack this domain, the D0 domain is not expected to interact directly with the HLA molecule. However, a study of the interaction of 3DL1 with B*5101 found that all three Ig-like domains (D0, D1 and D2) were necessary for binding suggesting that the D0 domain may contribute to the conformation of the ligand binding domain or may be important in ligand affinity (at least for B*5101) (Rojo et al, 1997). The modulatory role of D0 in 3DL1 binding is also supported by a study of the interaction of 3DL1 chimeric constructs containing point mutations in the D0 domain with Bw4 allotypes (Khakoo et al, 2002). Differences in this domain between *001 and *002 might be responsible for the variation in the interaction of these two 3DL1 allotypes with B*2705, although their reactivity with B*5101 was similar. In addition to variation in D0, *002 has the amino acid arginine at position 238 in the D2 domain in place of glycine in all other 3DL1 allotypes. Although this residue has not been directly implicated in ligand binding, this change from a small non-polar amino acid to one that is positively charged may influence binding or ligand specificity.

Our findings of the functional relevance of polymorphism within Bw4 and 3DL1 alleles is supported by a recent study of the interaction of two 3DL1 allotypes, *002 and *007 with B*2705 and B*5101. Although *002 was found to interact with both Bw4 allotypes, expression of B*5101 gave a greater level of protection than B*2705 from lysis by the NKL cell line retrovirally transduced with 3DL1*002 (Carr et al, 2005). In contrast, expression of B*2705 was found to give no protection from lysis by the *007 transduced NKL cell line. These data are in agreement with the results presented here for the specificity of the 3DL1*002 allotype and the findings with the *007 allotype are similar to our results with *001. Mutational analysis identified position 238 in the D2 domain and 320 in the transmembrane region as responsible for these differences. In contrast to this study and the work of Carr *et al*, Draghi *et al* found no difference in the inhibitory response of 3DL1 allotype to a single HLA-Bw4 allotype. B*5801 expressing cells were found to inhibit *002 and *005 expressing NK cells to a similar degree (Draghi et al, 2005).

Interaction between short-term cultured NK cells and HLA Class I negative cell line (parental 721.221) upregulated the activation marker CD69, activated NK cell cytotoxicity and, in the presence of IL-2, upregulated the IL-2 receptor α chain (CD25) and induced IFN- γ production. Production of IFN- γ was seen by up to 50% of NK cells, including a large proportion of CD56^{dim} cells. This is in contrast to the profile of IFN- γ producing cells reported in response to cytokine stimulation where CD56^{bright} cells are the main source of IFN- γ (Cooper et al, 2001). Our results are in agreement with a recent report that, in response to appropriate target cells, CD56^{dim} cells are capable of producing IFN- γ (Anfossi et al, 2006). Expression of a 3DL1 ligand by these cells was capable of preventing the induction of each of these activatory responses. Cytotoxicity and cytokine secretion are two of the main functional responses of NK cells and can be regulated independently e.g. ligation of KIR2DL4 by its HLA-G ligand induces IFN- γ but not cytotoxicity (Rajagopalan et al, 2001). The ability of 3DL1 ligation to inhibit IFN- γ after stimulation with susceptible target cells can be attributed to the dephosphorylation of ZAP70 and Syk protein kinases by SHP-1 (Plas et al, 1996), as IFN- γ production in response to target cell stimulation is absent in Zap70^{-/-}Syk^{-/-} mice (Colucci et al, 2002). While NK cell cytotoxicity is maintained in these mice, studies of human NK cells suggest that Syk is an important mediator of

cytotoxicity (Brumbaugh et al, 1997; Jiang et al, 2002). The deactivation of membrane proximal signals such as the ITAM-activated Syk and ZAP70 kinase thus allows for KIR ligation to inhibit a number of NK cell functions.

In addition to the inhibitory signal received through KIR ligation, NK cell activation is also controlled by HLA expression through CD94/NKG2A and LILRB1 receptors (Cosman et al, 1997; Lee et al, 1998). Our analysis of the relative contribution of KIR and CD94/NKG2A to the protection of a target cell bearing a single HLA allotype suggests that CD94/NKG2A provides relatively weak protection from lysis. Cells expressing B*3801, which provides a peptide to support HLA-E expression and thus a ligand for CD94, showed approximately 25% reduction in lysis by KIR⁺ NK cells relative to parental cell lysis or lysis in the presence of a CD94 blocking antibody. *In vivo*, a target cell would be expected to express two or more HLA molecules that support inhibition through CD94. In contrast to the KIR which bind to specific subsets of HLA, inhibitory signals are induced through CD94/NKG2A and LILRB1 due to the expression of a broad range of HLA-A, -B and -C allotypes. This difference, in combination with the clonal expression of these receptors, allows for specific subsets of NK cells to respond to different challenges. Loss of individual HLA allotypes, e.g. HLA-Bw4 allows activation of a subset of NK cells that depend on KIR specific for that HLA for inhibition, but would not affect NK cells expressing CD94/NKG2A. Loading of HLA-E with a peptide derived from the stress protein hsp60 has been shown to prevent recognition of CD94/NKG2A and may provide a mechanism for activation of CD94/NKG2A⁺ NK cells under stress conditions (Michaelsson et al, 2002).

3DL1, and other KIR family members, show a high level of allelic polymorphism and the number of described alleles continues to increase. As our knowledge of the genetics of the KIR genes increases, our understanding of the functional consequences of this variation lags behind. Using a number of complementary experimental approaches, we have described a role for polymorphism both within HLA-Bw4 and 3DL1 alleles in their interaction. Thus expression of 3DL1 allotypes contributes to the qualitative spectrum of NK cell responses to HLA-Bw4 ligation. An individual's KIR genotype, phenotype and HLA type may all influence the nature and extent of an immune response to infection or cellular stress.

Chapter Five

3DS1 expression and function

5.1 Introduction

Among the KIR family of NK cell receptors, there are several KIR which transmit activatory signals to NK cells. By comparison with other activatory KIR, the 3DL1 allele 3DS1 is believed to function as an activatory receptor. The expression, ligand specificity and function of 3DS1 are not well understood but several disease association studies have generated interest in uncovering its role in NK cell function.

5.1.1 2D Activatory KIR

Due to the lack of functional information on 3DS1, much of our understanding of this receptor comes from comparison with the activatory 2D KIR. An individual may express up to seven KIR family members that are believed to have activatory function - 2DS1-5, 2DL4 and 3DS1. In contrast to inhibitory KIR that transmit inhibitory signals through their cytoplasmic ITIM motifs, short tailed activatory KIR transmit positive signals via interaction with the adaptor protein DAP12 (Figure 5.1.1). 2DL4 is an unusual activatory KIR as it has a long cytoplasmic tail and interacts with and signals through the FcR γ protein (Kikuchi-Maki et al, 2005).

DAP12 is a transmembrane protein that exists as a disulfide-linked homodimer and contains a single ITAM signalling domain (Husain et al, 2002; Rajagopalan et al, 2001). An aspartic acid residue in the transmembrane domain allows it to associate with short-tailed KIR. Activation and phosphorylation of DAP12 lead to association with ZAP-70 and Syk protein tyrosine kinases (Lanier et al, 1998; McVicar et al, 1998). Signalling through DAP12-associated receptors leads to an increase in intracellular calcium, activation of the MAP kinase pathway and the induction of NK cell cytotoxicity and cytokine production (McVicar et al, 1998).

As many of the available anti-2DS antibodies also bind to 2DL KIR, assessment of the expression of these activatory KIR by flow cytometry is not possible. However, RT-PCR analysis of NK cell clones (Valiante et al, 1997) and single NK cells (Husain et al, 2002)

show that 2DS KIR are expressed by a variable number of NK cells (<10% (2DS1) to >70% (2DS4) in individual donors) and may be expressed by fewer NK cells than long tailed KIR. While initial reports suggested that 2DL4 was expressed by all NK cells (Valiante et al, 1997), this view has recently been challenged (Husain et al, 2002; Rajagopalan et al, 2001).

Sequence alignment of the 2D activatory KIR reveals that 2DS1 and 2DS2 show a high degree of similarity in the extracellular domain to the inhibitory 2DL1 and 2DL2/3 respectively. 2DS4 resembles 2DS2, while 2DS3 and 2DS5 are more closely related to 2DS1. 2DL4 is a unique KIR with a long cytoplasmic tail KIR containing a single ITIM motif as well as a charged residue in the transmembrane region. Interaction with its ligand HLA-G results in the transduction of a positive signal via FcR γ protein (Kikuchi-Maki et al, 2005). The high degree of homology to inhibitory KIR in the extracellular domain of 2DS1, 2DS2 and, to a lesser extent, 2DS4 suggested that they might share ligand specificity (Bianconi et al, 1996).

While recognition of HLA-C by the 2DS KIR has been supported by increased killing of target cells expressing HLA-C (Moretta et al, 1995), direct binding has been more difficult to prove (Vales-Gomez et al, 1998). Soluble Ig fusion proteins of 2DL1 and 2DS1 revealed that the inhibitory receptor displays a much greater affinity for its HLA-C ligands than 2DS1 (Vales-Gomez et al, 1998). This difference in binding affinity was mapped to position 70 of the KIR molecule, with substitution of the threonine of 2DL1 for the lysine of 2DS1 diminishing the binding of the long tailed KIR (Bianconi et al, 1997). Similarly, the binding of 2DS2 to HLA-C is of much lower affinity than that of the corresponding inhibitory KIR (2DL2/3). This receptor differs from 2DL2/3 by only 3/4 amino acids in the extracellular domain - substitution at position 45 (a tyrosine in 2DS2) for phenylalanine was found to enhance the affinity of the 2DS KIR for HLA-C ligand (Vales-Gomez et al, 1998; Winter et al, 1998). The two residues (45 and 70) in the short tailed KIR that are responsible for the reduced affinity are both positioned in loop structures involved in the interaction with the β -strands of HLA-C. 2DS4, which differs from 2DL KIR by 23 amino

acids in the extracellular domain, has also been reported to bind to HLA-C, but with low affinity (Katz et al, 2001). The ligand specificity of 2DS3 and 2DS5 is unknown.

The weak nature of the binding of 2DS KIR to HLA-C calls into question the physiological significance of this interaction. It has been suggested that this interaction may be controlled by additional factors, including the up-regulation of HLA on target cells by IFN- γ during infection, or restriction by the profile of peptide presented. EBV infection has been found to increase the cell surface expression of HLA and in turn the binding of 2DS1 tetramers (Stewart et al, 2005). These 2DS1 tetramers were also used to investigate the peptide specificity of the short tailed KIR, which was found to closely resemble that of 2DL1. This study was able to reproduce an earlier finding that C1R cells (which express Cw4) were lysed due to 2DS1 ligation, but despite the recognition of HLA-Cw6⁺ cells by 2DS1 tetramer, 721.221 cells expressing Cw6 were not lysed in a 2DS1-dependent manner. This result suggested that ligation of 2DS1 may not in itself lead to NK cell cytotoxicity and that the simultaneous engagement of additional activatory receptors may be necessary. A recent report has suggested a non-HLA ligand for 2DS4 based on the binding of soluble 2DS4-Ig protein to HLA-negative melanoma cells. This binding was found to be functional and to increase the lysis of these cells. Although the ligand was not identified, treatment of the cells with the proteinase K enzyme abrogated the binding of 2DS4-Ig, suggesting that it is a protein molecule. The residues involved in the recognition of the melanoma cell by 2DS4 are distinct from those involved in HLA-C binding (Katz et al, 2004).

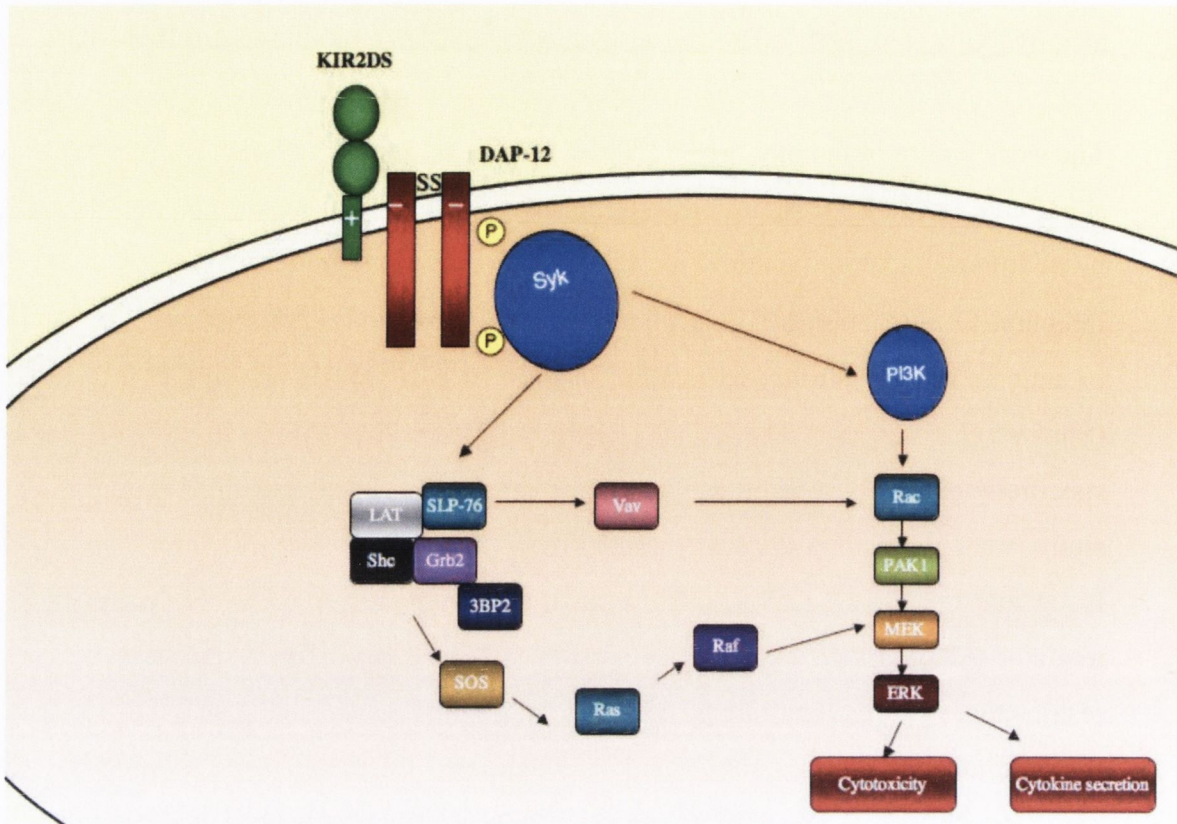


Figure 5.1.1 Signalling by Short tailed Activatory KIR

Activatory KIR induce signals in NK cells through interaction with the adaptor protein DAP12 via the charged amino acid in the transmembrane domain. Recruitment of the protein tyrosine kinase Syk induces a signalling cascade that results in the activation of the MAP kinases ERK1 and 2 and the induction of cytotoxicity and cytokine production.

5.1.2 3DS1

Pairs of inhibitory and activatory 2D KIR exist as the products of separate genes encoded by the LRC on chromosome 19. In contrast, the 3D KIR 3DL1 and 3DS1 are alleles of the same gene. Thus, if 3DS1 does function as an activatory receptor, an individual may encode either an inhibitory or activatory protein at the same locus. The short-tailed 3DS1 is a common allele of 3DL1, found in approximately 40% of Caucasian populations. It is highly similar to long-tailed 3DL1 allotypes in the extracellular domain, differing by only 6 amino acids and contains the positively charged amino acid lysine in the transmembrane region, characteristic of activatory KIR.

5.1.3 NK cells in HIV Infection

NK cells have long been known to play an important role in certain viral infections but their role in the control of HIV is not well understood. Several studies have correlated NK cell receptor genotype, cell number or activity to control of the virus or resistance to infection. NK cells are believed to contribute to the anti-HIV response both through the production of soluble mediators and lysis of appropriate target cells.

NK cells can produce a large range of chemokines including XCL1, CCL1, CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), CCL22 AND CXCL8 (1L-8) (Robertson, 2002). Some of these chemokines including RANTES, MIP-1 α and MIP-1 β bind to the CCR5 receptor, required by R5 strains of HIV virus for entry into the target cell. NK cell culture supernatant containing these chemokines has been shown to suppress endogenous HIV replication in autologous CD4⁺ T cells due to the presence of CC-chemokines (Fehniger et al, 1998; Oliva et al, 1998).

NK cells may also target infected cells for lysis, both through natural lysis due to the down-regulation of certain HLA class I molecules and antibody dependent cellular cytotoxicity (ADCC). HLA expression on the surface of HIV-infected cells is modulated by the viral protein nef. This protein has been shown to down-regulate HLA-A and -B but has no

significant effect on HLA-C and -E (Cohen et al, 1999). It has been suggested that this selective down-regulation allows the virally infected cells to escape from CTL mediated lysis while maintaining inhibition of NK cell activity. NK cells commonly express inhibitory receptors including CD94/NKG2A and 2DL that are triggered by HLA-E and -C. The subset of NK cells that do not express these receptors have been shown to mediate killing of autologous HIV-infected T cell blasts, and this effect is not seen in T cells infected with virus incapable of down-regulating HLA (Bonaparte and Barker, 2004). NK cells that depend on HLA-B to inhibit their activation (e.g. via 3DL1 ligation) may play an important role in this setting.

The role of NK cells in HIV is also supported by studies of HIV-exposed uninfected (EU) individuals. NK cells derived from a group of Colombian sexually HIV EU individuals showed increased IFN- γ production (Montoya et al, 2006). Similarly in a group of Vietnamese highly EU intravenous drug users, NK cell activity was increased, both in terms of increased cell lysis and the production of cytokines such as IFN- γ , TNF- α and CC chemokines (Scott-Algara et al, 2003). A follow-up study of this cohort revealed that NK cells in these individuals are characterised by high-level constitutive activation as measured by degranulation activity (CD107a) and expression of CD69 and CD161 (Ravet et al, 2007). In addition, the ratio of NKG2C⁺ cells to NKG2A⁺ cells was increased in some of these individuals, favouring an activatory rather than inhibitory response to HLA-E. Analysis of 3DL1 RNA transcripts by real time PCR quantification revealed that, in some EUs, a reduction in inhibitory 3DL1 transcripts together with an increase in 3DS1 transcript gave a high 3DS1/3DL1 ratio. This would also be predicted to enhance NK cell activation. Taken together these data suggest that a strong NK cell response at the time of exposure or very early in infection may protect these individuals from infection.

In addition to evidence supporting the role of NK cells in control of HIV, HIV also appears to alter NK cell function. A number of phenotypic changes in cell surface receptor expression on NK cells have been reported in HIV, including an increase in inhibitory receptors such as LILRB1 and KIR, and a decrease in the triggering NCRs (Costa et al, 2001; Kottlilil et al, 2004; Mavilio et al, 2003). Reduction in the expression of the NCRs

(NKp30, NKp44 and NKp46) is associated with diminished lysis of different tumour cell lines and may be in part responsible for the decrease in NK cell cytotoxicity and the development of opportunistic tumours in HIV patients (De Maria et al, 2003).

A subgroup of HIV patients termed “long term non progressors” (LTNP) show delayed disease progression (Easterbrook, 1999). This group of patients control viral load and maintain high CD4 counts for many years after infection in the absence of antiretroviral therapy. Some contributing viral factors have been identified (e.g. nef deletions resulting in decreased infectivity (Geffin et al, 2000)), but in the majority of cases, it is the response of the immune system of the infected individual that controls the disease. Several host genetic factors have been associated with LTNP including HLA-B*27 (den Uyl et al, 2004), HLA-B*57 (Altfeld et al, 2003) and mutations in the chemokine receptor CCR5 (Huang et al, 1996). More recently a genetic study has implicated 3DS1 with delayed progression to AIDS in HIV infected patients (Martin et al, 2002). A study of over 1,000 HIV-infected individuals found that individuals with 3DS1, only in the presence of HLA-Bw4 allotypes with isoleucine at position 80 (Ile80) remained disease free for longer. The protective effect was greatest when outcomes considered CD4 counts less than $200/\text{mm}^3$ (RH = 0.58). In contrast, in the absence of HLA-Bw4 Ile80, 3DS1 homozygosity was significantly associated with more rapid progression to AIDS (CD4<200, RH =1.5). A follow-up study found that the 3DS1/Bw4Ile80 genotype was protective against the development of opportunistic infections but not AIDS-related malignancies (Qi et al, 2006). This result raised the possibility that the effect seen for 3DS1/Bw4Ile80 on progression to AIDS may be due to the protection against opportunistic infections rather than direct control of the virus. However, 3DS1/Bw4Ile80 individuals were found to have a lower mean viral load set point (the average of viral loads determined between six months and three years after infection) than individuals without this genotype. These data suggest that 3DS1/Bw4Ile80 acts early in the course of infection to control viral replication in addition to its later protective effect against opportunistic infections.

In this chapter, we investigate the expression, ligand specificity and function of 3DS1 in NK cells. The data generated by genetic analysis suggest that 3DS1 acts as a receptor for certain HLA-Bw4 allotype leading to functional activation of 3DS1⁺ cells. However, much

of the basic analysis of 3DS1 expression and function is lacking. The current interpretation of the genetic associations is dependent on cell surface expression of the 3DS1 receptor but this has not been demonstrated due to the lack of a specific serological reagent. In addition, the activatory nature of the receptor has been inferred from sequence similarity to 2D activatory KIR in the transmembrane/cytosolic domains but has not been definitively demonstrated. The function and phenotype of NK cells in HIV infection was also examined, with particular interest in the cohort of HIV-infected individuals who show natural immunity to the virus.

5.2 Aims

The aims of this chapter were

- Characterise the cell surface expression of 3DS1
- Investigate the interaction between 3DS1 and HLA-Bw4 allotypes
- Characterise the function and phenotype of NK cells in HIV LTNP patients

5.3 Results

5.3.1 Generation of FLAG-tagged 3DS1 construct

To address the question of cell surface expression of 3DS1, a FLAG-tagged construct was generated. Addition of a FLAG-tag at the N terminal (extracellular) portion of the molecule would allow for staining of cell surface 3DS1 using an anti-FLAG antibody. The correct position of the FLAG tag in the mature 3DS1 protein required insertion of the FLAG sequence between the leader sequence (which is cleaved and absent in the mature protein) and the first immunoglobulin domain. The cloning strategy is outlined in Figure 5.1.

A full-length 3DS1 sequence was generated from a 3DS1 homozygous donor using a proofreading polymerase with the primer sequences and PCR conditions outlined in Tables 2.7 and 2.8. The PCR product consisted of two bands – one of the correct size (1200 bp) and a lower band that corresponds to a 2D KIR (Figure 5.2). The correct band was excised, gel purified and cloned into the sequencing vector pCR®II-TOPO. The sequence of the construct was verified by commercial sequencing (MWG). An error free clone was then subcloned (using the restriction sites for *NotI* and *EcoR1*) into the pcDEFIII vector.

The FLAG sequence was added by primer addition to the 3' end of the 3DS1 leader sequence and the 5' end of the 3DS1 D0 sequence using the primer sequences and PCR conditions outlined in Tables 2.7 and 2.9. The PCR products generated were small (116 bp and 81 bp) and were visualized on a 2 % MetaPhor agarose gel (Figure 5.3). Recombinant PCR using these products as template with conditions and primer sequences outlined in Tables 2.7 and 2.9 generated a product consisting of the 3DS1 leader-FLAG-D0 sequence as a single band of 170 bp (Figure 5.4). This product was cloned into the pCR®II-TOPO vector and the sequence of the construct and the presence of the FLAG tag were verified by commercial sequencing (Figure 5.5).

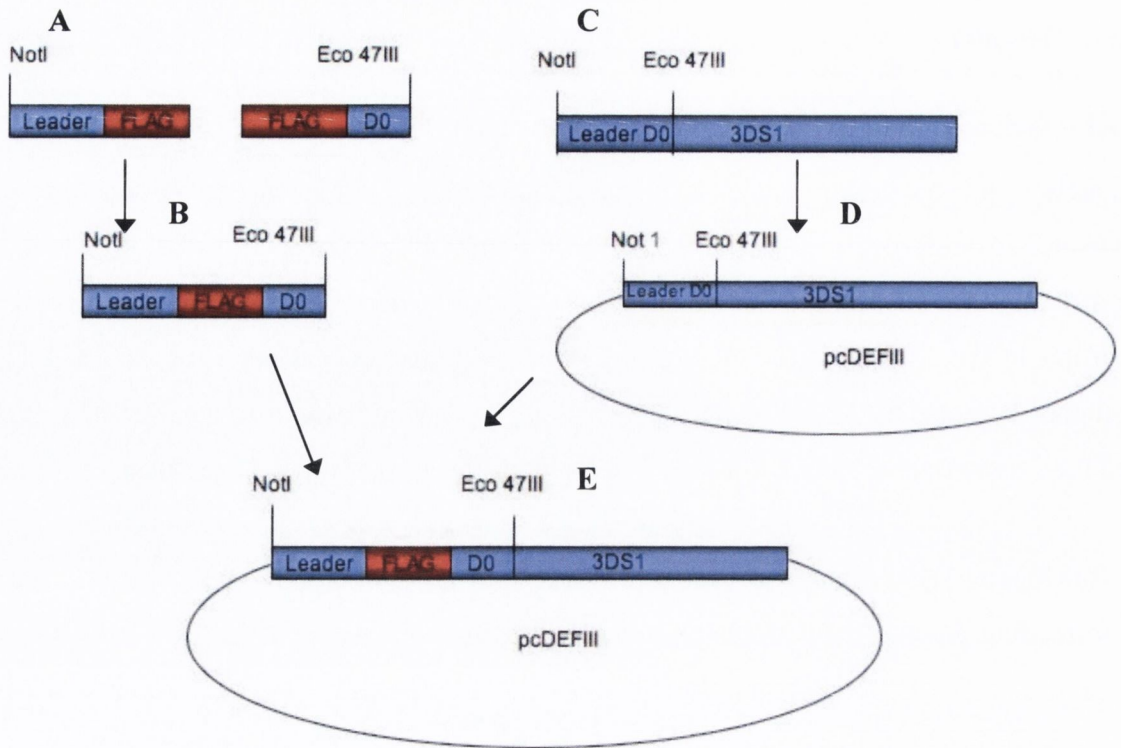


Figure 5.1 Cloning Strategy for the generation of a FLAG-tagged 3DS1

To generate a mature 3DS1 protein containing the FLAG sequence at the N terminus, the FLAG sequence needed to be inserted after the leader sequence of the protein. A, Leader and D0 sequences were amplified by PCR. The FLAG sequence and *Not* I restriction site were introduced by primer additions. B, Recombinant PCR was then used to generate a leaderFLAGD0 sequence. C, In parallel, full length 3DS1 was amplified. *Not* I and *Eco* R1 restriction sites were introduced by primer additions. D, 3DS1 sequence was cloned into a sequencing vector, sequenced and an error free clone was subcloned into the mammalian expression vector pcDEFIII. E, Digestion of leaderFLAGD0 sequence and 3DS1 sequences with *Not* I and *Eco* 47III allowed the excision of the wild type leaderD0 sequence and its replacement with the leaderFLAGD0 sequence

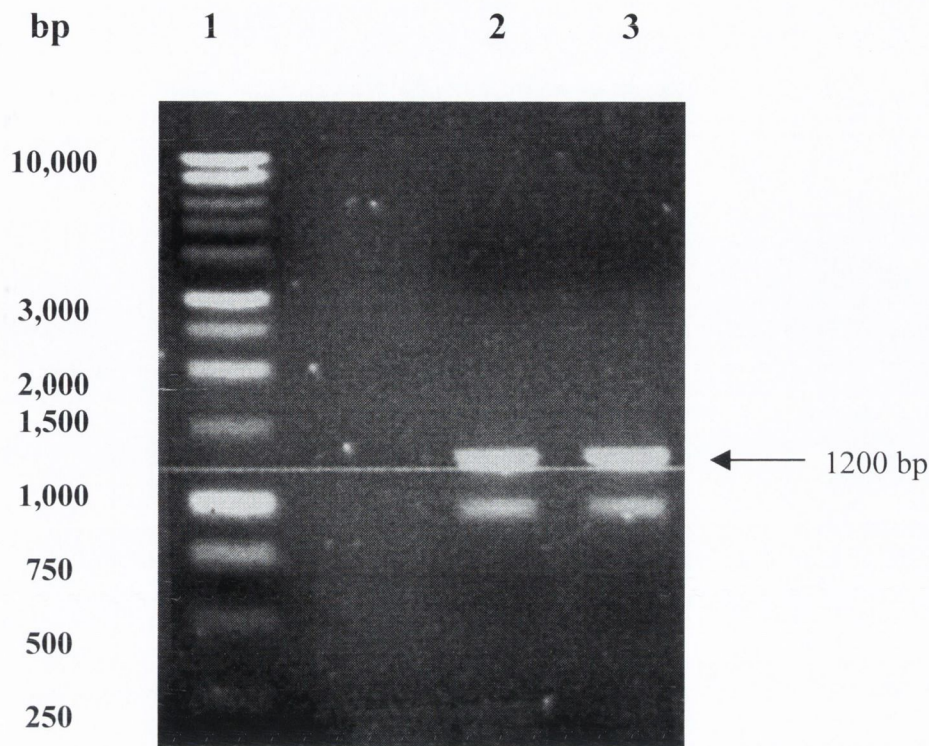


Figure 5.2 Full length 3DS1 construct was generated by PCR

cDNA was produced from the RNA of two 3DS1 positive donors. Full-length 3DS1 construct was generated using the primers and cycling conditions outlined in Table 2.7 and 2.8 respectively. Lane 1 contains a DNA ladder, lanes 2 and 3 contain full length 3DS1 amplified from cDNA generated from two donors. Upper band is present at the expected size (1,200 bp).

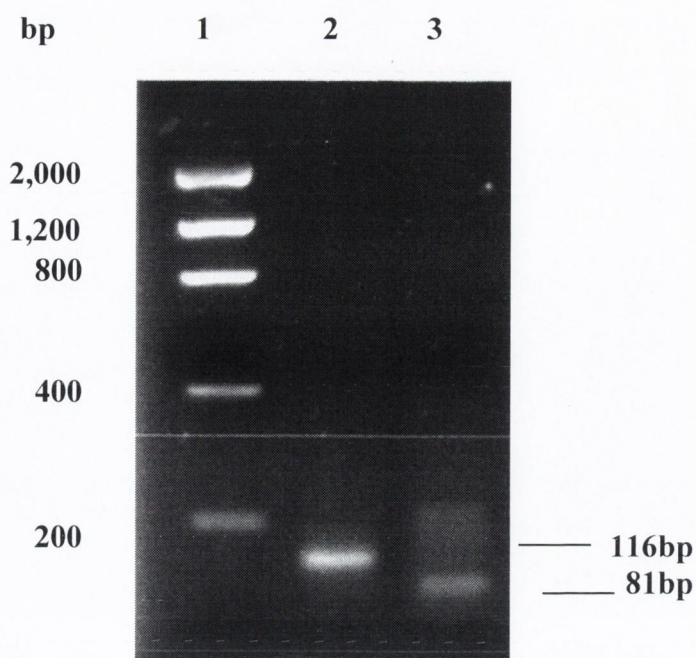


Figure 5.3 Leader-FLAG and FLAG-D0 were generated by PCR

cDNA was generated from the RNA of a 3DS1 positive donor. The 3DS1 leader (lane 2) and D0 sequences (lane 3) were amplified by PCR. The FLAG sequence was added to the 3' end of the leader sequence and the 5' end of the D0 sequences by primer additions using the primers and cycling conditions outlined in Tables 2.4 and 2.5. Lane 1 contains a DNA ladder.

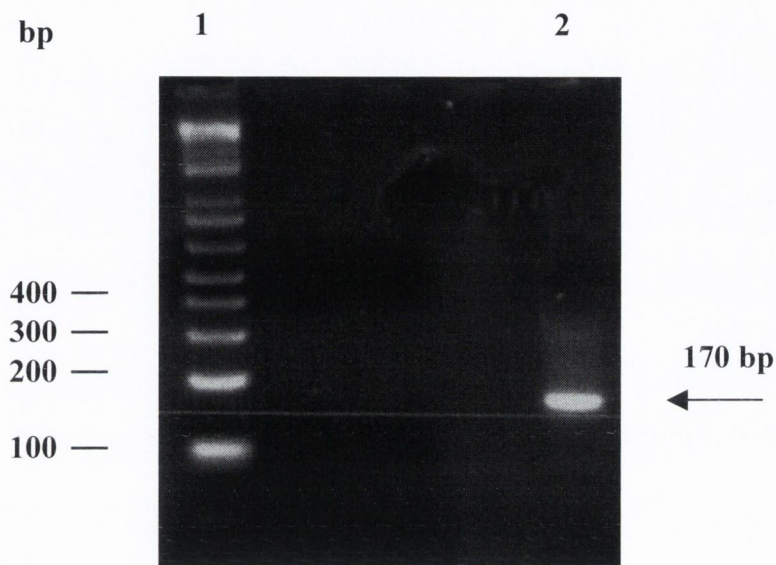


Figure 5.4 Leader-FLAG-D0 construct was generated by recombinant PCR

Leader-FLAG and FLAG-D0 PCR products were used as a template for the generation of leader-FLAG-D0 construct using the primers and cycling conditions described in Tables 2.3 and 2.4 respectively. Lane 1 contains a DNA ladder, lane 2 contains the recombinant PCR product.

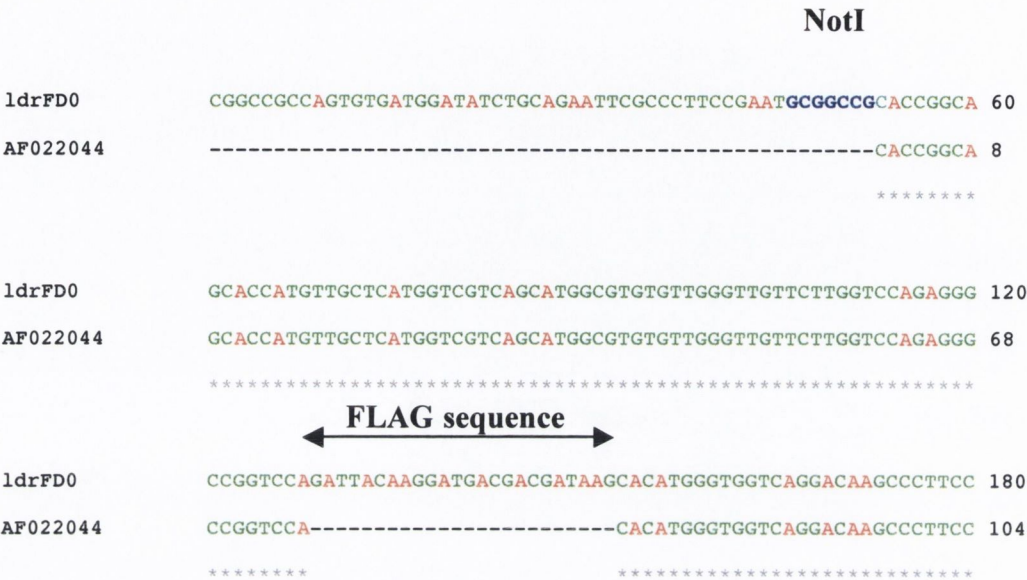


Figure 5.5 Presence of FLAG insert was verified by sequencing

Leader-FLAG-D0 construct was cloned into the sequencing vector pCR®II-TOPO and prepared for commercial sequencing by MWG using the T7 primer. The insert sequence obtained (ldrFD0) was aligned with 3DS1 sequence (accession number AF022044) using ClustalW alignment program. *Not* I restriction sites and FLAG insert are highlighted.

The recombinant 3DS1 leader-FLAG-D0 product and the full-length 3DS1 pcDEFIII construct were digested using the *Not* I and *Eco* 47III restriction enzymes. The digested 3DS1 leader-FLAG-D0 was then ligated into the digested 3DS1 pcDEFIII plasmid (see Figure 5.1). Plasmid DNA containing full-length 3DS1 sequence with a FLAG epitope at the N terminus was harvested from transformed competent *E. coli* cells using a Qiagen MidiPrep.

5.3.2 3DS1 cell surface expression

The 3DS1FLAG plasmid was transfected into the Jurkat T and the Thp-1 monocyte cell line by electroporation. The cells were maintained in medium supplemented with the selection agent G418 for 4 days and assessed for expression of the 3DS1 construct using an anti-FLAG antibody. Expression of 3DS1 was detected at the cell surface of both cell lines (Figure 5.6 and data not shown) providing clear evidence that 3DS1 is expressed at the cell surface.

5.3.3 3DS1 recognition by Z27 antibody

PBMCs from 3DL1 allele typed donors (Chapter 3) were routinely stained with two anti-3DL1 antibodies – clone DX9 and Z27 which were originally believed to have the same specificity. However, the pattern of reactivity of the Z27 clone was found to differ from that of the DX9 clone, with the Z27 showing an additional very dim peak present in some donors. Over 60 genotyped donors were analysed for the pattern of Z27 reactivity and a very dim Z27 peak was found to be present only in individuals who were positive for 3DS1 (Figure 5.7), suggesting that this dim peak was due to binding of Z27 to 3DS1.

To confirm the specificity of the Z27 antibody for 3DS1, the 3DS1ECD Jurkat cell line that had been stably transfected with the extracellular domain of 3DS1 fused to the CD3 zeta signalling chain (see Chapter 4) was stained with Z27. The 3DS1 transfectant, but not the parental Jurkat cell line stained with Z27 (Figure 5.8).

To examine the expression of 3DS1 on primary NK cell, NK cells were isolated from PBMCs and stained with the Z27 antibody. Z27 dim and Z27 negative cells were cell

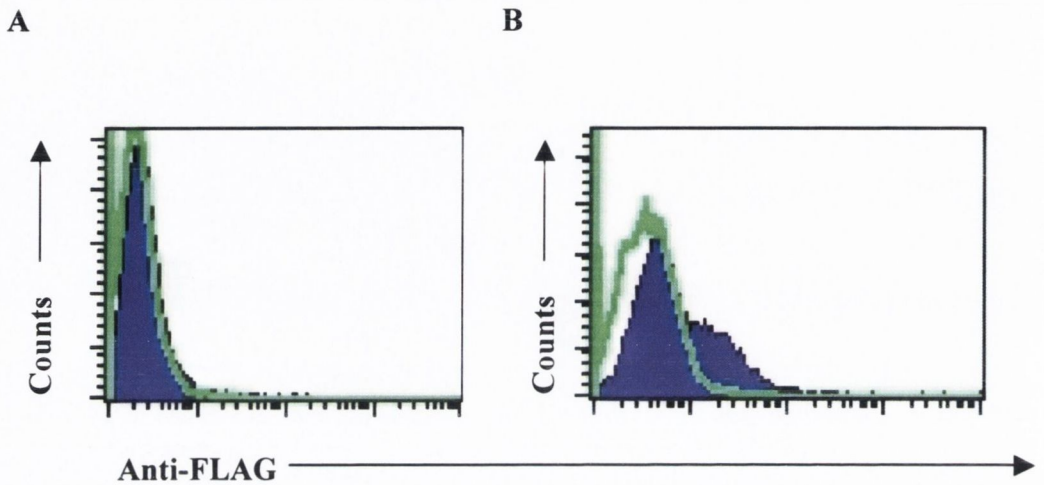


Figure 5.6 3DS1 is expressed at the cell surface

Flow cytometric analysis of Jurkat T cells transfected with FLAG-tagged 3DS1 construct (filled purple histogram, B) or mock transfected (A). Green histogram represents cells stained with an isotype-matched control antibody. Cells were maintained in RPMI supplemented with 2 mg/ml G418 for 7 days and then stained with anti-FLAG FITC antibody.

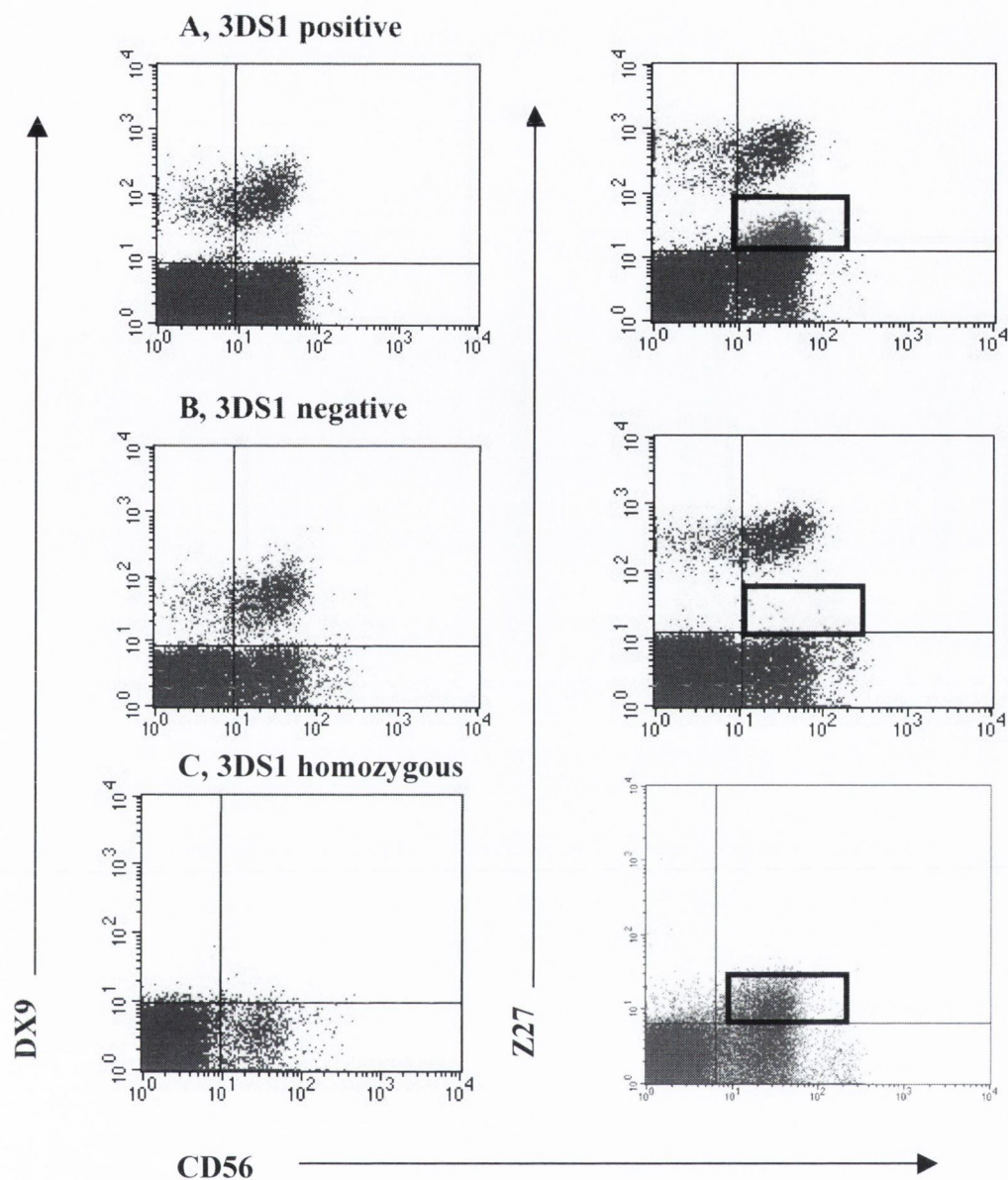


Figure 5.7 Z27 recognises 3DS1 on the surface of NK cells

PBMCs were stained using anti-CD56 FITC and DX9 PE (left panel) or Z27 PE (right panel) and analysed by flow cytometry after gating on the lymphocyte population. Results shown are representative of different genotypes including 3DS1 positive (A), 3DS1 negative (B) and 3DS1 homozygous donors (C). Results are representative of over 60 phenotyped donors.

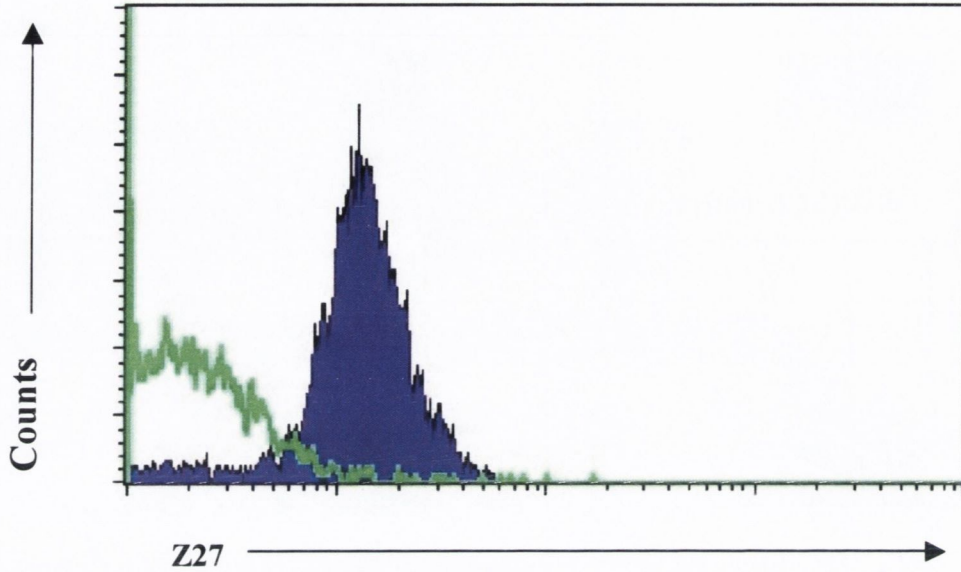


Figure 5.8 **Z27 recognises 3DS1 on the surface of 3DS1ECD Jurkat cells**

Jurkat cells stably expressing 3DS1ECD were stained with Z27 antibody and analysed by flow cytometry. The purple filled histogram shows transfected cells stained with antibody; the green empty histogram show untransfected controls. Results shown are representative of 3 independent experiments.

sorted and RNA was isolated from these two populations in three donors. The presence of 2DL4 (present in all NK cells, cDNA quality control) and 3DS1 mRNA was assessed by RT-PCR analysis using the primers and PCR conditions outlined in Table 2.5 and 2.6. 2DL4 was found to be present in both Z27 dim and Z27 negative populations for all donors (Figure 5.9). 3DS1 mRNA was found in Z27 dim but not in Z27 negative populations from each of the three donors (Figure 5.9).

These results prove that the Z27 antibody recognises 3DS1 at the cell surface and that Z27 dim staining populations represent 3DS1⁺ cells.

5.3.4 3DS1 expression profile

The identification of an antibody that recognizes 3DS1 allowed for analysis of the expression profile of 3DS1. We analysed the percentage of NK (CD56⁺CD3⁻) and T (CD3⁺CD56⁻) cells expressing 3DS1 in the peripheral blood of donors who possess the 3DS1 allotype. The percentage of NK cells expressing 3DS1 was variable ranging from 4.26% to 27.9% (mean 15.5%) of the peripheral blood NK cells staining Z27 dim (Figure 5.10). 0.2% to 3.6% (mean 1%) of peripheral blood T cells expressed 3DS1. Expression of 3DS1 on CD4⁺ and CD8⁺ T cell subsets was also examined. CD8⁺, but not CD4⁺ T cells express 3DS1 at the cell surface (Figure 5.11).

5.3.5 3DS1 acts as an activatory KIR

By comparison with other short-tailed KIR, ligation of 3DS1 is predicted to activate NK cells. To test this hypothesis, sorted 3DS1⁺ and 3DS1⁻ NK cells were isolated from three donors and stimulated for 18 hours by crosslinking of CD56 (negative control), Z27 or CD16 (positive control). Expression of CD69 was then examined by flow cytometry (Figure 5.12). As expected both 3DS1⁺ and 3DS1⁻ NK population increased CD69 expression after crosslinking of CD16. Crosslinking of 3DS1 using the Z27 antibody lead to activation of the 3DS1⁺ but not 3DS1⁻ NK cells, with over 1.5 fold increase in the percentage of CD69⁺ cells. Thus signalling through 3DS1 leads to the activation of NK cells.

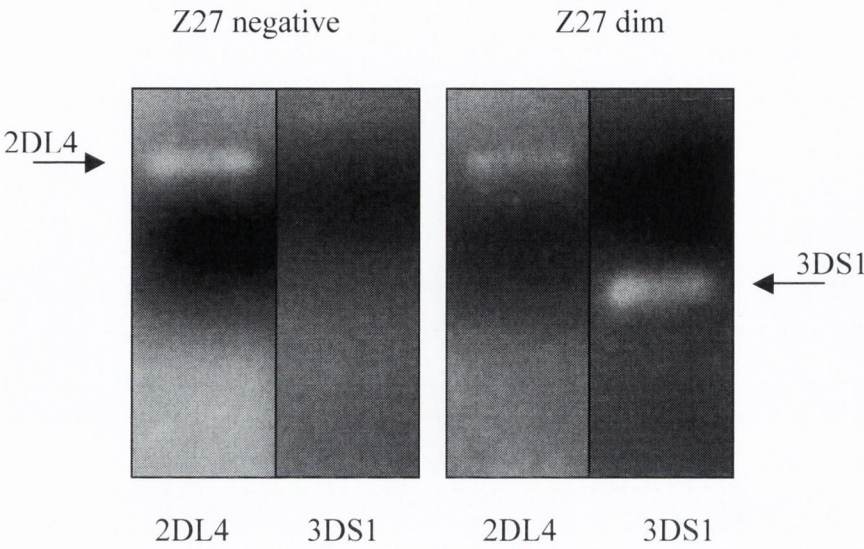


Figure 5.9 Sorted Z27dim cells express 3DS1

cDNA was generated from sorted Z27-negative and Z27-dim staining NK cell populations. RT-PCR was used to examine expression of 2DL4 (positive control for quality of cDNA) and 3DS1.

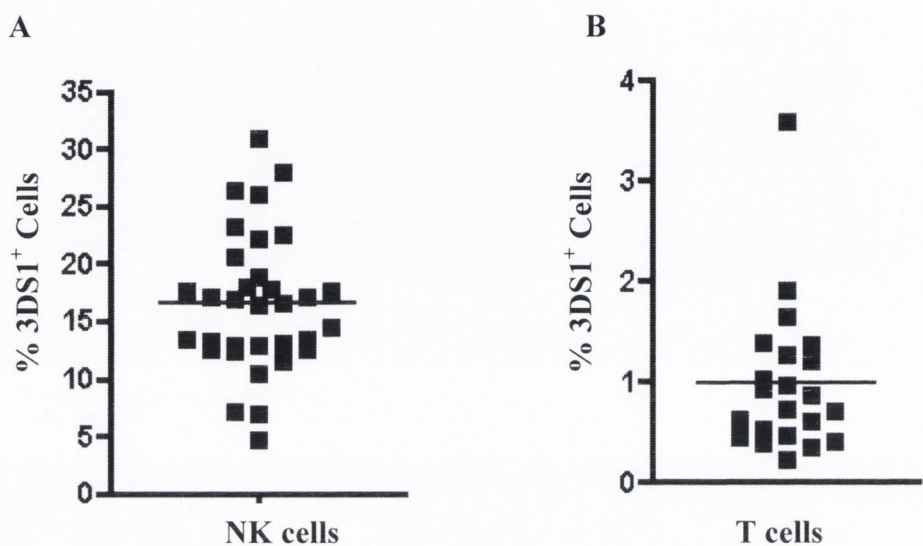


Figure 5.10 Expression of 3DS1 on NK and T cells

Isolated PBMCs were stained with anti-CD56 FITC, anti-CD3 PerCP and Z27 PE antibodies. The frequency of 3DS1 positive (Z27dim) NK cells (CD56⁺CD3⁻, A) and T cells (CD3⁺CD56⁻, B) was quantified by flow cytometry after gating on lymphocyte cells.

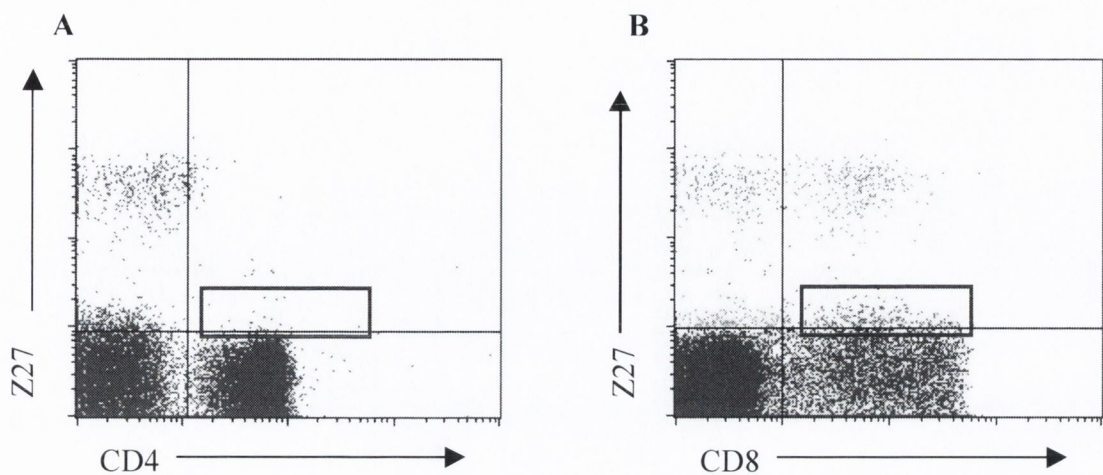


Figure 5.11 3DS1 is expressed on CD8⁺ but not CD4⁺ T cells

Isolated PBMCs were stained with anti-CD4, anti-CD8 and Z27 antibodies and analysed by flow cytometry. 3DS1 positive cells were identified as a Z27-dim staining population on CD4⁺ T cells (A) and CD8⁺ T cells (B).

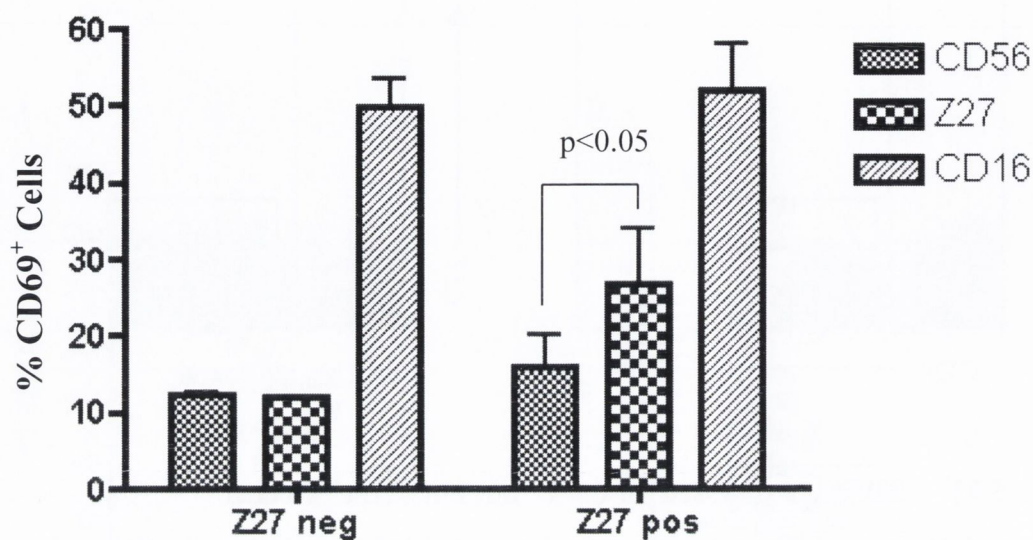


Figure 5.12 Crosslinking of 3DS1 leads to NK cell activation

Freshly isolated NK cells were sorted based on the expression of 3DS1 (Z27 dim) and the 3DS1 receptor was triggered by crosslinking with the Z27 antibody. Cells were analysed for CD69 expression by flow cytometry 18 hours post-stimulation. Data above is from one donor representative of three; error bars show standard deviation. CD69 expression in the presence of control Ig and Z27 antibodies was compared using a Student's T-test.

5.3.6 3DS1 ligand specificity

Due to high similarity in the ligand-binding domain, 3DS1 and other (inhibitory) allotypes of 3DL1 were predicted to have an overlapping ligand affinity. 3DS1 interaction with HLA-Bw4 was assessed in a similar manner to that used for the other 3DL1 allotypes (Chapter 4).

Jurkat T cells, stably transfected with a construct consisting of the extracellular domain of 3DS1 fused to the CD3 zeta chain (and GFP protein) were transiently transfected with an NFAT reporter plasmid and stimulated with a panel of 721.221 cell transfectants (see Figure 4.1). Stimulation of the cells with CD3/CD28 microbeads produced an NFAT signal but no interaction between 3DS1 and any of the HLA-Bw4 allotypes tested was seen (Figure 5.13A). Crosslinking of 3DS1 using plate bound Z27 antibody was capable of generating a signal through the chimeric construct and gave an NFAT signal (Figure 5.13B). Similar results were seen with 3DS1ECD Jurkat cells that were sorted on the basis of GFP expression to generate a population expressing the 3DS1 construct at a high surface density. The ratio of stimulator to responder cells (typically 0.2:1) was also varied (from 5:1 to 0.1:1) to no effect (data not shown).

3DS1⁺ and 3DS1⁻ NK cells from three donors were sorted on the basis of Z27 staining and polyclonal cultures were expanded in IL-2 containing medium. These cells were used as effector cells against the panel of 721.221 cell line transfectants in a standard chromium release assay. Although there was variation in the degree of lysis of each cell line, this did not differ between 3DS1⁺ and 3DS1⁻ NK cell populations and blocking of 3DS1 using the Z27 antibody had no effect (Figure 5.14).

5.3.7 Tetramer binding studies

The peptide bound by HLA-Bw4 allotypes has been shown to influence recognition by 3DL1 allotypes and we speculated that recognition of HLA-Bw4 by 3DS1 might be restricted by the presented peptide. B*2705 tetramers (a complex of four HLA-B*2705 molecules folded in the presence of a single defined peptide) are a useful tool for analysis of peptide specificity.

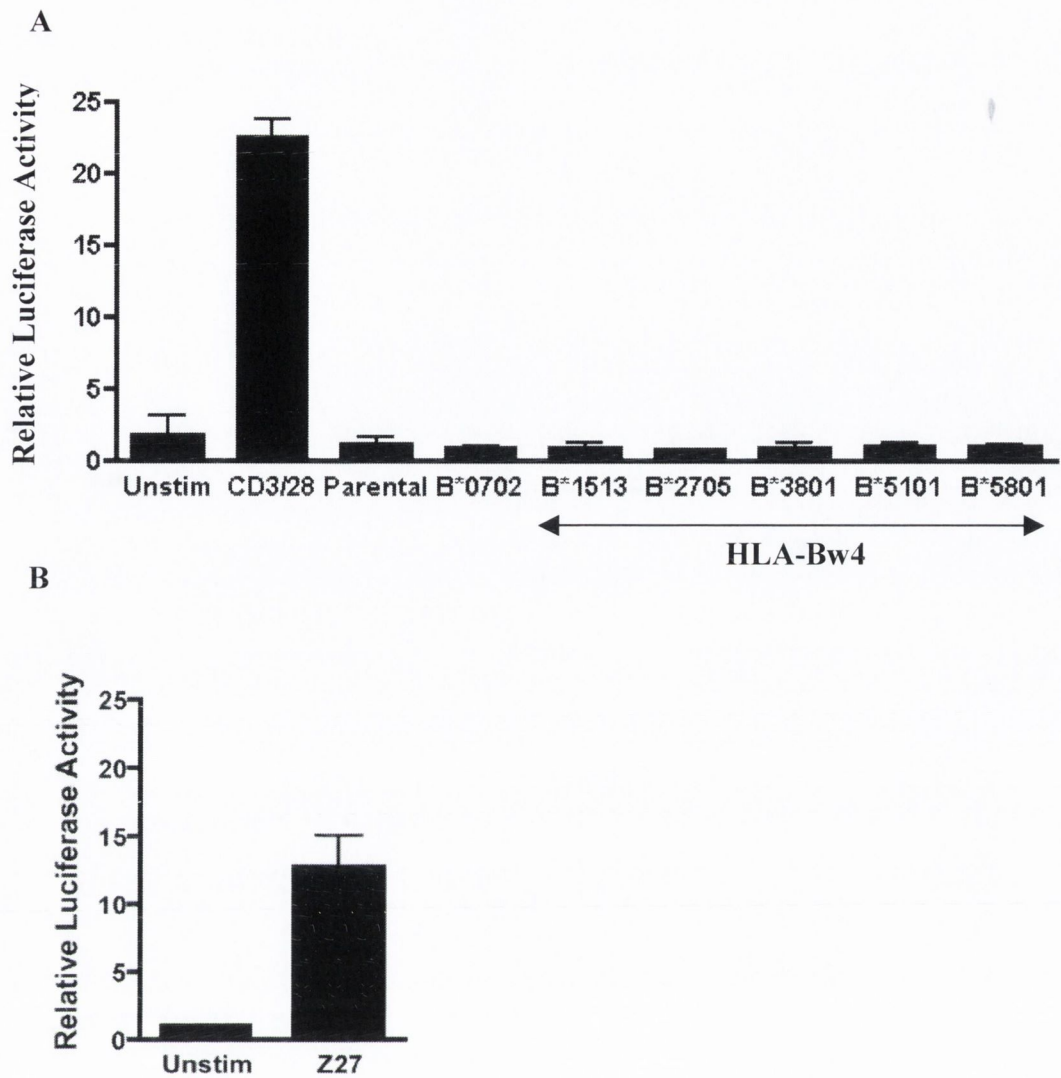


Figure 5.13 3DS1ECD Jurkat cells do not recognise HLA-B on EBV transformed B cells

A, Jurkat cells expressing 3DS1ECD were stimulated with 721.221 cells (0.2X) and a panel of its transfectants. Firefly luciferase activity was measured after 18 hours as a read-out of NFAT activation. CD3/CD28 Dynabeads® (3 beads/cell) were used as a positive control to activate NFAT in the Jurkat cell line. Crosslinking of 3DS1 with Z27 antibody (2 µg/ml) was used as a positive control to show functional signal transduction through the chimeric 3DS1 construct (panel B). Results are representative of five (721.221 stimulation) or three (Z27 crosslinking) independent experiments. Error bars show SD.

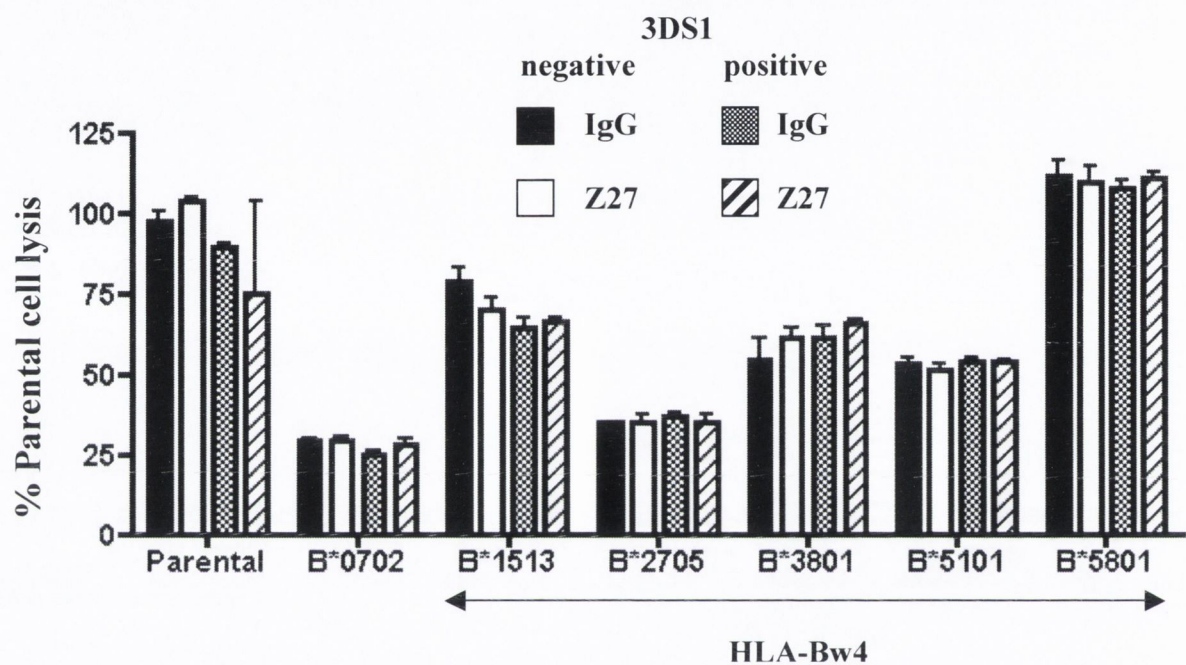


Figure 5.14 3DS1 positive NK cells are not activated by HLA-B

3DS1 positive cells were identified by Z27 staining, sorted, expanded and used as effectors against a panel of 721.221 transfectants in a 4 h Cr release cytotoxicity assay. Cytotoxicity is presented as percentage killing of 721.221 parental cell line. Results shown are representative of three donors. Error bars show SD.

B*2705 monomers, a gift from Dr. Simon Kollnberger (Weatherall Institute of Molecular Medicine, Oxford, UK) were incubated with extravidin PE to promote the formation of B*27 tetramers. The correct formation and folding of the tetramers was tested by examining their binding to LILRB1. For this purpose a Baf3 cell line transfected with LILRB1 was used. This cell line expresses LILRB1 at a high cell surface density at the cell surface (Figure 5.15). All tetramers used bound to the Baf3 LILRB1 transfectant cell line but not to the parental Baf3 cell line, showing that the tetramers have adopted the correct folding (Figure 5.16).

3DL1*002, 3DS1 and parental Jurkat cell lines were stained using the B*2705 tetramers (Figure 5.17). The parental Jurkat cell line did not show any binding with any tetramer used (Figure 5.17A). 3DL1*002 Jurkat cell line showed strong binding to B*2705 tetramers folded in the presence of an RKSTEL self peptide (see Table 2.2). In contrast, 3DS1 Jurkat did not show significant binding to this tetramer. The 3DL1*002 Jurkat cell line showed a weaker level of binding to a B*2705 tetramer folded in the presence of a flu-derived peptide. The 3DS1 Jurkat cell line also bound to this tetramer, with the staining showing all cells binding, giving a distinct positive peak (Figure 5.17C).

To test for the specificity of the interaction between 3DS1 and B*2705 in the presence of the flu peptide the cells were incubated with the blocking Z27 antibody prior to staining with the tetramer. Incubation with Z27 was not capable of blocking the interaction (Figure 5.18B), suggesting that, despite the binding to 3DS1 transfected but not parental Jurkat cells (Figure 5.18A), the binding seen was not due to specific interaction between 3DS1 and the B*2705 tetramer. To examine the effect of non-specific protein interactions, 3DS1 Jurkat cells were stained with HLA-A2 tetramer, which it is not expected to bind. The HLA-A2 tetramer showed a significant level of binding to the 3DS1 transfectant, equalling the level of binding seen by the B*2705 flu tetramer (Figure 5.19).

While the tetramer approach to identifying a 3DS1 ligand did not yield any definite results, the approach shows some promise. If the interactions seen between 3DS1 and the B*2705 tetramers were purely non-specific protein interaction, it would not be expected that there

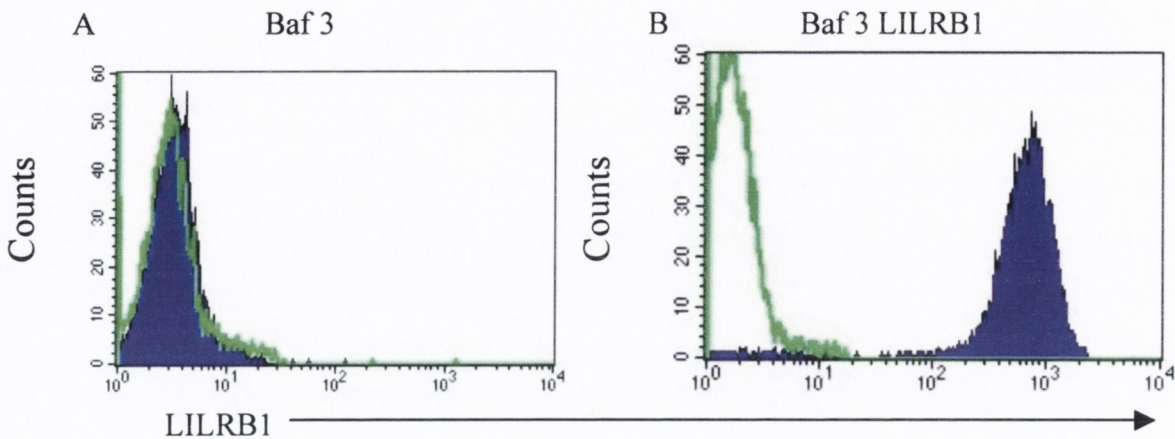


Figure 5.15 LILRB1 expresssion on Baf3 transfectants

Expression of LILRB1 on parental Baf3 and LILRB1 Baf3 transfectants was monitored by flow cytometry. A, Baf3 cells were stained with anti-LILRB1 antibody (purple fill); green histogram represents control isotype binding. B, Baf3 LILRB1 transfectant cells were stained with anti-LILRB1 antibody (purple filled histogram); green histogram represents control isotype binding.

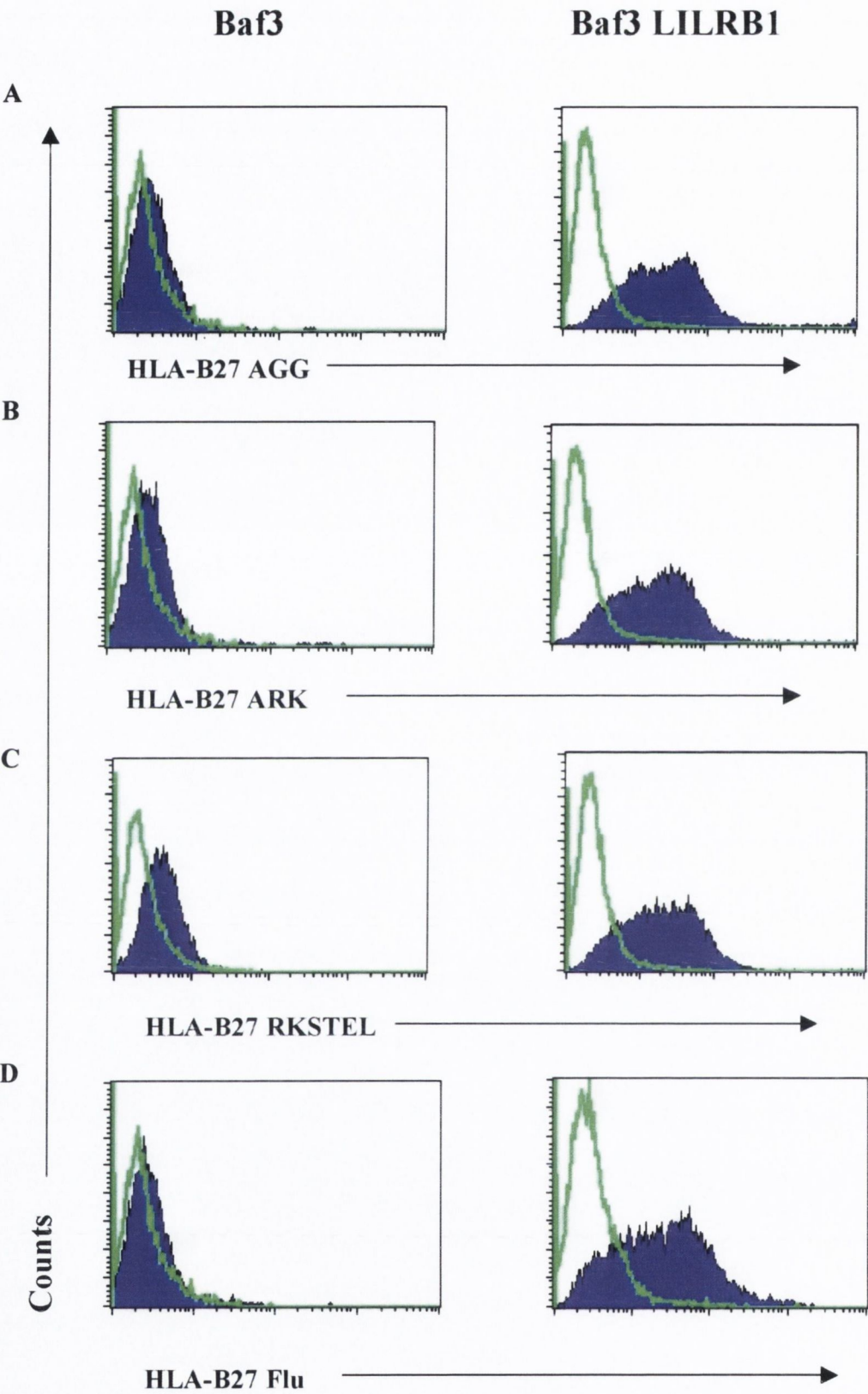


Figure 5.16 HLA tetramers bind LILRB1

Parental Baf3 (left panel) and a LILRB1 transfectant (right panel) were stained with HLA-B27 tetramers - HLA-B27 AGG (panel A), ARK (panel B), RKSTEL (panel C), and flu NP (panel D). Purple filled histograms represent tetramer staining. Green histograms represent control staining with extravidin PE.

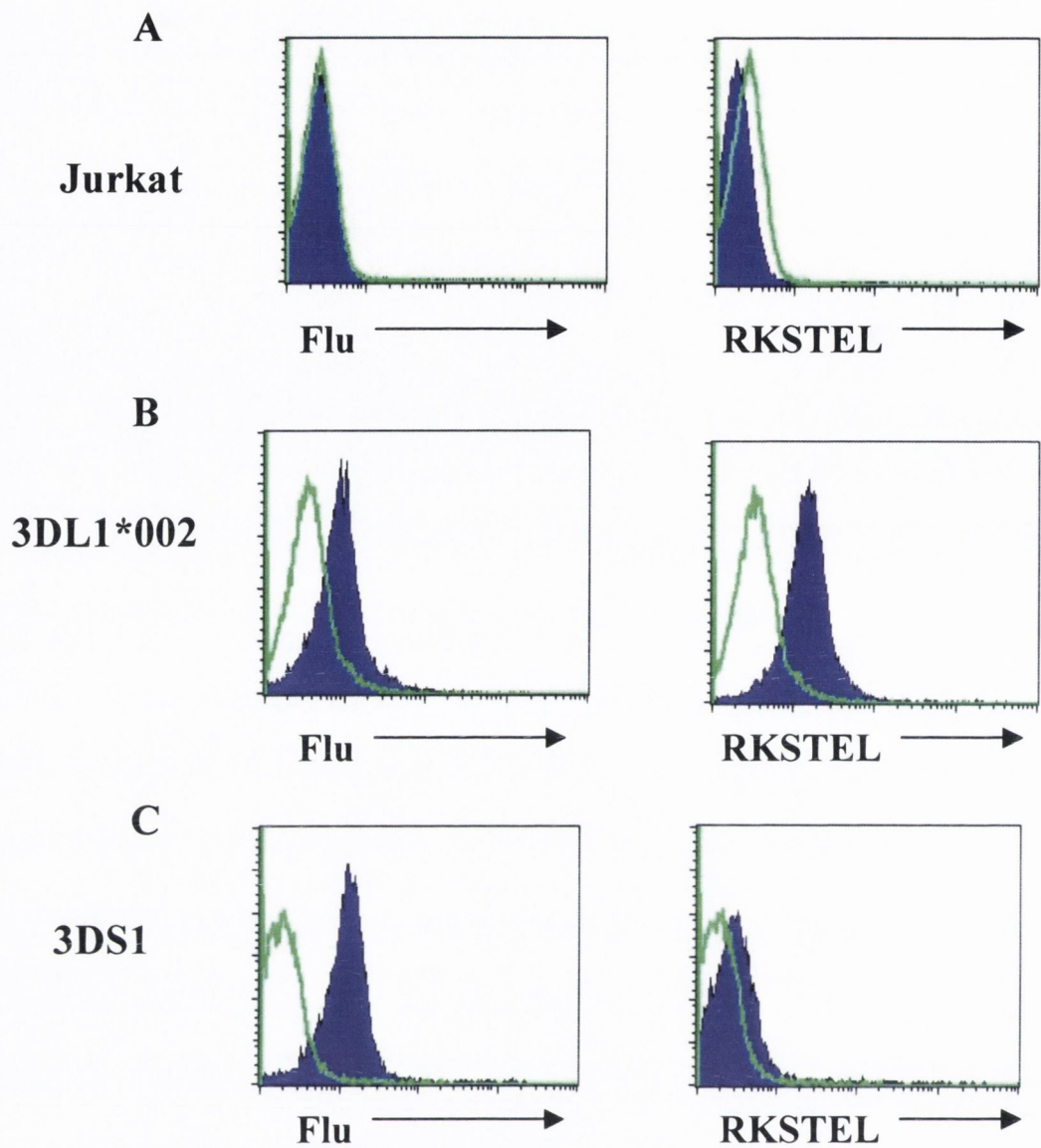


Figure 5.17 3DL1*002 and 3DS1 show different affinity for HLA-B27 tetramers

Jurkat (A), 3DL1*002 (B) and 3DS1 (C) cell lines were stained with HLA-B27 flu (left panel) and HLA-B27 RKSTEL (right panel). Purple filled histograms represent tetramer staining. Green histograms represent extravidin PE binding.

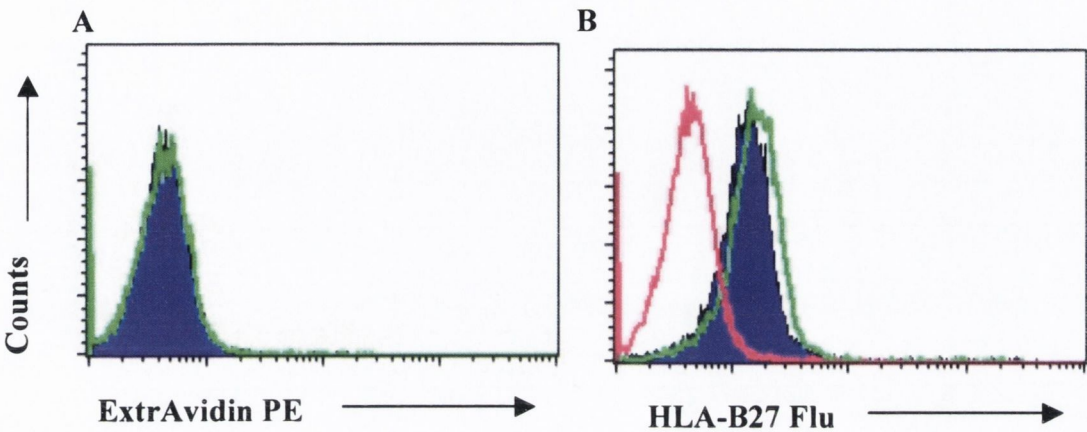


Figure 5.18 Binding of HLA-B27 tetramers to 3DS1 was not inhibited by Z27 antibody

3DS1 cells were stained with extravidin PE (panel A, purple filled histogram) or HLA-B27 Flu tetramers (panel B, purple fill). Cells were incubated with Z27 antibody (10µg/ml) on ice for 15 minutes prior to staining with tetramer (green histogram). Extravidin PE staining is shown in pink overlay.

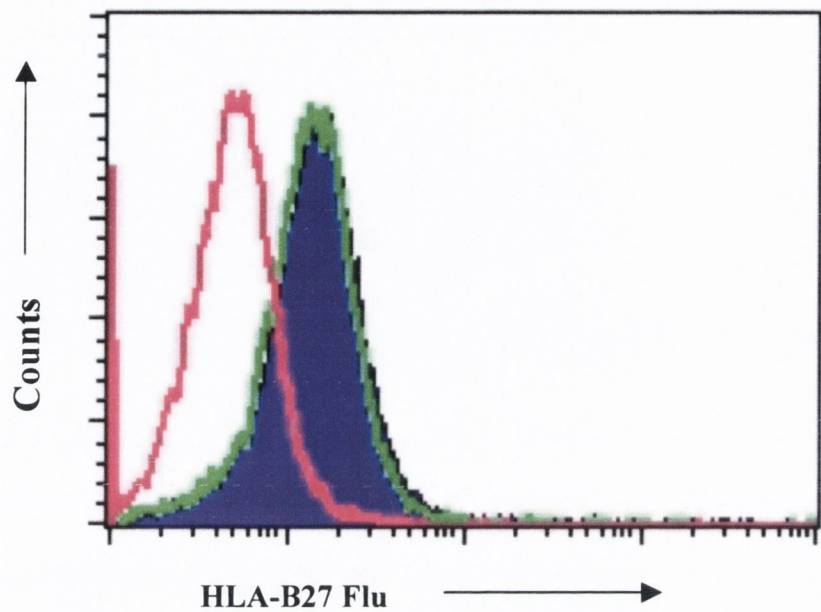


Figure 5.19 3DS1 cell line shows significant staining with HLA-A2 tetramer.

3DS1 cell line was stained with HLA-B27 flu tetramers (purple filled histogram). Cells were also stained with a control HLA-A2 CMV tetramer (green histogram) and with extravidin PE alone (pink overlay).

would be distinct differences between the level of binding in the parental, 3DL1*002 and 3DS1 Jurkat cells, as was seen in this study. Titration of the tetramer concentration to minimise non-specific interactions in conjunction with control staining with an irrelevant HLA tetramer may prove useful in optimizing this approach in the future.

5.3.8 NK cell function and phenotype in HIV and HIV LTNP

To examine the function of NK cells in HIV infection and in particular in HIV LTNP we examined NK cell function and phenotype in control, HIV viremic and HIV LTNP. Control samples were collected from low risk exposure patients of the St James GUIDE clinic who tested negative for HIV and other sexually transmitted infections. HIV viremic patients were patients who had a high viral load and were not at the time (or in the previous two years) on any anti-retroviral treatment. HIV LTNP patients were defined as asymptomatic HIV infected for ten or more years with a HIV viral load of less than 5,000 copies/ml and a CD4 cell count greater than $300 \times 10^6/\text{dL}$ in the absence of antiretroviral therapy. All HIV patients were infected with HIV-1 strain of the virus. The details on patient groups are shown in Table 5.1.

5.3.8.1 NK cell function in HIV and HIV LTNP

NK cell cytotoxicity within the PBMC population was measured in control HIV negative (n=8), HIV viremic, not undergoing treatment (n=8) and HIV LTNP (n=9) patients. At an E:T of 100:1 control patients show a large range (8.9% to 44.5% maximum cell lysis) of cytotoxic capacity (Figure 5.20A). HIV viremic patients show a reduced level of killing relative to control levels ($p < 0.05$). HIV LTNP patients show a greater level of cytotoxicity than viremic HIV, although this does not reach the levels of control patients (Figure 5.20A). Cytotoxicity was also measured at E:T of 50:1 and 25:1. The same trend is seen at these ratios – HIV viremic patients show reduced killing relative to controls and HIV LTNP patients show a level of cytotoxicity intermediate between control and HIV viremic levels (Figure 5.20B). The same trend was seen when the data was expressed as lytic units (LU20) values (data not shown).

	n	Median HIV viral load (copies/ml)	Median CD4 cell counts (x 106/dl)	Mean Years since HIV diagnosis
Viremic HIV	8	56,105 (22,000-500,000)	306 (58-1086)	6 (1-18)
HIV LTNP	9	793 (<50-4,500)	507 (321-769)	17 (10-22)
Control	8	N/A	N/A	N/A

Table 5.1 Characteristics of viremic HIV, HIV LTNP and control patients.

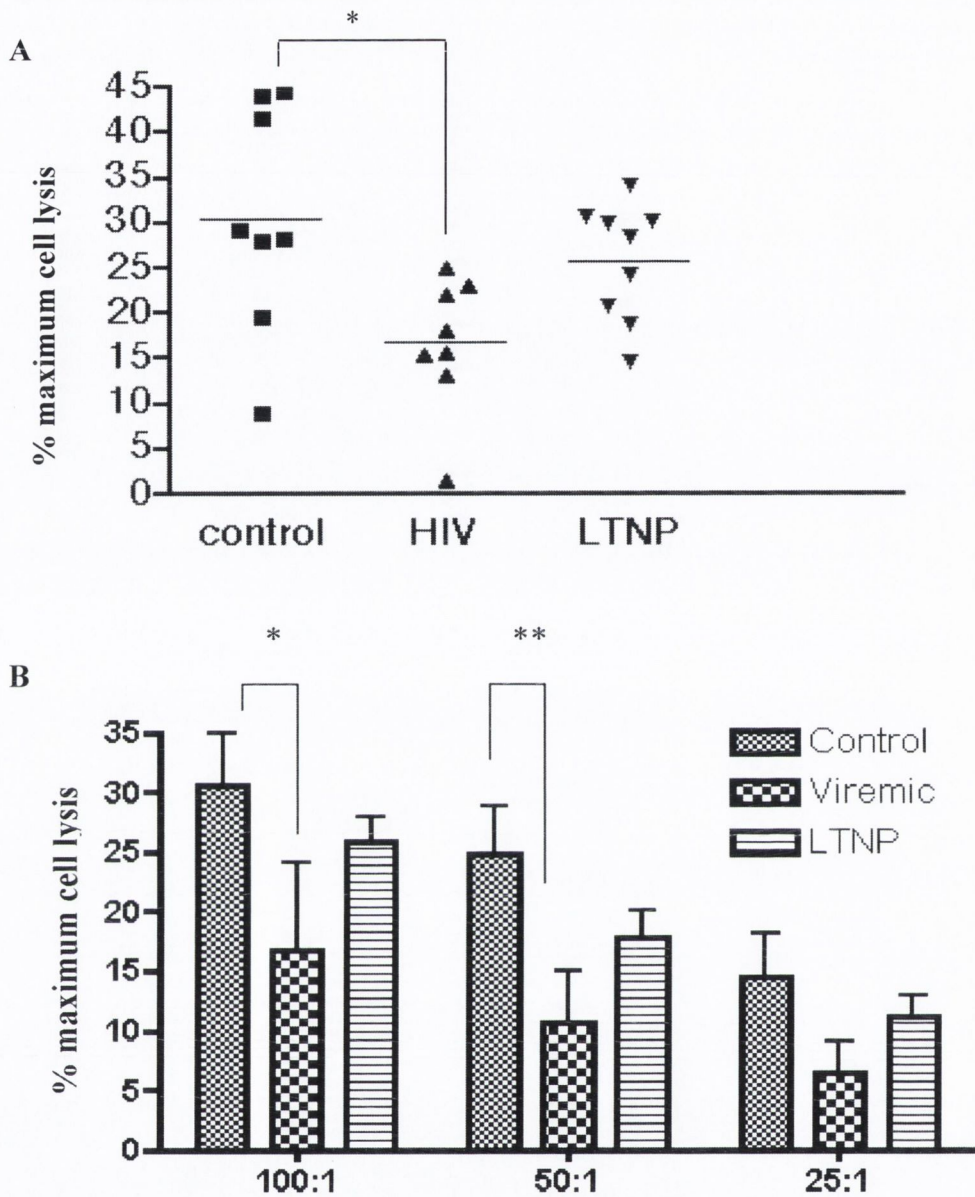


Figure 5.20 NK cell cytotoxicity is maintained in LTNPs

PBMCs were isolated from the blood of control, HIV and HIV LTNP patients. Cytotoxicity was assessed using a standard 4hr ^{51}Cr release assay against K562 targets at an effector: target of 100:1, 50:1 and 25:1. **A**, shows the range of values obtained at an effector:target ratio of 100:1. **B**, The average value at each effector:target ratio is shown, error bars represent standard error of the mean. *, $p < 0.05$; **, $p < 0.01$.

5.3.8.2 Percentage NK cells

The percentage of NK cells ($CD56^+CD3^-$) present within the lymphocyte gate in control, HIV viremic and HIV LTNP patients was measured. Both HIV and HIV LTNP patients show a reduction in the percentage of NK cells relative to control, although the reduction is slightly less for HIV LTNP (Figure 5.21). This pattern of reduction is seen in both the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets (data not shown). The level of $CD56^-CD16^+$ NK cells previously reported to be expanded during HIV was not found to differ among the three groups (data not shown). There was no correlation between the percentage of NK cells and the level of cytotoxicity, suggesting the observed reduction in killing seen is not due solely to a decreased percentage of NK cells (data not shown).

5.3.8.2 3DS1 in HIV and HIV LTNP

We were interested in 3DS1 function in HIV and HIV LTNP due to the genetic association reported between the presence of 3DS1 and certain HLA-Bw4 alleles and a delayed progression to AIDS. 3DS1 was present in 3/8 control, 2/8 HIV viremic and 2/9 HIV LTNP as measured by Z27 dim staining and KIR genotyping (Figure 5.22). However, the numbers of patients positive for 3DS1 in each group was too low to allow any meaningful analysis.

5.3.8.3 Expression of NK cell associated receptors in HIV and HIV LTNP

The expression of a number of NK cell receptors was examined on the NK cells ($CD56^+CD3^-$) and T cells ($CD3^+CD56^-$) of control, HIV viremic and HIV LTNP patients. Figure 5.23 shows a representative dot plot for the expression of each receptor on NK cells. The anti-KIR antibodies (Figure 5.23A-C) stained a variable portion of the $CD56^{dim}$ NK cells, but did not stain $CD56^{bright}$ cells. The NCRs, NKp30 and NKp46, were found on the majority of NK cells, while NKp44 did not show significant staining in any of the samples (Figure 5.23D-F). CD94 was expressed on a large percentage of NK cells, with brighter expression seen on $CD56^{bright}$ cells (Figure 5.23G). A smaller percentage of NK cells were positive for NKG2A, this receptor was expressed by a greater percentage of $CD56^{bright}$ NK

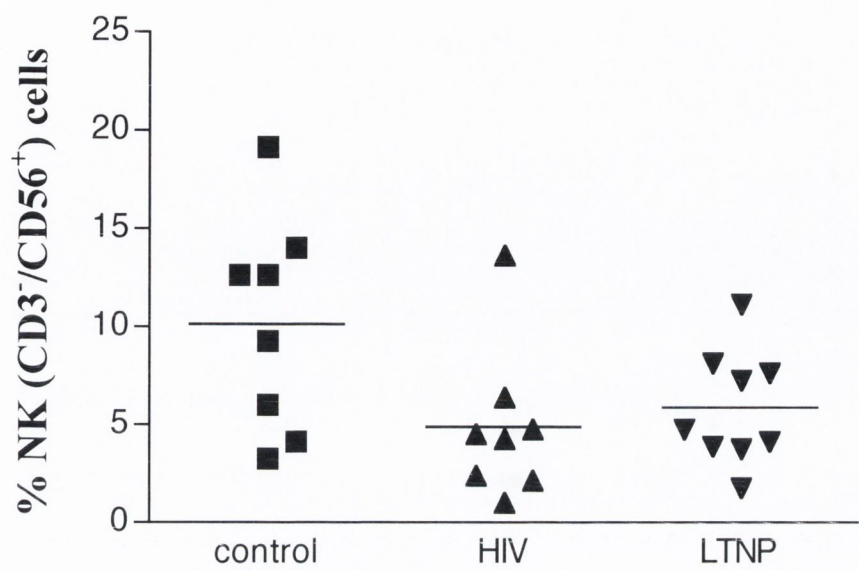
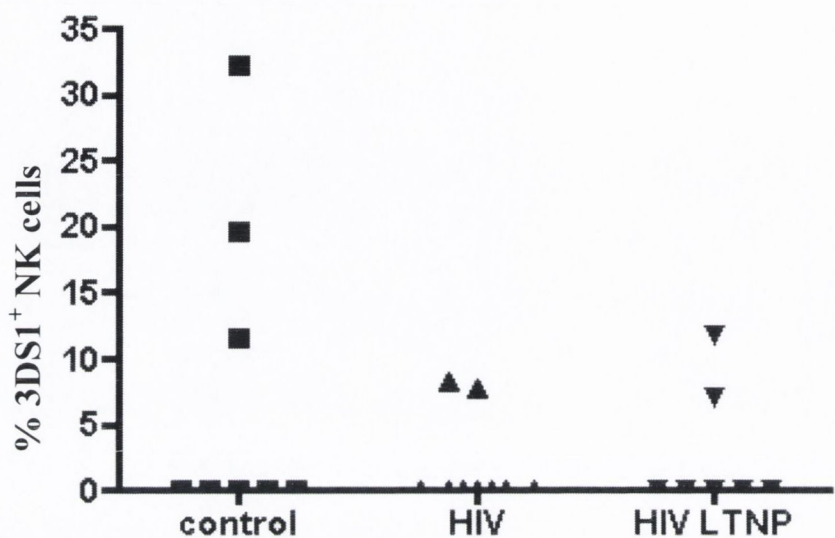


Figure 5.21 Percentage of NK cells is reduced in HIV and HIV LTNP.
PBMCs were isolated from the blood of control, HIV and HIV LTNP patients. Percentage of NK cells (CD56⁺/CD3⁺ lymphocytes) was assessed by flow cytometry.



	control	HIV	HIV LTNP
n	3	2	2
mean	21.11	8.02	9.41

Figure 5.22 3DS1 expression in HIV

Gated NK cells (CD56⁺/CD3⁻ lymphocytes) from control, HIV and HIV LTNP patients were examined for expression of 3DS1 using the Z27 anti-KIR antibody. Table shows mean percentage of positive NK cells for donors who express 3DS1.

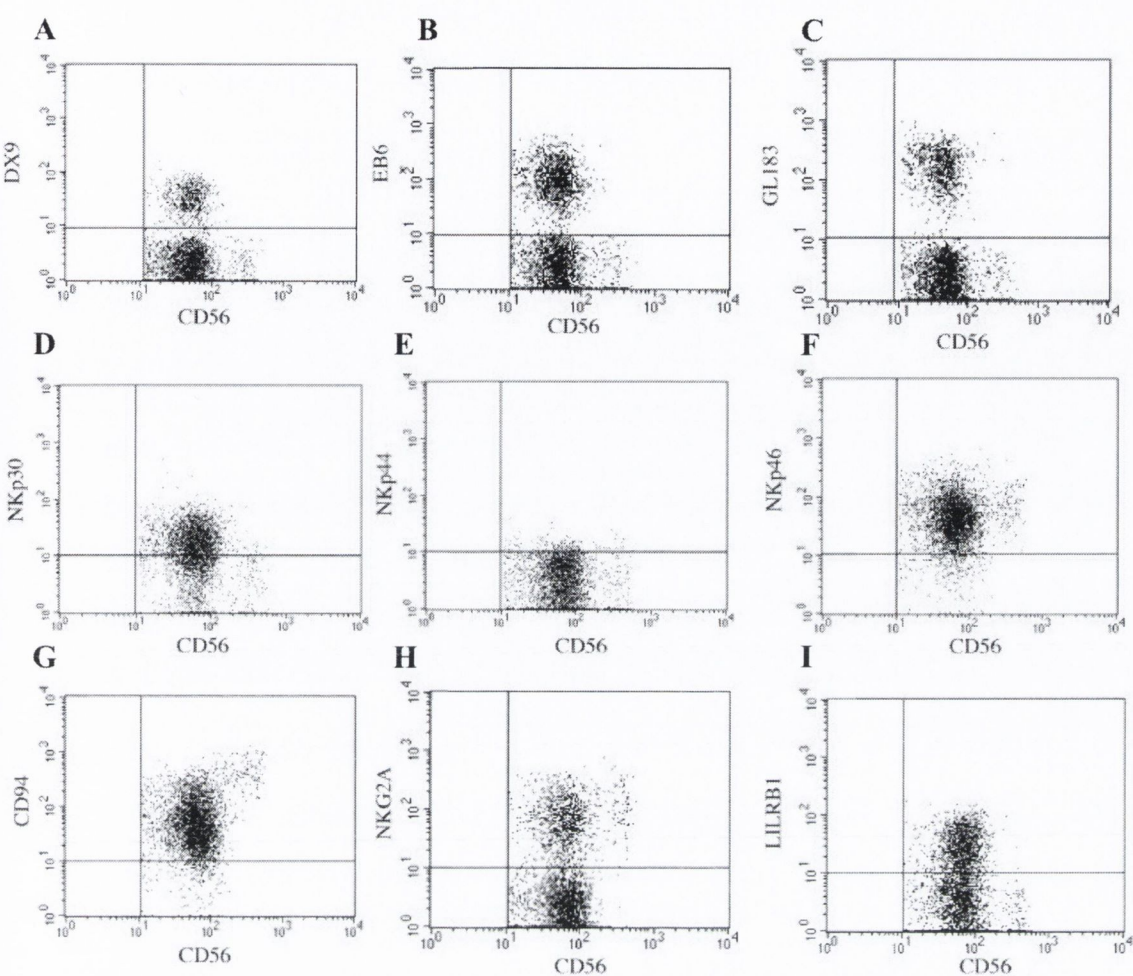


Figure 5.23 NK cell receptor expression in HIV

Gated NK cells (CD56⁺/CD3⁻ lymphocytes) from healthy controls were examined for expression of A, 3DL1 (DX9 antibody); B, KIR2DL1/2, 2DS1 (EB6 antibody); C, KIR2DL2/3/2DS2 (GL183 antibody) ; D, NKp30; E, NKp44; F, NKp46; G, CD94; H, NKG2A and I, LILRB1.

cells than CD56^{dim} NK cells (Figure 5.23H). LILRB1 was expressed by a variable percentage of CD56^{dim} cells and by a very low percentage of CD56^{bright} NK cells.

The percentage of NK cells expressing KIR showed large variation and was broadly similar between the three groups, although there was a trend for lower expression on NK cells from viremic and LTNP HIV patients relative to healthy controls for both 2DL1/S1 and 3DL1 (Figure 5.24). Conversely, on T cells (CD3⁺CD56⁻) there was a slight trend for increased expression of 3DL1 and 2DL1/S1 in the viremic HIV group relative to healthy controls (Figure 5.25).

Expression of both NKp30 and NKp46 (found exclusively on NK cells) were reduced in viremic HIV patients relative to controls (NKp30; 40%±25 vs. 53%±25, NKp46; 66%±25 vs. 88%±23). HIV LTNP patients showed no trend towards normalization for these receptors and indeed levels of NKp30 expression were further reduced in this group (Figure 5.26). NKp44 is not expressed on non-activated NK cells and levels of expression were low and similar in the three groups examined (data not shown).

CD94 expression on NK cells was high in the control group and was similar in both HIV groups (Figure 5.27A). CD94 expression on T cells was lower than on NK cells and shows an increase in viremic HIV (Figure 5.27B). This increase was also seen in the HIV LTNP group (Figure 5.27B). NKG2A expression levels are greater on NK than T cells and showed some interdonor variation but were similar across the three groups (Figure 5.28A, B).

LILRB1 is expressed primarily on monocytic cells, but is also found on both NK and T cells. In healthy controls 27%±10 of NK cells expressed LILRB1 and this significantly increased in both HIV viremic (65%±9, $p<0.001$) and LTNP (63%±22, $p<0.001$) groups (Figure 5.29A). A smaller percentage of T cells express LILRB1 and a similar trend towards increased expression of LILRB1 in HIV viremic and HIV LTNP was seen in T cells (Figure 5.29B).

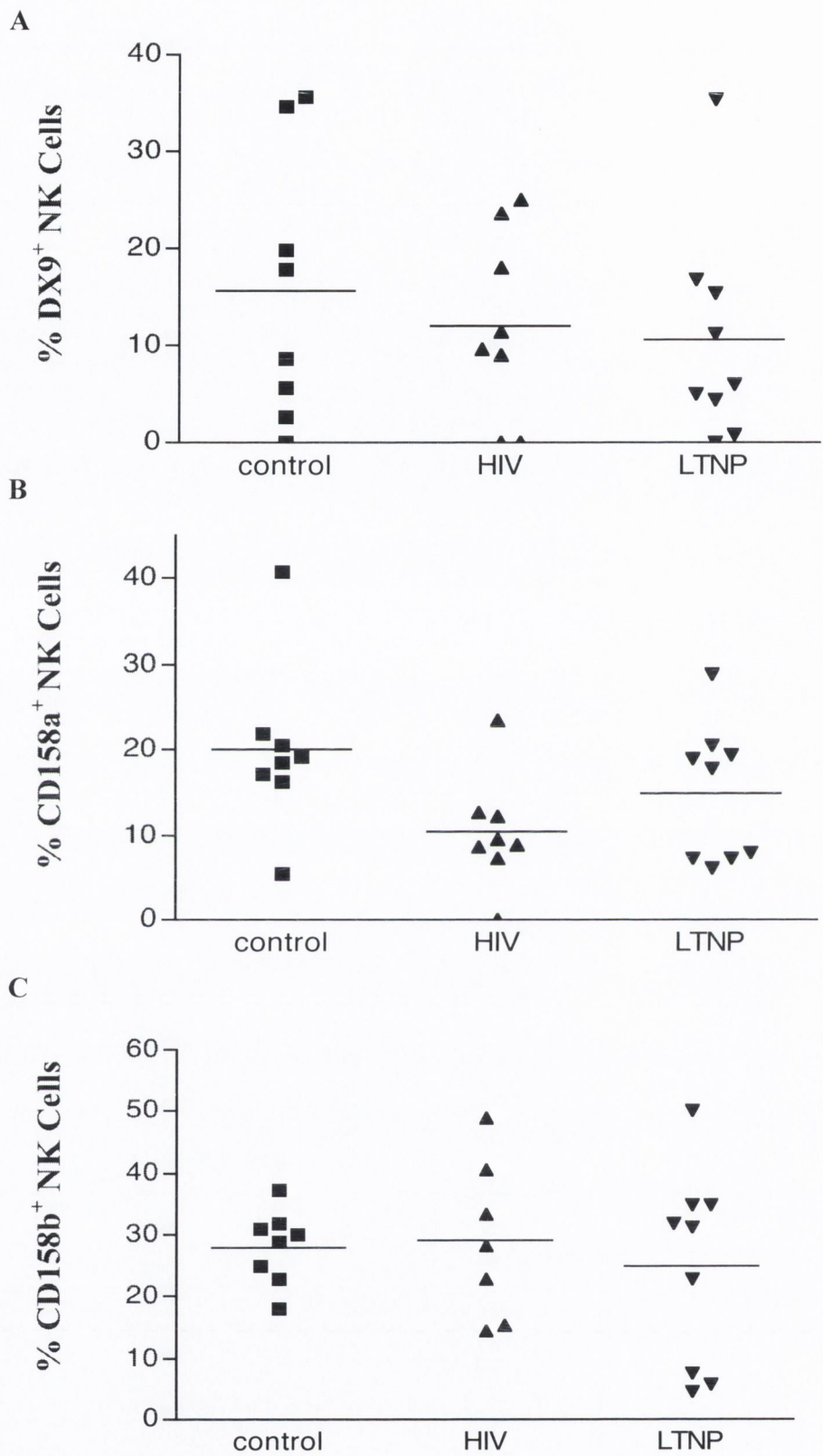


Figure 5.24 KIR expression on NK cells

Gated NK cells ($CD56^+/CD3^-$ lymphocytes) from control, HIV and HIV LTNP patients were examined for expression of KIR molecules using DX9 (A, anti-3DL1), EB6 (B, anti-KIR2DL1/KIR2DS1) and GL183 (C, anti KIR2DL2/KIR2DL3/KIR2DS2).

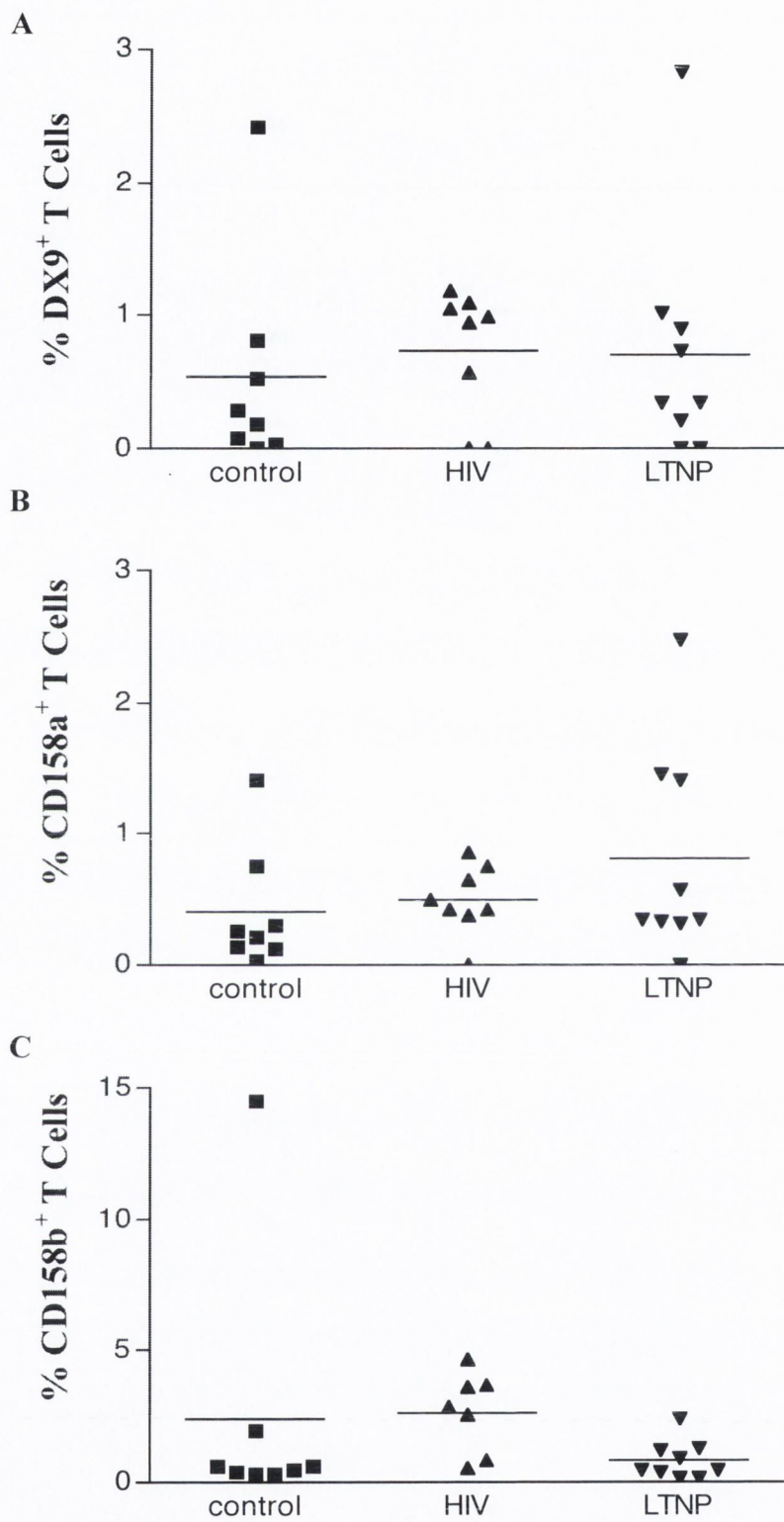


Figure 5.25 KIR expression on T cells

Gated T cells ($CD3^+/CD56^-$ lymphocytes) from control, HIV and HIV LTNP patients were examined for expression of KIR molecules using DX9 (A, anti-3DL1), EB6 (B, anti-KIR2DL1/KIR2DS1) and GL183 (C, anti KIR2DL2/KIR2DL3/KIR2DS2).

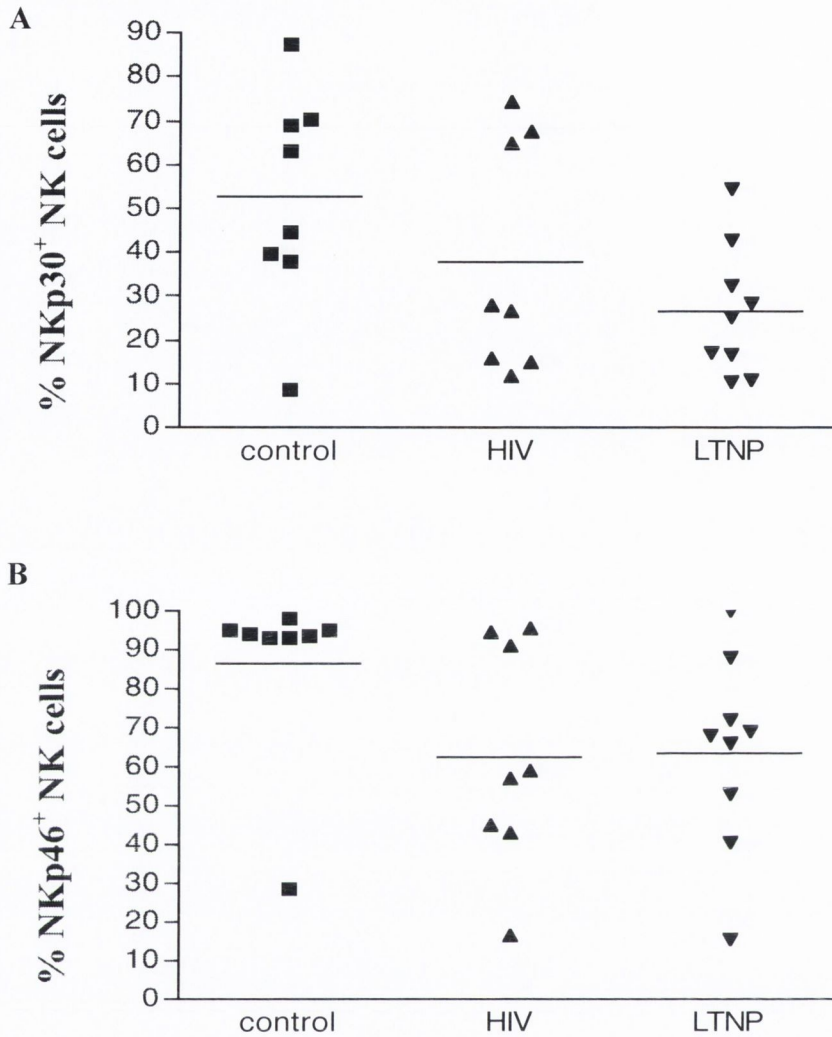


Figure 5.26 Decreased NCR expression on NK cells in HIV infection

Gated NK cells (CD56⁺/CD3⁻ lymphocytes) from control, HIV and HIV LTNP patients were examined for expression of NCR molecules using anti-NKp30 (A, clone Z25) and anti-NKp46 (B, clone BAB281).

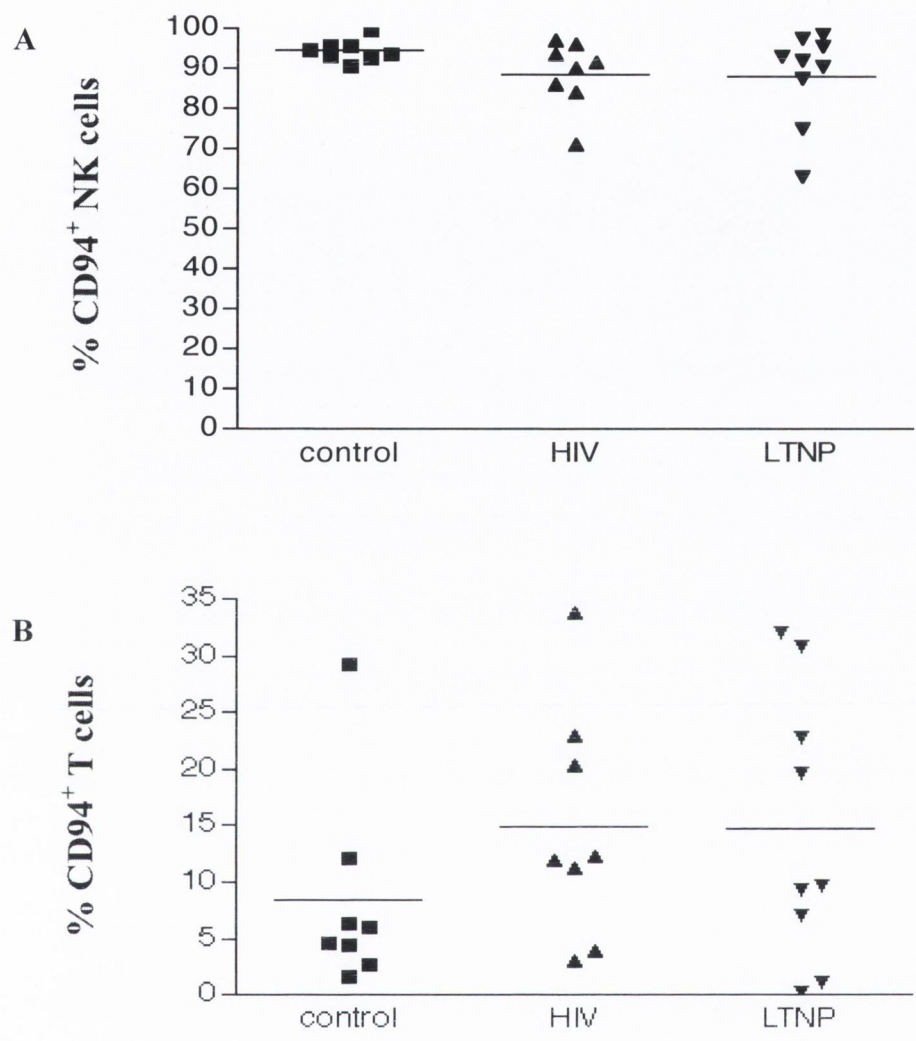


Figure 5.27 CD94 expression on NK and T cells

Gated NK cells (CD56⁺/ CD3⁻ lymphocytes, A) and T cells (CD3⁺/CD56⁻, B) from control, HIV and HIV LTNP patients were examined for expression of CD94 molecule using antibody clone Z199.

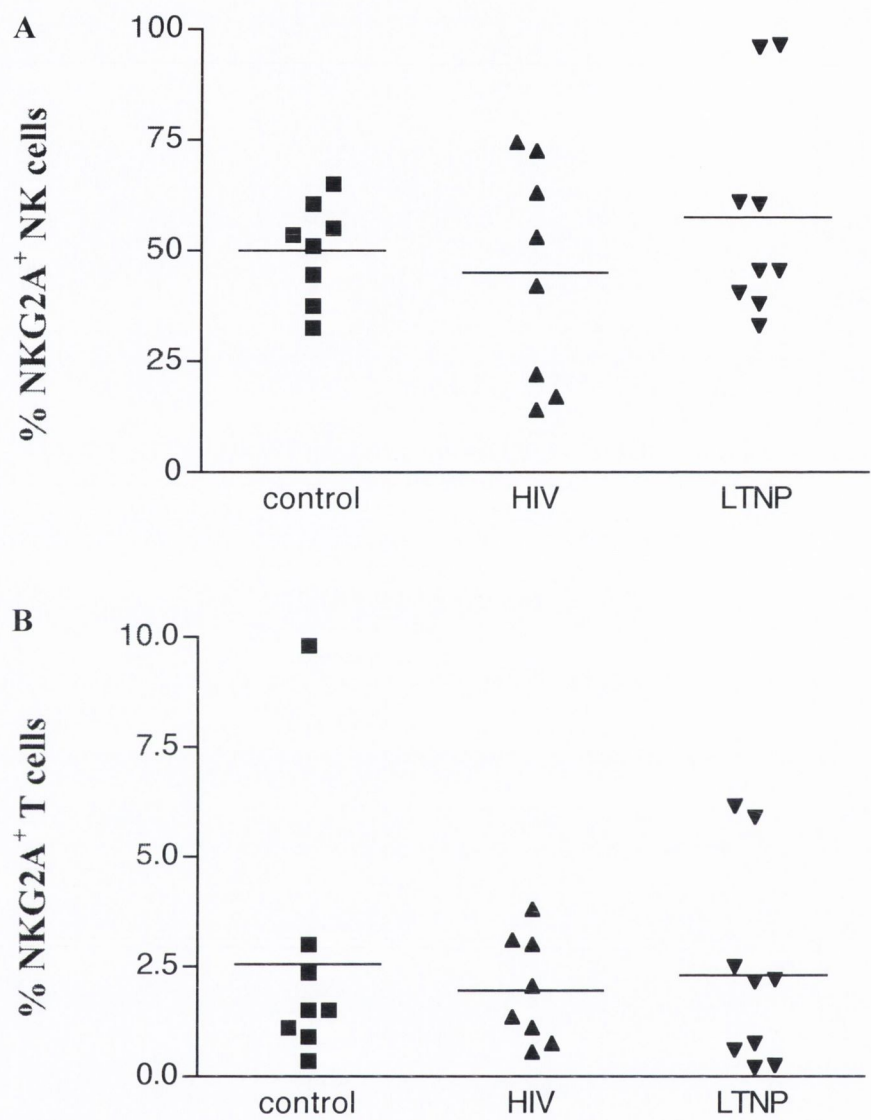


Figure 5.28 NKG2A expression on NK and T cells

Gated NK cells (CD56⁺/CD3⁻ lymphocytes, A) and T cells (CD3⁺/CD56⁻, B) from control, HIV and HIV LTNP patients were examined for expression of NKG2A molecule using antibody clone HP-3D9.

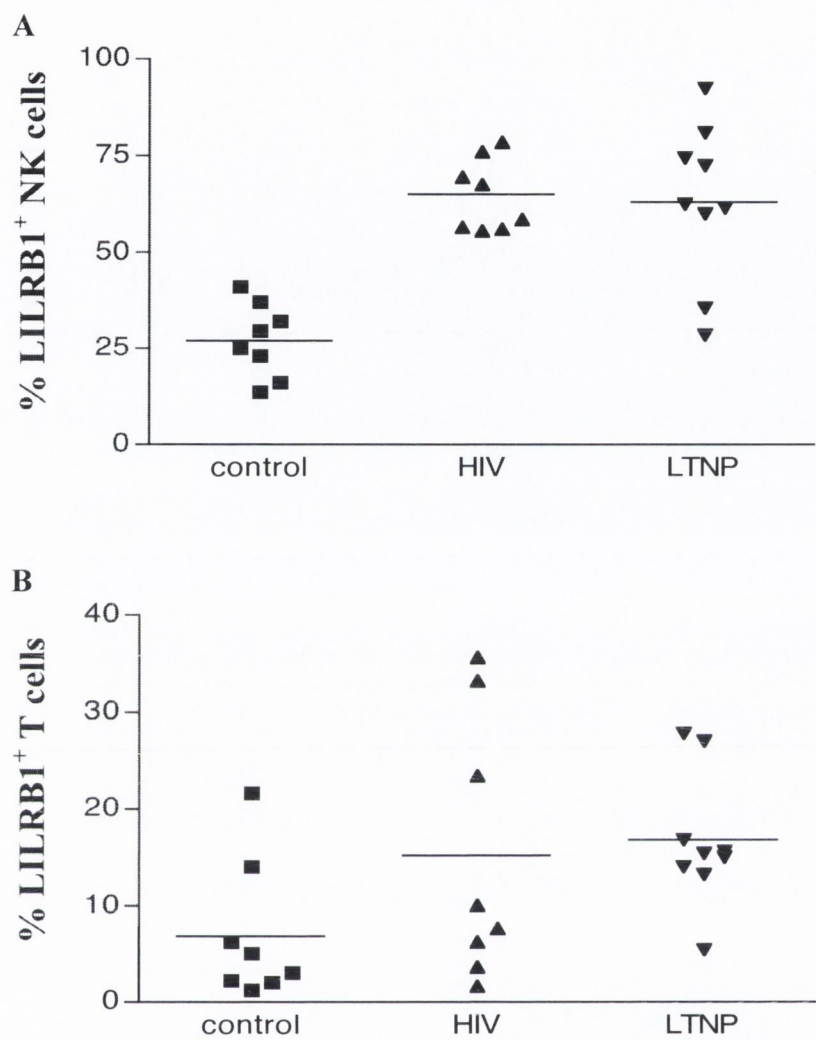


Figure 5.29 Increased LILRB1 expression on NK and T cells in HIV infection

Gated NK cells (CD56⁺/CD3⁻ lymphocytes, upper panel) and T cells (CD3⁺/CD56⁻, lower panel) from control, HIV and HIV LTNP patients were examined for expression of LILRB1 molecule using antibody clone HP-F1.

5.4 Discussion

In this chapter the expression of 3DS1 on the cell surface of NK cells and CD8⁺ T cells and the recognition of this receptor by the monoclonal antibody Z27 were characterised. While triggering of 3DS1 via Z27 cross-linking lead to activation of NK cells, 3DS1 did not interact with HLA-Bw4 on the surface of 721.221 cell lines. Analysis of NK cells from HIV LTNP patients revealed that these cells show enhanced cytotoxicity relative to viremic HIV patients and have an altered cell surface phenotype.

5.4.1 3DS1

The use of a FLAG-tagged 3DS1 construct allowed for definitive demonstration of the cell surface expression of 3DS1. As 3DS1 is a type I membrane protein with a leader sequence that is absent from the mature protein, insertion of the FLAG tag between the leader sequence and the D0 domain resulted in the FLAG tag at the N terminus of the expressed receptor. Generation of the FLAG-tagged construct was achieved through primer additions, recombinant PCR and restriction enzyme digestion. Due to the high degree of sequence similarity between 3DS1 and both other 3DL1 allotypes and other KIR (2D and 3D) sequences, specific primers that amplified only 3DS1 could not be designed. The use of homozygous 3DS1⁺ donors and the size difference in the product generated from 2D and 3D KIR helped to minimise and identified incorrect PCR products prior to sequencing. The cell surface expression of some, but not all, activatory KIR has been reported to be dependent on the expression of the associated signalling adaptor DAP12 (Blery et al, 1997; Lanier et al, 1998). However, we found the 3DS1 was expressed on the surface of both a cell line expressing DAP12 (THP-1) and cell line lacking DAP12 (Jurkat). A recent report supports our findings on the cell surface expression of 3DS1. This study also showed that co-expression of DAP12 leads to an enhancement of cell surface expression on the murine Baf3 B cell line (Carr et al, 2007).

The strong genetic association previously reported between 3DS1 and HLA-Bw4Ile80 allotypes suggested that 3DS1 is expressed at the cell surface where it interacts functionally with Bw4Ile80 but this study was limited to genetic analysis and there was no functional

data to support this interpretation. Indeed, the other 3DL1 allotype that does not react with the DX9 antibody (*004) is retained intracellularly due to amino acid substitutions in the D0 and D1 domains (Pando et al, 2003). Expression of 3DS1 at the cell surface supports the interpretation of the data from genetic association studies that 3DS1 may interact functionally with HLA-B at the cell surface.

Identification of the Z27 antibody as a serological reagent capable of detecting 3DS1 provided a useful tool for assessing the level and extent of 3DS1 expression. 3DS1 is expressed on an average of 17% of peripheral blood NK cells, higher than that seen for *002 and *005 (Chapter 3). 3DS1 expression is characterised by a very dim intensity staining with Z27. Different long tailed allotypes of 3DL1 show varying intensities of staining with this antibody (and DX9, Chapter 3) reflecting different levels of cell surface expression. However, staining of 3DS1ECD Jurkat cells with Z27 also gave a peak with lower intensity than that seen for 3DL1ECD Jurkat cells despite similar expression levels of the chimeric construct (as measured by GFP expression). This suggests that the low intensity peak seen with 3DS1 is not due solely to the level of cell surface expression but may be due, in part, to reduced affinity of the antibody for 3DS1. Although Z27 and DX9 antibodies show very similar staining profiles for inhibitory 3DL1 allotypes, their exact epitopes differ (Khakoo et al, 2002). Tyrosine at position 200 has been defined as an amino acid important for DX9 but not Z27 recognition of 3DL1. This residue is common to all 3DL1/S1 allotypes and therefore can not be directly involved in Z27 discrimination of 3DS1. It is possible that amino acid residue 199, in close proximity, may contribute to differential antibody recognition as 3DS1 contains leucine at this site, while all other 3DL1 allotypes contain proline.

3DS1 is also expressed on CD8⁺ but not CD4⁺ T cells. This is a typical feature of KIR expression on T cells, although expression of activatory KIR on CD4⁺ cells has been reported in healthy individuals (van Bergen et al, 2004) and expansion of this subset is found in certain disease states e.g. rheumatoid arthritis (Namekawa et al, 2000) and acute coronary syndrome (Nakajima et al, 2003).

The data generated by several genetic studies, which show an effect of the presence of the 3DS1 gene only in the presence of Bw4Ile80 allele, argue for a functional interaction between the products of these two loci. 3DS1 is protective against progression to AIDS and the development of opportunistic infections in HIV patients only when present in combination with Bw4Ile80 (Martin et al, 2002; Qi et al, 2006). Similarly, the presence of Bw4 in association with 3DS1 is protective against the development of cervical neoplasia (Carrington et al, 2005). 3DS1 in the presence of Bw4Ile80 but not Bw4Thr80 is also protective against the development of hepatocellular carcinoma in Hepatitis C patients (Lopez-Vazquez et al, 2005). However, attempts to define an interaction between 3DS1 and Bw4 allotypes using both the transfected Jurkat system and polyclonally expanded 3DS1⁺ NK cells were unsuccessful. It is possible that there is no interaction between these two proteins and that the interpretation of the genetic data is incorrect. The association seen may reflect the interaction of the products of genes in linkage disequilibrium with 3DS1 and HLA-B. Both 3DS1 (chromosome 19) and HLA-B (chromosome 6) exists in clusters containing many other candidate genes implicated in the immune system.

Comparison of the amino acid sequences of 2DL/HLA-C and 3DS1/HLA-B suggests that most of the residues that play an important role in their interaction are conserved (Figure 5.4.1). This supports the analysis of the genetic data and suggests that 3DS1 may be capable of recognising HLA-B. If these two gene products do interact then several factors may account for the lack of interaction seen in this study, including technical limitations, 3DS1 polymorphism and the role of presented peptide. The assays used to probe the interaction of 3DS1 and Bw4 included the Jurkat transfectant system, lysis of Bw4⁺ 721.221 cells by 3DS1⁺ effector cells and tetramer staining of 3DS1⁺ cells. Although some of these approaches have been successfully used to measure that interaction of 2D KIR with HLA-C, they may not be sensitive enough to detect the interaction between 3DS1 and HLA-B. The panel of Bw4 allotypes used contained representative members of several HLA-B families and included both Ile80 and Thr80 allotypes but may still have been too restrictive to see the interaction. Five 3DS1 alleles (3DS1*010-*014) have been reported in GenBank (L76661, X97233, U73396, AF022044, AJ417558) but we believe that the lack of interaction seen is unlikely to reflect the allotype used in this study. The allele used

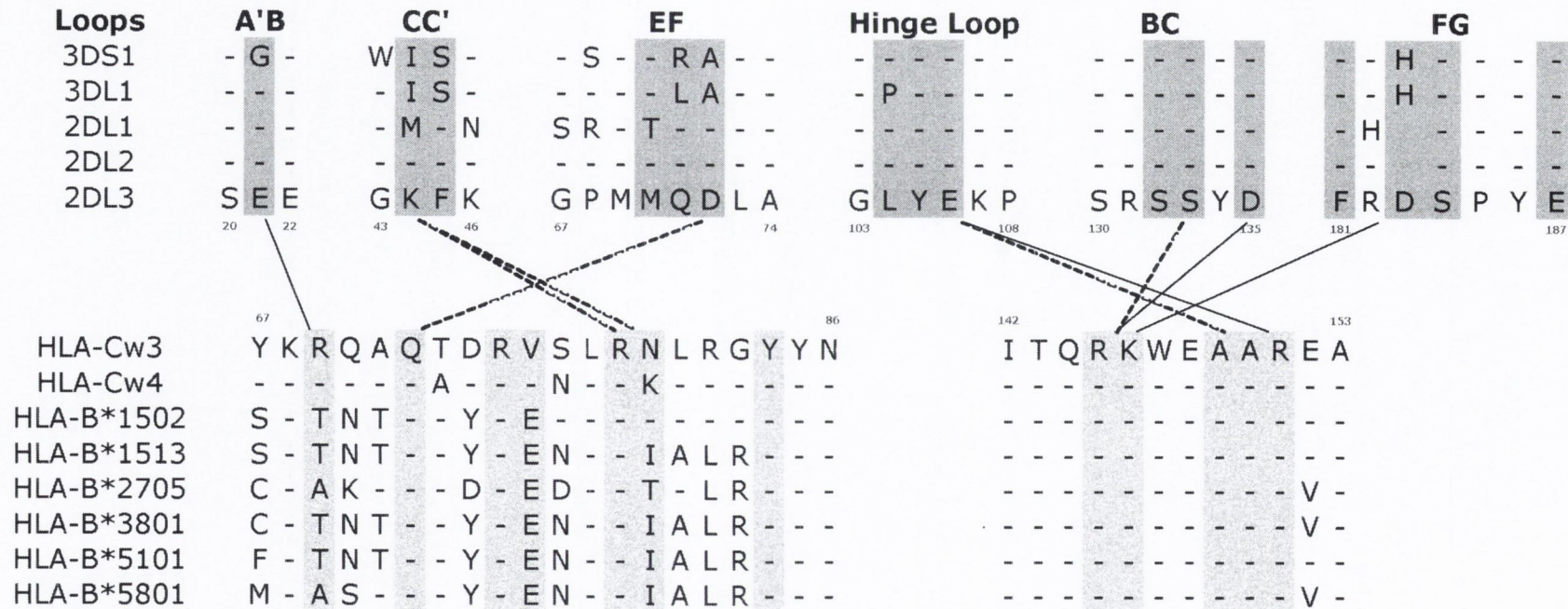


Figure 5.4.1 A comparison of the HLA binding regions of 2D and 3D KIR

A sequence alignment of KIR (including 3DL1 and 3DS1) and HLA binding regions based on the crystal structure of 2DL in association with HLA-C. The solid lines represent salt bridges; dotted lines represent hydrogen bonds and contact residues are shaded. Adapted from Boyington et al, 2001.

(3DS1*013) is the predominant allele in Caucasian populations and the only allele present in the HIV cohort that was used in the genetic study by Martin *et al.*

The interaction between 3DS1 and Bw4 may be restricted by the presented peptide. The recognition of HLA ligand by both inhibitory and activatory KIR is influenced by the bound peptide. In particular, the interaction between 3DL2 and HLA-A3 or HLA-A11 has been shown to be highly peptide specific; in a panel of HLA-peptide complexes only HLA-A3 or HLA-A11 tetramers folded in the presence of an EBV, but not self or HIV, peptides allowing binding (Hansasuta et al, 2004). The presentation of specific, perhaps infection or stress associated peptides by HLA-Bw4 may be required for recognition and this would allow control of activation of NK cells via 3DS1 ligation. To examine the role of peptide in Bw4 recognition by 3DS1, we used B*2705 (a Bw4 Thr80 allotype) tetramers folded in the presence of a panel of self and pathogen-derived peptides. Evidence in support of an interaction between B*27 and 3DS1 comes again from a genetic study, showing that 3DS1 contributes to ankylosing spondylitis (Lopez-Larrea et al, 2006). This inflammatory condition is closely associated with B*27, with B*27 present in 90% of sufferers compared to 5-10% in control populations (Konieczna et al, 1983). It was not possible to verify the results obtained by specific blocking of the interaction in this study, possibly due to the protocol used but this approach shows promise for the identification of a 3DS1 ligand. The study by Carr *et al* using soluble 3DS1-Ig fusion protein also failed to find any interaction between 3DS1 and the HLA-Bw4 allotypes B*2705, B*5701 and B*5801. In addition, a second study using tetramers consisting of a panel of HLA-Bw4 Ile80 allotypes folded in the presence of HIV-specific tetramers failed to find any interaction with 3DS1 (Gillespie et al, 2007).

3DS1 is believed to act as an activatory receptor based on its similarity to other activatory KIR. Here, we show that cross linking of the 3DS1 receptor leads to activation (up-regulation of CD69) of freshly isolated, unstimulated NK cells. CD69 is used as an activation marker on NK and T cells and is rapidly upregulated in response to stimuli. Carr *et al* (2007) have shown that ligation of 3DS1 on 3DS1-transduced NKL cells and polyclonally expanded NK cells (both IL-2 stimulated) leads to the induction of

cytotoxicity and IFN- γ production. Thus resting NK cells are activated by the engagement of 3DS1 and, in the presence of IL-2, this leads to the activation of two of the key effector mechanisms of NK cells: cytotoxicity and cytokine production.

5.4.2 NK cells in HIV infection

The repertoire of receptors expressed on NK cells differs greatly among individuals in the population and this variability is predicted to influence the nature of the NK cell response to pathogen. While NK cells have long been established as crucial mediators of certain anti-viral responses, there is emerging evidence of a role for NK cells in the response to HIV. We investigated NK cell activity and cell surface phenotype in viremic HIV and HIV LTNP groups.

NK cell killing was decreased in viremic HIV patients relative to controls in agreement with several reports in the literature, although the opposite has also been observed (Alter et al, 2004). Importantly, we see higher activity in HIV LTNP patients (relative to viremic HIV), who have levels of killing close to that of controls, in spite of a decrease in the percentage of NK cells in these individuals. HIV LTNP individuals offer a unique insight into the immune system of the minority of individuals who control the spread of HIV and provide an opportunity to study a successful immune response to this virus. NK cell activity (as measured by cytotoxicity) was maintained in these patients, suggesting that this immune parameter may be allowing these individuals to control the virus. As a component of the innate immune system, NK cells are primed to respond immediately to invading virus and it may be the initial, early control of viral replication that permits eventual control of the infection (both by NK cells themselves and by the development of a protective adaptive immune response). The low numbers of HIV LTNP patients prevented any meaningful functional analysis of the role of 3DS1 in the control of HIV in these patients.

The difference seen in cytotoxic activity of NK cells in HIV LTNP relative to viremic HIV patients lead us to investigate if NK cell surface phenotype correlated with functional outcome. Perturbation of NK cell receptor repertoire has been reported in HIV infection, affecting both inhibitory receptors (Kottilil et al, 2004; Mavilio et al, 2003) and activatory

NCRs. We observed decreased expression of NKp46 and NKp30 in viremic HIV patients as previously reported (De Maria et al, 2003), but this was seen equally in the HIV LTNP group. Increased levels of inhibition of NK cells have also been proposed as a reason for decreased NK cell activity in HIV. We did not observe any increase in KIR or NKG2A expression on NK cells during HIV, however levels of LILRB1 positive NK cells were significantly increased in HIV viremic patients in agreement with previous reports (Mavilio et al, 2003). This increase in LILRB1 expression was also seen in HIV LTNP patients and, as LILRB1 is an inhibitory receptor, it is an unlikely candidate to explain the increase in cytotoxicity. The reduction in NCR expression and the lack of reduction (and in some cases increase) in expression of the inhibitory receptors examined, in both HIV groups, suggests that maintenance of NK cell killing in HIV LTNP cannot be attributed to these factors. In addition to cell surface receptor expression, NK cell cytotoxicity is also governed by a range of factors, including the expression of adhesion and costimulatory molecules, levels of granzyme and perforin and the cytokine environment, and differences seen in levels of killing in the HIV groups examined here may reflect alterations in any one of these regulatory factors.

The data generated here are consistent with a model in which increased NK cell activity maintains HIV infection under control in LTNP; however this model does not consider the effects that HIV infection itself may have on NK cells. NK cell parameters examined could potentially be altered both by the immune environment created during HIV infection and by direct interaction with HIV proteins such as gp120, which has been shown to suppress proliferation, activity and survival of NK cells (Kottilil et al, 2006). It is difficult to conclude if our data from LTNP patients reflect differences in NK cell biology that lead to control of HIV, or a beneficial effect of low viral load on NK cell function. It has been reported that many of the alterations of the immune system, including those of NK cells are dependent on viremia and can be restored by suppression of viral load with highly active antiretroviral treatment (HAART) (Kottilil et al, 2004; Mavilio et al, 2003).

Other components of the immune system such as CD8⁺ T cells have also been shown to contribute to control of HIV progression in some cohorts (Beattie et al, 2002; Lopalco,

2004) and indeed we observed a trend towards increased expression of KIR on T cells of both HIV patient groups, possibly due to the activation of T cells in the response to HIV viremia. KIR expression on HIV specific CD8⁺ T cells has been shown to limit their cytolytic function and may contribute to disease progression by inhibiting the elimination of infected cells by cytotoxic T cells (De Maria et al, 1997).

Expression of activatory KIR (including 3DS1) on NK cell subsets may provide a mechanism for NK cell activation during infection or cellular transformation. Variation within a population in the expression of these receptors and their ligands is predicted to give an immunological advantage to certain individuals. The immune response to HIV is complex and it is likely that more than one component of the immune system is required to mount an effective immune response, with their synergistic cooperation allowing for control of viremia in LTNP. In this respect it is possible that the role of NK cells may be one in which they act early in infection to limit viral spread, eliminate infected cells and help in the generation of an appropriate adaptive immune response.

Chapter Six

General Discussion

6.1 Introduction

The function of the immune system is to provide protection from a virtually limitless array of pathogenic bacteria, viruses and parasites as well as preventing the development of malignancies. In order to achieve this the cells of the immune system must be capable of responding to a wide array of “danger signals”. The greater the ability of an individual’s immune system to detect and respond to disease or malignancy, the greater the chance that individual will survive the many immunological challenges that will be encountered. Within the innate immune system, NK cells are capable of responding to the down-regulation of HLA, alterations in peptide presentation by HLA and up-regulation of stress proteins as well as responding to pro-inflammatory cytokines and direct activation by pathogen associated molecular patterns (PAMPs). Due to their specialised function, the genes of proteins involved in the immune system display a number of features, many of which are typified by the KIR gene family. These include polygeny, clustering of genes, rapid evolution, co-evolution with other immune genes, association with disease and high levels of polymorphism (Trowsdale and Parham, 2004). These features of immune genes allow for the generation of diversity within the immune system, increasing the probability that it will be capable of mounting an effective defence against the wide array of possible pathogens.

6.2 Diversity in the NK cell response – 3DL1

Within the NK cell compartment of an individual diversity can be achieved through the stochastic expression of receptors such that each NK cell expresses only a subset of the available receptors. This leads to the generation of subsets of NK cells that are differentially activated in response to different challenges. Thus for example, NK cells expressing CD94/NKG2A as the sole inhibitory receptor becomes activated in the event of down-regulation or alteration in HLA-E expression, while NK cells expressing NKG2D are activated upon expression of stress-induced ligands on target cells. Within a heterozygous individual, expression of two different 3DL1 allotypes adds diversity to the pool of NK cells due to the difference in the level of inhibition of different allotypes by class I

molecules. There is also evidence that suggests that different 3DL1 allotypes display distinct peptide specificities (Thananchai et al, 2007). The expansion of the specificity of the NK cell pool due to the expression of one, the other or both, of two different 3DL1 allotypes by subsets of NK cells is predicted to influence the NK cell response during NK cell development as well as in times of immune activation (Figure 6.1). The emerging model of NK cell development suggests that the activation threshold of an NK cell is set dependent on the level of inhibition generated by the profile of expressed inhibitory receptors (10th Meeting of the Society for Natural Immunity). In this way NK cell tolerance is maintained, as the level of inhibition received by the complement of expressed receptors is sufficient to prevent autoreactivity; NK cells which do not express an inhibitory receptor for self HLA are maintained in a hyporesponsive state (Anfossi et al, 2006). NK cells that express a 3DL1 allotype that is strongly inhibited by self Bw4 would then be expected to become more activated upon the loss of that inhibition (through down-regulation or shift in peptide presentation) than NK cells that expresses a 3DL1 allotype that is more weakly inhibited. This model of developmental “tuning” based on the functional differences of 3DL1 allotypes has been suggested to explain the genetic association seen for improved outcome in HIV with expression of both the activatory 3DS1 and the highly inhibitory allotypes of 3DL1 (Martin et al, 2007). Activation of NK cells might be achieved either through engagement of an activatory allotype and by the loss of a highly inhibitory allotype during infection.

Within a population, KIR diversity on NK cells is a result of haplotypic variation in gene number and content as well as allelic polymorphism (Shilling et al, 2002). Haplotypic variation results in the presence or absence of individual KIR genes and in turn the gain or loss of the specificity of that receptor e.g. individuals lacking 2DL1 do not possess a KIR specific for HLA-C2 allotypes. Allelic polymorphism has been found to result in quantitative rather than qualitative differences in the ability to respond to changes in HLA. In this work, the impact of polymorphism of the 3DL1 gene was explored. Different alleles of the gene encode proteins with pronounced functional differences. In the common allele 3DS1, the resulting protein possesses activatory rather than inhibitory signalling potential while another common allele in Caucasian populations, *004, encodes a protein that is

retained intracellularly and is not available at the cell surface for interaction with HLA-B. These examples represent the extreme cases of the functional consequences of allelic polymorphism, but analysis of the function of allotypes that are not dramatically different from each other in terms of signalling capacity or localisation revealed that genetic polymorphism here also results in functional differences. Using the common allotypes (*001, *002, *005 and 3DS1) and a sample of HLA-Bw4 molecules, we defined a role for the polymorphism of both Bw4 allotypes and 3DL1 allotypes in influencing their interaction. The functional consequences of the allelic variability of the 3DL1 gene add another layer of diversity to the NK cell response within an individual and within the population as a whole. Allelic polymorphism can also lead to a loss of function e.g. as 3DL1*004 is not expressed at the cell surface it is expected that if it does interact with HLA it does not do so in the same manner as other 3DL1 allotypes (Pando et al, 2003). 2DL4 is also localised primarily within the cell but this localisation does not prevent it from playing a role in the activation of NK cells. In this case the HLA ligand is the soluble form of the HLA-G molecule (Rajagopalan et al, 2006). Low levels of 2DL4 present at the cell surface bind soluble HLA-G and this complex is then internalised into the endosomes of NK cells. It is possible that 3DL1*004 interacts with a ligand in a similar manner.

Expression of different 3DL1 allotypes within a population would also be expected to contribute to functional heterogeneity at this level. The expression of several 3DL1 allotypes differs across population sets. *004 is a common allele (~20%) in Caucasian and other populations but is not found in the Japanese population (Yawata et al, 2006); similarly the frequency of 3DS1 in African American populations (~18%) is much lower than that seen in European Americans (~37%) (Martin et al, 2007). The difference in prevalence of these 3DL1 alleles in different populations may represent the selection pressure imposed by differences in the pathogenic challenges in different environments. Both inhibitory and activatory KIR could potentially be either beneficial or disadvantageous depending on the nature of the challenge faced. Thus, 3DS1 may have been selected for in populations that moved out of Africa due to some beneficial role, or may have been selected against in African populations, if expression lead to decreased survival. *004 is of particular interest, as it is widely believed to be a loss-of-function

mutation. Expression of this allotype may have conferred a disadvantageous phenotype such as providing an inhibitory receptor for a viral ligand. Individuals not expressing this allele, or expressing a mutant form that was not available at the cell surface would have a selective advantage in this event leading to the loss of this allele (as in the Japanese population) or in the selection of an allele encoding an inactivated protein. In the murine system, there is a well-defined example of a pathogen-driven evolution of an NK cell receptor (Arase and Lanier, 2002). It is believed that the MCMV protein m157 originally conferred protection to virally infected cells by engaging an inhibitory Ly49 receptor (Ly49I) on NK cells. Exposure to this pathogen then drove the selection of an activating Ly49 receptor (Ly49H) with the same ligand specificity but with activating potential (Arase et al, 2002). This resulted in the generation of an activating NK cell receptor that directly recognises a viral protein (Smith et al, 2002) and expression of Ly49H confers resistance to MCMV in certain mouse strains e.g. C57BL/6.

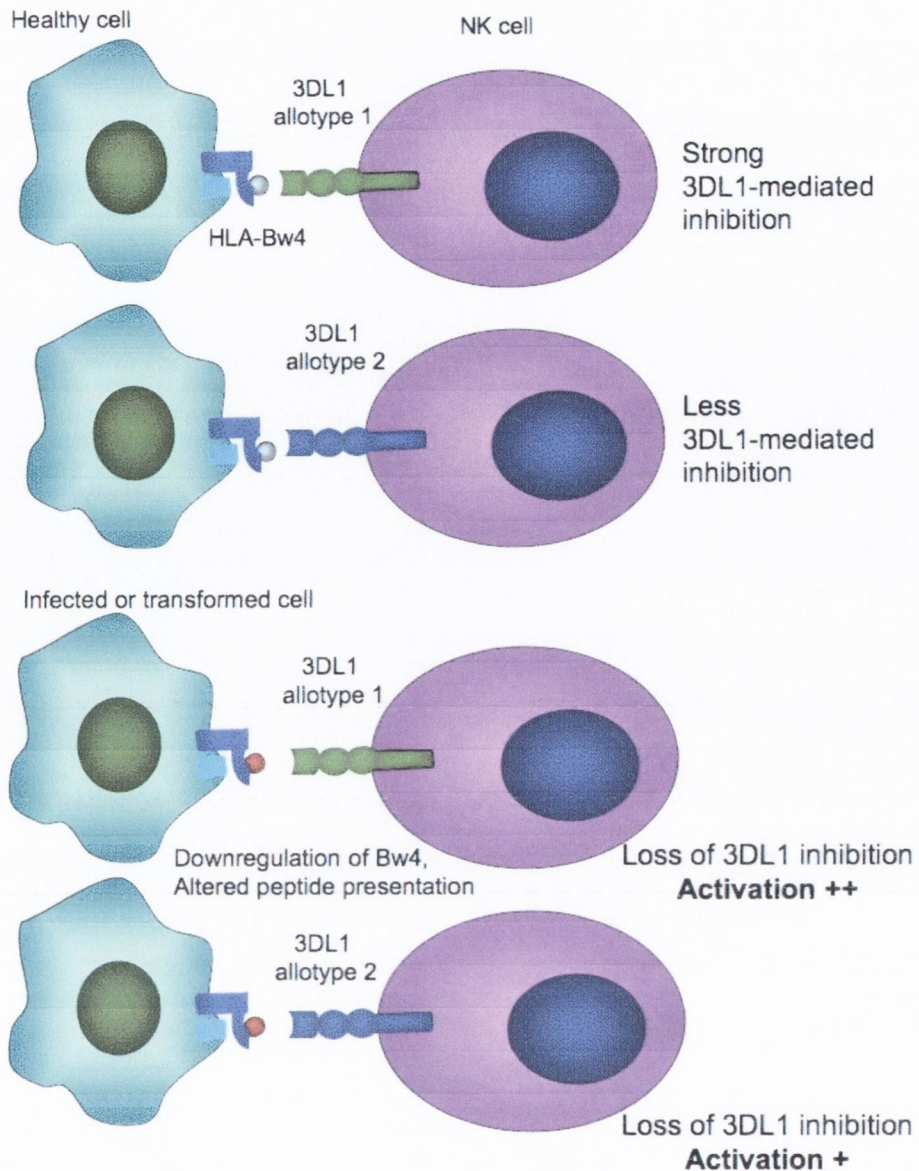


Figure 6.1 3DL1 genetic polymorphism contributes to NK cell functional heterogeneity

Different 3DL1 allotypes can be inhibited to a greater or lesser degree by a single HLA-Bw4 allotype. The loss of Bw4-mediated inhibition (due to alteration in the pool of presented peptide or down-regulation of HLA) may lead to the activation of NK cells expressing 3DL1. NK cells that express a 3DL1 allotype strongly inhibited by the Bw4 allotype are expected to be activated to a greater extent than NK cells expressing a 3DL1 allotype that is more weakly inhibited.

6.3 Activatory KIR

The variable expression of activatory KIR genes, the large proportion of individuals with no known functional activatory KIR and the weak interaction of activatory KIR with their known ligands have called into question the functional relevance of these receptors. The origin and natural history of activatory KIR has been examined using phylogenetic analysis and evolutionary reconstruction (Abi-Rached and Parham, 2005). These receptors have evolved from their inhibitory counterparts through the introduction of two amino acid changes; one to introduce the positively charged residue to the transmembrane domain and a second to introduce a stop codon before the ITIM sequence in the cytoplasmic domain. The substitutions involved only directly affected the signalling function of the receptors suggesting that a selective pressure drove this evolution. Analysis of current activatory sequences suggests that in the evolution and expansion of these receptors after their initial introduction there has been a purifying selection to preserve their signalling capacity. Three species (bonobo, orangutan and human) have evolved an activatory KIR with 3Ig domains (Pp-3DSA, Popy-3DS2 and human 3DS1). Analysis of the genetic distances between these three receptors suggests that they are not orthologues but have arisen independently in these three species. This convergent evolution towards the acquisition of a 3D activatory KIR suggests that each of these three species faced a selective challenge where this receptor provided an evolutionary advantage.

The recurrent acquisition and loss of activatory KIR in human and activatory Ly49 in mice suggests that these receptors may play an important role in immune defence at certain times but their presence may be detrimental in other situations. This hypothesis is supported by disease association studies where expression of activatory KIR has been associated with resolution of infection (e.g. 3DS1 in HIV and Hepatitis C) but also with the development of autoimmune conditions (e.g. 2DS1 with psoriatic arthritis, psoriasis vulgaris and idiopathic bronchiectasis). Thus in times of infection, expression of these receptors can be beneficial by activating NK cells, but in the absence of disease these receptors may become inappropriately activated leading to autoimmune conditions – the result of the immune system's "friendly fire". A similar explanation has been used by the hygiene hypothesis to explain the inverse correlation between the prevalence of childhood infections and the

development of allergic diseases. The immune response in asthma – a Th2 response with raised IgE levels with eosinophil and mast cell recruitment are the same responses seen in the control of extracellular parasites such as Helminth infection still common in Africa and South America. In developed countries, where the immune system of children develops in the absence of exposure to these challenges, it has been suggested that there is an error in the “tuning” of the immune response such that it now mounts an excessive and inappropriate response to harmless allergens (Yazdanbakhsh et al, 2002). In addition to the development of autoimmune conditions, activatory KIR may also play a role in an excessive immune response following infection. The association of 3DS1 expression with the development of cervical neoplasia, particularly in the absence of inhibitory KIR/HLA pairings (Carrington et al, 2005), suggests that excessive inflammation following human papillomavirus infection leads to cellular damage and may contribute to the development of malignancy.

In light of the potential detrimental effects of inappropriate activation of any component of the immune system, it is perhaps to be expected that any interaction between 3DS1 and HLA-B is controlled and cannot be detected under steady-state conditions. In spite of the consistent protective effect of 3DS1, only in association with Bw4, seen in a number of disease association studies, a direct interaction between the two proteins has not been detected. In this work the cell surface expression of 3DS1 was definitively demonstrated, along with the potential of triggering this receptor to activate NK cells. However interaction of this receptor with Bw4 (including Bw4 Ile80) allotypes was not detected. However, the role of the 3DS1/Bw4 Ile80 interaction in HIV infection is supported by functional data that shows that NK cells from 3DS1⁺/Bw4 Ile80⁺ individuals have a greater capacity to suppress viral replication in autologous HIV-infected CD4⁺ T cells than 3DS1⁻ individuals (Galit Alter, 10th Meeting of the Society for Natural Immunity). In the same study, a 3DS1⁺ NKL cell line was significantly more activated by HIV⁺Bw4⁺CD4⁺ T cells than by uninfected Bw4⁺ or HIV⁺Bw6⁺ cells. These data strongly support the interpretation of the genetic data that 3DS1 can be functionally activated by HLA-Bw4 allotypes with isoleucine at position 80 in the context of HIV infection. This interaction may be controlled at the level of the peptide presented by the HLA molecule in a manner analogous to that

seen for 3DL2 and HLA-A3 and -A11 presenting EBV-derived peptide (Hansasuta et al, 2004). In this model, HIV infection of a Bw4 Ile 80⁺ cell would result in the presentation of a peptide that would be permissive for 3DS1 binding. Much interest has focused on defining a specific HIV-derived peptide that controls the interaction between 3DS1 and Bw4 and Bw4Ile80 tetramers folded in the presence of HIV peptides have been tested for their interaction with 3DS1 expressing cells. Despite the use of three different Bw4Ile80 allotypes (including B*5701) presenting a range of HIV-derived peptides that differed at the P7 and P8 positions known to be important for KIR recognition, no interaction with 3DS1 could be detected (Gillespie et al, 2007). As the HIV virus is a recently emerged human pathogen, it does not make sense that 3DS1 has evolved solely to present a HIV-specific peptide. While it is possible that the 3DS1 specifically recognises Bw4 in the context of a HIV-derived peptide, a more attractive hypothesis is that 3DS1 is activated upon presentation of peptides from host-derived stress proteins. Restriction of 3DS1 activation to occasions of cellular stress would prevent inappropriate activation, but would also allow NK cells to respond to damaged or infected cells without strict pathogen specificity. There is growing support for the idea that the immune system is capable of responding to danger signals not only from “non-self” sources (PAMPs) but also from endogenous sources, collectively known as danger associated molecular patterns (DAMPs; (Matzinger, 2007). Examples of endogenously derived DAMPs include heat shock protein 70, hyaluronan and High Mobility Growth Box 1 (HMGB1) protein. HMGB1 is a nuclear protein that is released from necrotic cells and binds to a number of receptors to activate inflammation including TLR2 and RAGE (receptor for advanced glycation end-products) (Raucci et al, 2007). HMGB1 has been shown to act as a costimulatory molecule for NK cells and increases IFN- γ production from monocyte-stimulated NK cells (DeMarco et al, 2005). Thus NK cell are capable of responding to the presence of an endogenous signal of cellular damage or distress. During HIV infection, this signal may take the form of altered peptide presentation (a reflection of altered protein synthesis within the cells). A study of the profile of peptide presentation in HIV infection identified 15 different host proteins that were differentially presented by HIV⁺ cells (Hickman et al, 2003). This change in protein expression in an infected cell could skew the profile of peptides presented by that cell in such a manner as to allow interaction with, and activation of 3DS1⁺ cells (Figure 6.2).

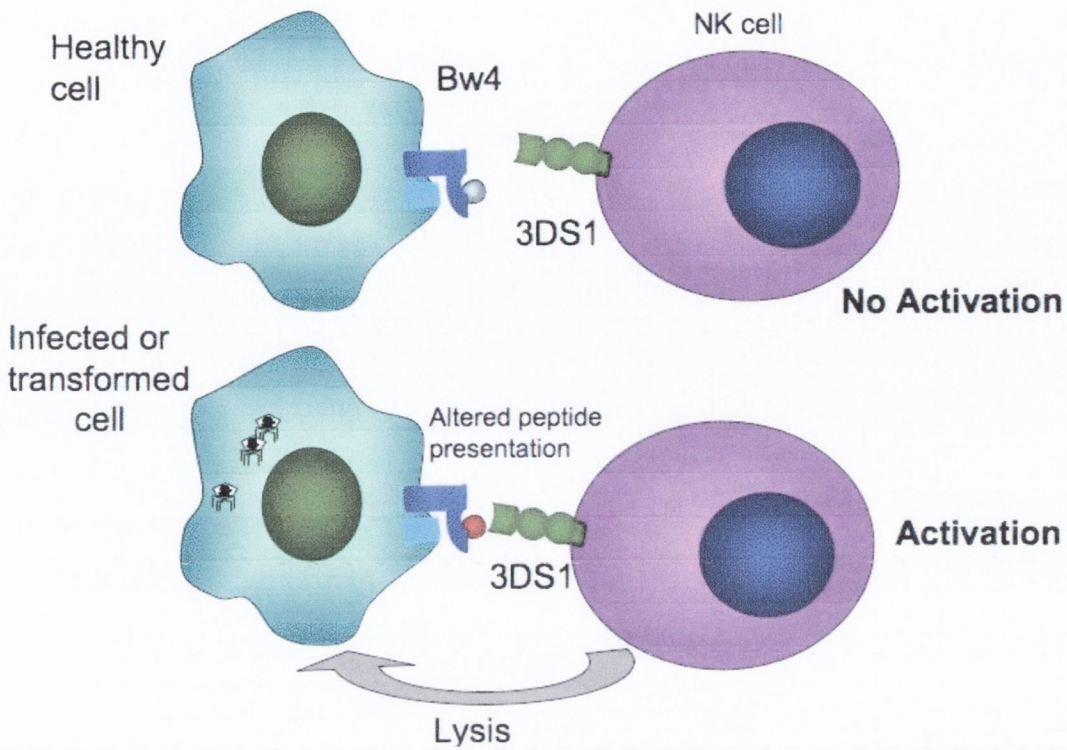


Figure 6.2 The role of 3DS1 in NK cell activation

3DS1 does not interact with Bw4 allotypes on the surface of healthy cells. Alterations in the profile of presented peptides during a viral infection or other time of cellular stress may allow recognition by 3DS1 and the activation of 3DS1⁺ NK cells. Controlled activation by 3DS1 in this manner would allow for the elimination of infected or malignant cells while protecting healthy cells from NK cell lysis.

6.4 Control of KIR expression

Diversity is achieved by several different mechanisms within the human immune system. One of the classical examples of how this is achieved is the generation of highly diverse T cell receptors through somatic gene recombination that allows T cells to respond to a huge array of non self peptides presented by MHC molecules. While in comparison to the level of variation seen within the adaptive immune system, the innate immune system shows more limited diversity, expression on innate cells of members of newly identified families of receptors (including the TLR and NOD family) adds to the diversity of this response. The expression of a random combination of inhibitory and activatory receptors by individual NK cells leads to the generation of NK cell subsets that are differentially activated in different circumstances. The stochastic expression of the majority of KIR genes (including 3DL1), as well as differences in the level of cell surface expression of 3DL1 allotypes contribute to the NK cell repertoire. Expression of KIR genes is controlled by the methylation status of the promoter with silenced KIR genes being methylated (Chan et al, 2003; Santourlidis et al, 2002). In spite of very high sequence similarity in the promoter region, the 3DL1 gene can be expressed in a monoallelic fashion. In human NK cell clones, this monoallelic expression is associated with demethylation of the expressed allele (Chan et al, 2003) and the methylated allele remains untranscribed in spite of the presence of all *trans*-acting factors required for transcription of the other allele. There have been conflicting reports on the frequency of monoallelic expression of 3DL1 alleles, with one group reporting that 90% of 3DL1⁺ NK cells contain a single hypomethylated (and by inference expressed) allele (Chan et al, 2003), while others have suggested that there is preferential biallelic expression (Gardiner et al, 2001; Santourlidis et al, 2002).

The proximal promoter of KIR genes has recently been identified as having bidirectional activity and anti-sense activity from this promoter is predicted to generate double stranded RNA with the product of the distal KIR promoter (Davies et al, 2007). It has been suggested that this might lead to the recruitment of DNA methylases and thus KIR gene silencing (Morris et al, 2004). The expression levels and percentage of positive NK cells vary substantially for different 3DL1 alleles. Analysis of receptor cell surface density and

frequency of expression of different 3DL1 allotypes in this study confirmed previous reports that allotypes highly expressed on an individual NK cell were also expressed on a greater percentage of NK cells, with *001>*002>*005. Analysis of the promoter region of the *001 and *002 alleles reveals that the *002 promoter contains a single nucleotide change that generates an Sp1 binding site and results in decreased forward transcription (Davies et al, 2007). Reduced competition at the promoter then leads to a greater level of reverse activity resulting in a lower forward: reverse activity for *002 relative to *001. The *005 promoter contains a nucleotide substitution that disrupts a second Sp1 site and results in a more pronounced reduction in the forward: reverse activity of this promoter. In combination with data on the frequency of expression of individual 3DL1 alleles (*001>*002>*005), this suggests the relative activity of the forward and reverse promoter controls the level of gene activation and in turn the frequency of expression of KIR genes.

6.5 Rapid evolution of KIR genes

In addition to a high level of diversity, another key feature of immune genes is that they show rapid evolution. A comparison of mouse and human NK cell MHC-specific receptors reveals a large degree of functional conservation. Both species possess a lectin like receptor specific for non-classical MHC (HLA-E in human and Q1a in mice) that presents leader peptide derived from classical MHC molecules. In addition, both species possess a multigene family, containing both inhibitory and activatory members, specific for the polymorphic classical MHC molecules. These families (KIR in human and Ly49 in mice) have many common features including stochastic expression patterns, variation in gene number, allelic variation and similar signal transduction pathways. However, these functions are carried out by structurally different receptors encoded by distinct families of genes – the Ig superfamily members KIR and lectin like receptors Ly49. The independent and convergent evolution of these receptors systems performing the same function in humans and mice suggest that they play an important role in these organisms. Humans and rodents diverged over 100 million years ago from a common ancestor who contained ancestral CD94, NKG2 and Ly49 genes, while the absence of KIR genes in the rodent suggests that it evolved after the divergence of the two species. In humans the Ly49 family

is represented by a single gene, which is believed to encode a non-functional protein (Barten and Trowsdale, 1999). Use of these different gene families (Ly49 and KIR) for analogous functions in mice and humans may represent the response of these two species to their particular environmental and pathogen challenges.

In addition to humans, KIR genes have also been characterised in other primates including rhesus monkeys (*Macacca mulatta*), orangutans (*Pongo pygmaeus*) and chimpanzees (*Pan troglodytes*) (Guethlein et al, 2002; Hershberger et al, 2001; Khakoo et al, 2000; Rajalingam et al, 2001). A comparison of these species, in particular our closest living relative the common chimpanzee, suggests that the KIR genes are evolving very rapidly (Khakoo et al, 2000). These two species are believed to have diverged 5 million years ago and share approximately 98.4% sequence similarity in their genomes. Chimpanzees express orthologues of the human HLA-A, B and C genes but of the 16 KIR genes in the human only 4 orthologues (2DL4, 2DL5, 2DS4 and 3DL3) could be identified in the chimpanzee. The two 3 Ig KIR in humans, 3DL1 and 3DL2, are represented by a single sequence in the chimpanzee, Pt-3DL1/2. This chimpanzee receptor recognises both MHC-A and -B molecules and contains elements of the sequence of both human receptors along with a pattern of unique substitutions. Thus it appears that in the 5 million years of evolution between the divergence of human and chimpanzee there has been considerable change in the KIR genes. This evolution of the KIR family of genes appears to be occurring at an equal or faster rate than the evolution of the MHC class I genes.

The family of KIR genes present in human is believed to have arisen from a process of gene duplication and recombination, point mutation and inactivation of functional regions. Analysis of the genomic organisation of KIR genes suggests that gene duplication is an important mechanism of KIR diversification (Martin et al, 2000). Duplication of an ancestral gene allows preservation of one daughter copy with the original function while the second copy can be adapted to serve a novel function. Separate functional domains (ligand binding, transmembrane, signalling) of KIR proteins are encoded by separate exons. This exon structure of KIR genes facilitates the diversification of KIR genes through recombination as replacement of the signalling domain can generate a new KIR gene

encoding a protein with the same ligand specificity but with the opposite signalling capacity e.g. 3DL1/3DS1. A comparison of the allelic diversity of 3DL1 and 3DL2 revealed that there were distinct patterns of variation in these two KIR genes (Gardiner et al, 2001). 3DL1 contains more polymorphic residues than 3DL2 and 3DL1 alleles are more divergent from each other than are 3DL2 alleles. The nucleotide substitutions in 3DL1 alleles are a mixture of synonymous and non-synonymous substitutions and a large proportion of these substitutions are shared between alleles. In contrast, nucleotide substitutions in 3DL2 alleles are almost all non-synonymous and are shared to a lesser extent among alleles. These patterns suggest that while point mutation was important in the generation of allelic diversity for 3DL2, there is greater evidence for recombination in 3DL1. This method could also have given rise to the 3DS1 allele as outlined above. In this work, we have shown that these recombination events in the evolution of 3DL1 have resulted in the generation of functionally distinct receptors; alterations in function that result in a more protective immune response would have been selected for within the population.

KIR genes are rapidly evolving and have been estimated to have expanded at a rate of 0.52 genes/million years (Hao and Nei, 2004). Exposure to pathogens is likely to be the driving force behind the evolution of KIR genes, although as KIR genes have also been implicated in preeclampsia and recurrent spontaneous abortion, reproductive success may also play a role. The role of pathogen in driving the evolution of HLA class I genes has been documented e.g. in West Africa the B*5301 gene is found in high frequency where it confers resistance to severe malaria (Hill et al, 1991). Pathogen exposure may have shaped the KIR repertoire by two different mechanisms. For life threatening pathogens where a T cell response is crucial for the resolution of the infection, there is a selective pressure towards HLA class I molecule capable of presenting pathogen-derived peptide to T cells to initiate a T cell response. This may give rise to alteration in the HLA class I proteins that weaken their recognition with KIR receptors. The KIR receptors would then be under pressure to evolve to maintain their interaction with HLA. Alternatively, for pathogens where NK cells play a vital role in the control or elimination of the infection, there would be a direct selection pressure on KIR genes. This type of selection pressure is likely to

explain the differences in KIR between humans and chimpanzee as these two species have relatively conserved MHC protein. Chimpanzee KIR recognise and are functionally inhibited by human HLA class I alleles, but human KIR do not recognise chimpanzee MHC (Khakoo et al, 2000). Thus the evolution of human KIR cannot be solely explained by a need to “keep up” with MHC evolution.

6.6 KIR in human health and disease

Expression of the KIR family of receptors shows a large degree of variation within the population; to such an extent that there is evidence that healthy individuals may lack any single KIR. While the framework gene 2DL4 is expressed in the vast majority of individuals and was speculated to play an important role during pregnancy, a woman with several successful pregnancies has been documented who does not express this gene (Gomez-Lozano et al, 2003). 3DL1 is found in a very high percentage (~98%) of the population but its HLA ligand Bw4 is found in a smaller fraction such that the estimated frequency of people with a functional 3DL1/HLA pairing is closer to 60% (Du et al, 2007). In addition, while most people express 3DL1, there are two common allotypes that have opposite signalling capacity (3DS1) or altered cellular localisation (*004) and are not expected to function in a similar way to other allotypes. As such 3DL1 (or any other KIR) is not expected to play a dominant role in the response to any vital, lethal or persisting immunological challenge. There is increasing evidence however that expression of these receptor may be beneficial in the control of a number of diseases and malignancies, where the more subtle effects of KIR ligation may tip the balance of the immune response towards a favourable outcome.

6.7 Concluding Remarks

The focus of this study was the investigation of genetic polymorphism of the 3DL1 gene. In this work, we define a functional role for the genetic variation seen between 3DL1 alleles - with different 3DL1 allotypes generating different levels of inhibition upon interaction with HLA-Bw4 allotypes. Sequence variation within the Bw4 subfamily of HLA also impacts on the interaction with a given 3DL1 allotype. Thus expression of different 3DL1 and Bw4 allotypes contributes to the qualitative spectrum of NK cell responses upon 3DL1/Bw4 ligation. In addition, the demonstration that 3DS1 is expressed at the cell surface of NK cells represents a significant advancement in our understanding of its role in NK cell biology. The identification of a serological reagent capable of detecting 3DS1 and distinguishing it from other 3DL1 allotypes provides a useful tool to further probe the function of this receptor. 3DS1 was shown to act as an activatory receptor on NK cells but, despite the sequence similarity to 3DL1 and genetic associations found, it does not interact with Bw4.

6.8 Future Perspectives

This work, as well as studies by other groups, has clearly defined a functional role for the genetic polymorphism seen in long-tailed 3DL1 alleles. The role of other 3DL1 allotypes including 3DS1 and *004 in NK cell function is less clear. The nature of the ligand of 3DS1 remains a key unanswered question in the KIR field. If 3DS1 does recognise Bw4 as currently believed, it must do so only under a restricted set of conditions as several studies, including this one, have failed to detect an interaction. The current focus of research in this area is on the identification of specific, perhaps HIV- or stress protein- derived, peptides that when presented by Bw4 allow recognition by 3DS1. It would be of particular interest to determine if CD4⁺ HIV-infected T cells express a ligand for 3DS1, as would be expected if 3DS1 plays a role in the direct elimination of HIV-infected cells. The *004 allotype of 3DL1, common in many populations, is unusual among 3DL1 allotypes in that it is located almost exclusively within the endoplasmic reticulum. The lack of cell surface expression of this allotype suggests that it does not interact with Bw4 in a similar manner as other 3DL1

allotypes. Thus, *004 may represent a loss of function allotype. However, the *004 allele in association with Bw4 alleles has been associated with improved outcome in HIV. If this genetic association reflects a real functional interaction between these two gene products it would be of considerable interest to define the manner in which this occurs. Is the *004 protein expressed at the cell surface under certain conditions? Or, like 2DL4, does 3DL1*004 recognise a soluble HLA ligand that is endocytosed allowing intracellular interaction? NK cells play a central role in the immune response to a number of different challenges and KIR are important receptors involved in the control of NK cell activation. As such a greater understanding of the mechanisms that control NK cell activation and inhibition by these receptors will lead to a fuller picture of their role in health and disease.

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Appendix

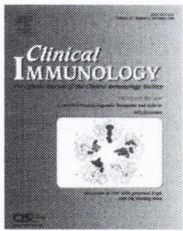
Peer reviewed publications

Peer Reviewed Publications

O'Connor, G. M., A. Holmes, F. Mulcahy, and C. M. Gardiner. Increased Natural Killer Cell Activity in HIV Long Term Non Progressor Patients. *Clin Immunol* (In Press, June 2007)

O'Connor, G. M., K. J. Guinan, R. T. Cunningham, D. Middleton, P. Parham, and C. M. Gardiner. 2007. Functional polymorphism of the KIR3DL1/S1 receptor on human NK cells. *J Immunol* 178:235-241.

O'Connor, G. M., O. M. Hart, and C. M. Gardiner. 2006. Putting the natural killer cell in its place. *Immunology* 117:1-10.



Natural Killer cells from long-term non-progressor HIV patients are characterized by altered phenotype and function

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Received 17 January 2007; accepted with revision 29 May 2007
Available online 3 July 2007

KEYWORDS
Natural killer cells;
HIV;
Long-term
non-progression;
Cytotoxicity

Abstract Natural killer (NK) cells are part of the innate immune system important in the control of viral infections and recent evidence suggests that they may play a role in the pathogenesis of HIV. Long-term non-progressor (LTNP) HIV patients who control replication of the virus and show a delayed disease progression have naturally occurring successful immune responses to HIV. We investigated a role for NK cells in these patients. In agreement with previous reports, NK cell cytotoxic activity was decreased in viremic HIV patients relative to healthy individuals ($p < 0.05$). Viremic HIV patients showed an altered cell surface phenotype, including a reduction in natural cytotoxicity receptor expression and an increase in leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) expression. These phenotypic changes were also present in LTNP patients; however, these patients showed increased levels of NK cell activity relative to viremic HIV patient group.
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Introduction

Natural killer (NK) cells are lymphocytes of the innate immune system important in the control of tumours and viral infections. NK cells play a role in the control of viral infection both through direct killing of infected target cells and the production of chemokines and cytokines which direct an appropriate adaptive immune response [1]. Activation of NK cells is governed by soluble factors as well as the interaction

of a number of inhibitory and activatory cell surface receptors [2]. Many of the inhibitory receptors, including killer inhibitory receptors (KIRs), leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) and CD94/NKG2A, are engaged by HLA molecules. Loss or downregulation of cell surface HLA molecules can target a virally infected cell for NK cell-mediated lysis due to loss of NK cell inhibition. Some of the most important activatory receptors include the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, although their physiological ligands are as yet unknown.

NK cells have been shown to be central to the resolution of certain viral infections including herpes viruses but the

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role of NK cells in HIV infection has not been fully elucidated. NK cell culture supernatant has been shown to suppress endogenous HIV replication in autologous CD4⁺ T cells. This is due to the presence of CC chemokines including RANTES, MIP-1 α and MIP-1 β , which bind to the CCR5 receptor, required by R5 strains of the virus for entry into the target cell [3]. NK cells may also target infected cells for lysis, both through natural lysis due to the downregulation of certain HLA class I molecules and antibody dependent cellular cytotoxicity (ADCC). Increased NK cell activity, both in terms of increased cell lysis and the production of cytokines such as IFN- γ , TNF- α and CC chemokines has been reported in a group of highly exposed, uninfected intravenous drug users [4]. In addition to evidence supporting the role of NK cells in control of HIV, HIV also appears to alter NK cell function. A number of phenotypic changes in cell surface receptor expression on NK cells have been reported in HIV, including an increase in inhibitory receptors such as LILRB1 and KIR, and a decrease in the triggering NCRs [5–7].

A subgroup of HIV patients termed "long-term non-progressors" (LTNP) show delayed disease progression [8]. This group of patients control viral load and maintain high CD4 counts for many years after infection in the absence of antiretroviral therapy. Some contributing viral factors have been identified e.g. nef deletions resulting in decreased infectivity [9], but in the majority of cases, it is the response of the immune system of the infected individual that controls the disease. Several host genetic factors have been associated with LTNP including HLA-B27 [10], HLA-57 [11] and mutations in the chemokine receptor CCR5 [12]. More recently, a study found a genetic association between the activating NK cell receptor KIR3DS1, in the presence of HLA-B molecules containing isoleucine at position 80, and delayed progression to AIDS in HIV infected patients [13]. We investigated the role of NK cells in the control of HIV in LTNP, by examining NK cell function (natural cytotoxicity) and cell surface phenotype of NK cells. We hypothesised that NK cells in these individuals may allow them to control HIV viral replication and remain disease-free.

Materials and methods

Patients

NK cell cytotoxicity and cell surface phenotype was examined in three groups; healthy HIV-negative individuals ($n=8$, hereafter referred to as controls); HIV-positive, HAART (highly active antiretroviral therapy)-negative patients (who had not received HAART in the previous 2 years; hereafter referred to as viremic HIV) ($n=8$) and HIV LTNP (defined as asymptomatic HIV infection for ten or more years with a HIV viral load of less than 5000 copies/ml and CD4 cell count greater than 300×10^6 /dl in the absence of antiretroviral therapy; hereafter referred to as HIV LTNP) ($n=9$). See Table 1. All HIV patients were infected with HIV-1. All patients gave written informed consent.

Cell surface phenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated from patients by Ficoll density gradient. PBMCs were stained

Table 1 Characteristics of viremic HIV, HIV LTNP and control patients

	<i>n</i>	Median HIV viral load (copies/ml)	Median CD4 cell count ($\times 10^6$ /dl)	Mean years since HIV diagnosis
Viremic HIV	8	56,105 (22,000–500,000)	306 (58–1086)	6 (1–18)
HIV LTNP	9	793 (<50–4500)	507 (321–769)	17 (10–22)
Control	8	N/A	N/A	N/A

with the following antibodies: anti-CD56 PE, anti-CD16 FITC, anti-CD3 PerCP, anti-KIR3DL1 PE (DX9), anti-KIR2DL1/2DS1 PE (EB6), anti-KIR2DL2/2DL3/2DS2 PE (GL183), anti-NKG2A PE (Z199), anti-CD94 PE (HP-3D9), anti-LILRB1 PE (HP-F1), anti-NKp30 PE (Z25), anti-NKp44 PE (Z231) and anti-NKp46 PE (BAB281). Cells were acquired on a FACScalibur flow cytometer with Cellquest software (Becton Dickinson, Mountain View, CA).

NK cell cytotoxicity

K562 target cells (1×10^6) were labelled with $50 \mu\text{Ci Na}^{51}\text{CrO}_4$ for 1 h at 37°C . Cells were then washed and incubated with freshly isolated PBMCs at an effector/target ratio of 100:1, 50:1 and 25:1. Cells were centrifuged (150 g, 5 min) and incubated for 4 h at 37°C . Cells were then centrifuged (300 g, 10 min) and a 25- μl sample of supernatant transferred to a flexiplate (Perkin Elmer, Wellesley, MA). Scintillation fluid (150 μl) was added and samples were assayed on a Microbeta Trilux scintillation counter (Perkin Elmer). Percentage cytotoxicity was calculated using the formula (Experimental – spon)/(maximum – spon) $\times 100\%$, where spon = release from targets incubated with medium alone and maximum = release from targets induced by 5% Triton X-100.

Statistics

Data were analysed using one-way ANOVA followed by Bonferroni's multiple comparison test: $*p < 0.05$ and $**p < 0.001$.

Results

NK cell subsets are altered during HIV infection

Alterations in NK cell subsets have been documented in HIV infection, including a reduction in the CD56⁺ population and an increase in the CD56⁺ CD16⁺ population [14,15]. We examined lymphocyte subsets identifying CD3, CD56 and CD16 positive populations in HIV infection. Viremic patients who were not undergoing antiviral therapy and LTNP patients were compared to normal healthy controls. As expected, the percentage of CD56⁺ lymphocytes was decreased in viremic HIV patients relative to healthy controls. While this decrease in CD56⁺ NK cells was also seen in the HIV LTNP group, it was not as pronounced (Fig. 1). Analysis of the CD56^{bright} NK cell subset gave similar results. No significant expansion in the

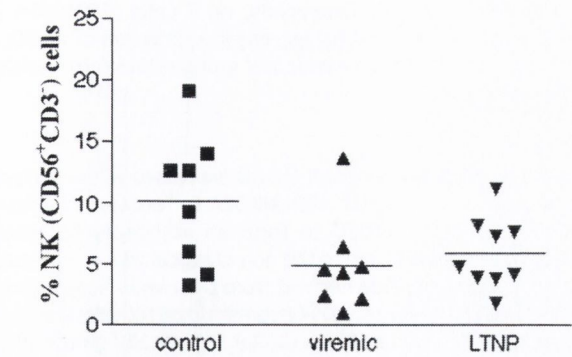


Figure 1 NK cells are altered during HIV infection. PBMCs were isolated from the blood of healthy control, viremic HIV and HIV LTNP patients. Percentage of NK cells (CD56⁺CD3⁻ lymphocytes) was assessed by flow cytometry.

CD56⁻/CD16⁺ subset was observed in any HIV group (3.07 ± 2.03% control, 3.20 ± 2.75% HIV, 3.75 ± 2.60% viremic HIV LTNP).

Long-term non-progression in HIV is associated with increased NK cell cytotoxicity

One of the primary roles of NK cells is their ability to lyse virally infected cells. We examined the cytotoxic potential of NK cells in HIV infection using the NK susceptible cell line K562 as a target. NK cells from healthy controls were able to lyse K562 cells efficiently at a range of effector to target ratios. In agreement with several published reports [16,17], viremic HIV patients showed significantly reduced killing in the same assay at an effector/target ratio of 100:1 (*p* < 0.05) and 50:1 (*p* < 0.01). In line with our hypothesis that NK cells may contribute to a better outcome in HIV infection, HIV LTNP patients showed increased levels of killing relative to

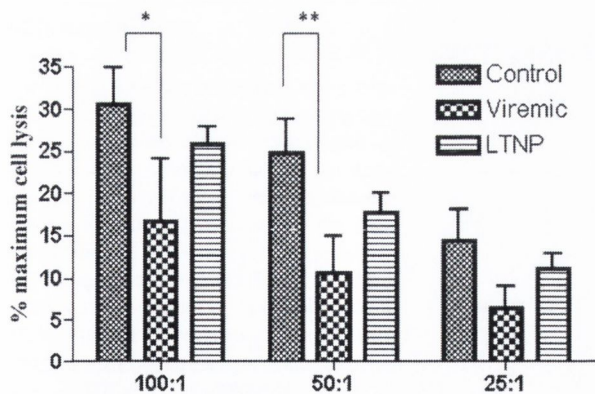


Figure 2 NK cell cytotoxicity is increased in HIV LTNP relative to viremic HIV patients. PBMCs were isolated from the blood of control, viremic HIV and HIV LTNP patients. Cytotoxicity was assessed using a standard 4-h ⁵¹Cr release assay against K562 targets at a range of effector to target ratios. Data shown is the average of 8 (control and viremic HIV) or 9 (LTNP) samples; error bars show SEM.

viremic HIV patients at each effector/target ratio, although levels did not reach those of healthy controls (Fig. 2).

Cell surface phenotype is altered in viremic and LTNP HIV patients

NK cell activation is governed by the balance of positive and negative signals integrated from cell surface receptors. These receptors have been a focus of much research in recent years and include several HLA-specific receptors as well NCRs. In light of the difference in NK cell cytotoxic activity in the patient groups, we hypothesized that this could reflect an altered cell surface phenotype. We therefore examined the expression of a number of these receptors.

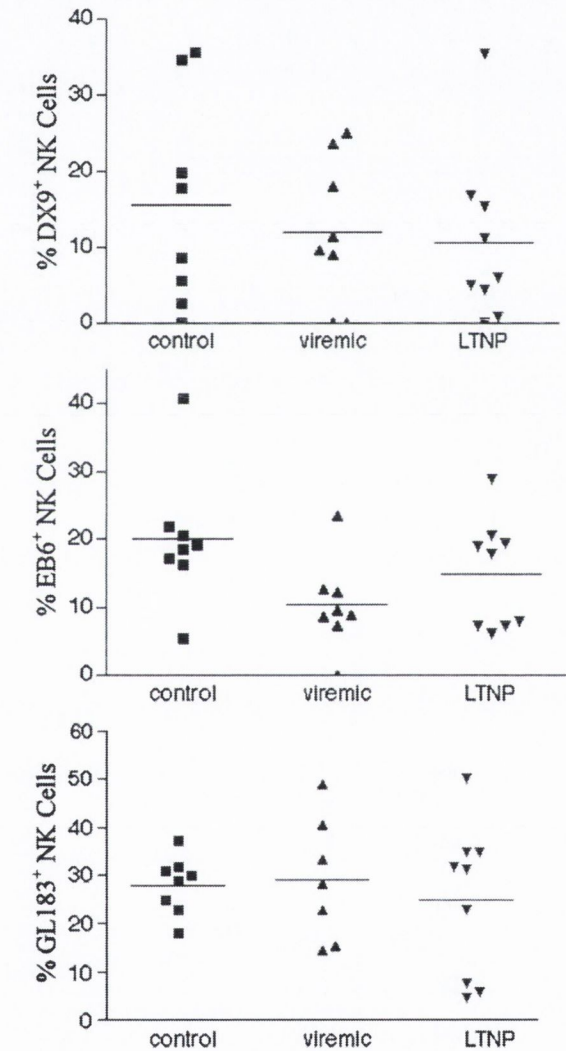


Figure 3 KIR expression on NK cells during HIV infection. PBMCs were isolated from the blood of healthy control, viremic HIV and HIV LTNP and examined by FACS analysis. Gated NK cells (CD56⁺CD3⁻ lymphocytes) were examined for expression of KIR molecules using DX9 (top panel, anti-KIR3DL1), EB6 (middle panel, anti-KIR2DL1/KIR2DS1) and GL183 (lower panel, anti-KIR2DL2/KIR2DL3/KIR2DS2).

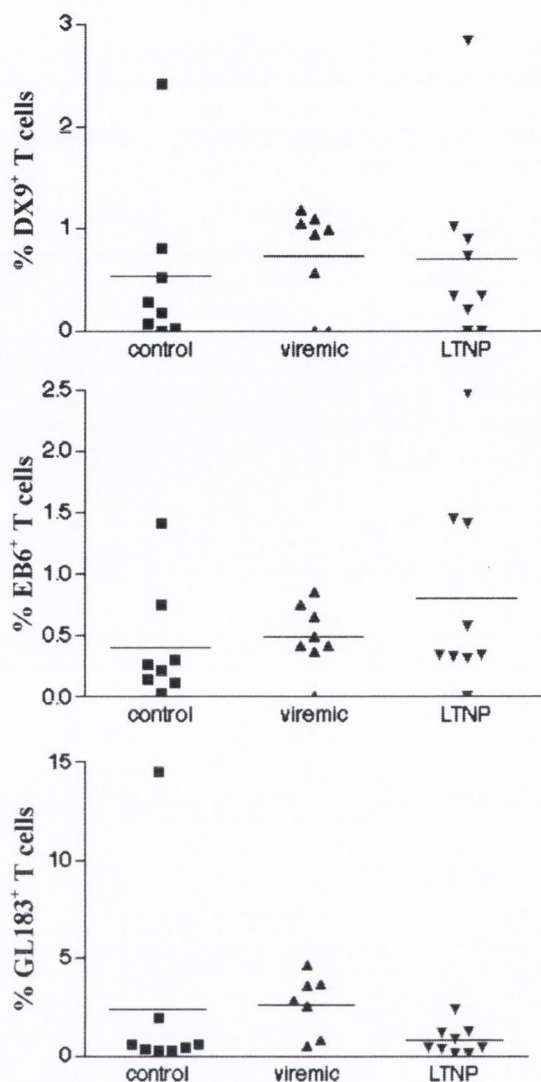


Figure 4 KIR expression on T cells. PBMCs were isolated from the blood of healthy control, viremic HIV and HIV LTNP and examined by FACS analysis. Gated T cells ($CD3^+/CD56^-$ lymphocytes) were examined for expression of KIR molecules using DX9 (top panel, anti-KIR3DL1), EB6 (middle panel, anti-KIR2DL1/KIR2DS1) and GL183 (lower panel, anti KIR2DL2/KIR2DL3/KIR2DS2).

HLA-specific receptors

Killer cell Immunoglobulin-like receptor (KIR) expression

The KIR family includes both inhibitory (KIR2DL, 3DL) and activatory (KIR2DS, 3DS1) receptors which have been shown to be specific for certain HLA class I molecules. HLA-C-specific KIR including KIR2DL1/2DS1 (specific for HLA-C containing lysine at position 80) and KIR2DL2/3 and KIR2DS2 (specific for HLA-C containing asparagine at position 80) were examined using EB6 and GL183 antibodies respectively. KIR3DL1, specific for HLA-Bw4, was examined using a DX9 antibody. The percentage of NK cells expressing KIR was broadly similar between the groups, although there was a trend for lower expression on NK cells from viremic and LTNP HIV patients relative to healthy controls for both KIR2DL1/S1

and KIR3DL1 (Fig. 3). Conversely, on T cells ($CD3^+/CD56^-$), there was a slight trend for increased expression of KIR3DL1 and KIR2DL1/S1 in the viremic HIV group relative to healthy controls (Fig. 4).

CD94 expression

CD94 is a lectin-like receptor that is expressed at the surface of NK cells in association with NKG2A to form an inhibitory receptor, or with NKG2C to form an activatory receptor. These heterodimers bind to the non-classical HLA-E molecule which presents peptide derived from the leader sequence of many class I HLA alleles. CD94 expression on NK cells was high in the control group and was similar in both HIV groups (Fig. 5). CD94 expression on T cells was lower than on NK cells and shows an increase in viremic HIV. This increase was also seen in the HIV LTNP group (Fig. 5). NKG2A expression levels on NK and T cells were also examined and were similar across the groups (data not shown).

LILRB1

LILRB1 is a inhibitory member of the leukocyte immunoglobulin-like receptor (LILR) superfamily that has been shown to bind to a number of HLA-A and HLA-B alleles. LILRB1 expression on both NK and T cells showed a similar pattern with increased expression in viremic HIV and HIV LTNP groups relative to healthy controls. In healthy controls $27.16 \pm 9.67\%$ of NK cells expressed LILRB1 and this significantly increased in both HIV viremic ($65.41 \pm 8.88\%$, $p < 0.001$) and LTNP

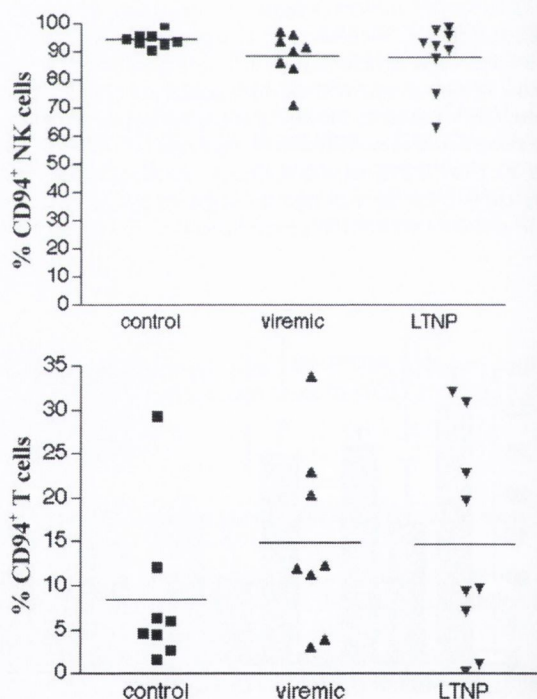


Figure 5 CD94 expression is increased on T cells in viremic HIV and HIV LTNP patients. PBMCs were isolated from the blood of healthy control, viremic HIV and HIV LTNP and examined by FACS analysis. Gated NK cells ($CD56^+/CD3^-$ lymphocytes, upper panel) and T cells ($CD3^+/CD56^-$, lower panel) were examined for expression of CD94 molecule using antibody clone HP-3D9.

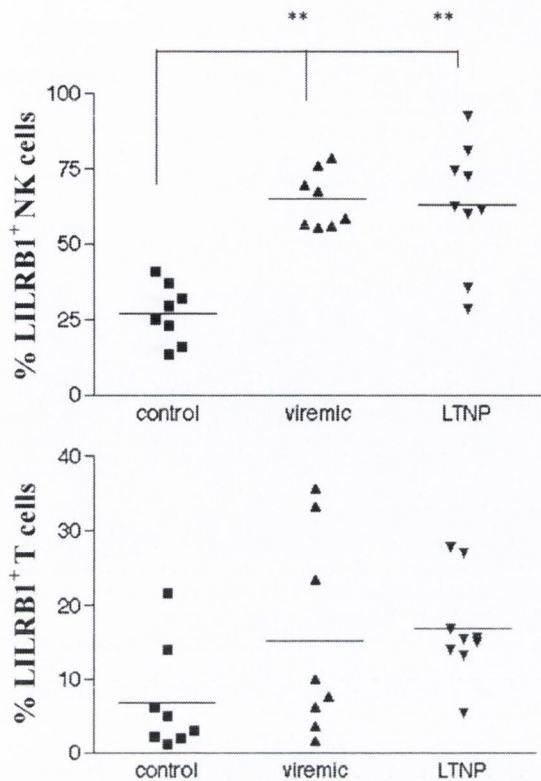


Figure 6 LILRB1 expression is increased on both NK and T cells in viremic HIV and HIV LTNP patients. PBMCs were isolated from the blood of healthy control, viremic HIV and HIV LTNP and examined by FACS analysis. Gated NK cells (CD56⁺CD3⁺ lymphocytes, upper panel) and T cells (CD3⁺CD56⁺, lower panel) were examined for expression of LILRB1 molecule using antibody clone HP-F1. ***p*<0.001.

(63.42±21.97%, *p*<0.001) groups. A similar trend was seen in T cells with cells expressing LILRB1 (Fig. 6).

NCR expression

The NCR activatory receptors are preferentially expressed on NK cells. In agreement with reports in the literature [16], we found that levels of both NKp30 and NKp46 were reduced in viremic HIV patients relative to controls (NKp30; 39.63±25.14% vs. 52.62±24.71%, NKp46; 66.12±5.10% vs. 88.3±23.4%). Based on the cytotoxicity data of the three groups, we predicted that the NK cells of HIV LTNP individuals would have increased expression of these receptors relative to viremic HIV patients. However, we found that HIV LTNP patients showed no trend towards normalization and indeed levels of NKp30 expression were further reduced in this group (Fig. 7). NKp44 is not expressed on non-activated NK cells and levels of expression were low and similar in the three groups examined (data not shown).

Discussion

NK cells are an attractive subject for study in HIV infection due to their long established role in the control of viral

infections, both through the lysis of virally infected cells and the production of immunomodulatory cytokines that help the generation of an appropriate adaptive immune response. NK cells are also producers of CC chemokines, which compete with some HIV strains for binding to cell surface receptors on target cells providing another possible mechanism by which NK cells may contribute to the anti-HIV response. In support of a role for NK cells in HIV, several studies have correlated NK cell receptor genotype [13], cell number or activity to LTNP and resistance to infection [4,18].

We investigated NK cell activity and cell surface phenotype in viremic HIV and HIV LTNP groups. We observed a decrease in NK cell killing in viremic HIV patients relative to controls in agreement with several reports in the literature, although the opposite has also been observed [19]. Importantly, we see higher activity in HIV LTNP patients (relative to viremic HIV), who have levels of killing close to that of controls, in spite of a decrease in the percentage of NK cells in these individuals. HIV LTNP individuals offer an unique insight into the immune system of the minority of individuals who control the spread of HIV and provide an opportunity to study a successful immune response to this virus. NK cell activity (as measured by cytotoxicity) was maintained in these patients, suggesting that this immune parameter may be allowing these individuals to control the virus. As a component of the innate immune system, NK cells are primed to respond immediately to invading virus and it may be the initial, early control of viral replication that permits

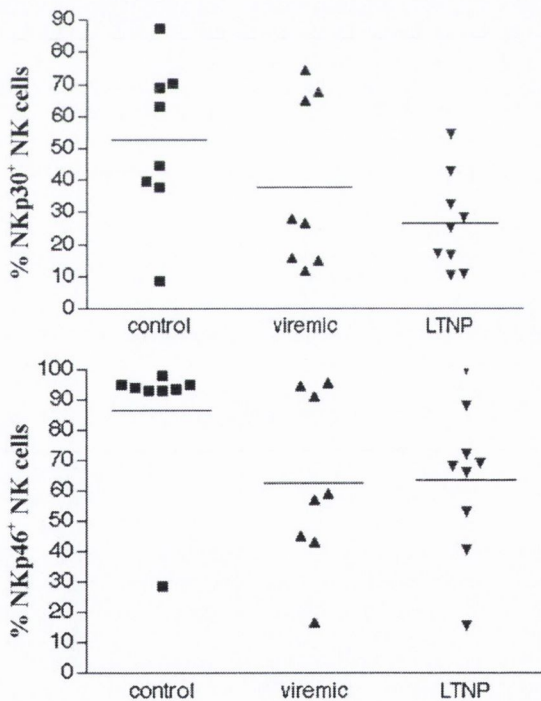


Figure 7 NCR expression is reduced on NK cells in viremic HIV and HIV LTNP patients. PBMCs were isolated from the blood of healthy control, viremic HIV and HIV LTNP and examined by FACS analysis. Gated NK cells (CD56⁺CD3⁺ lymphocytes) were examined for expression of NCR molecules using anti-NKp30 (top panel, clone Z25) and anti-NKp46 (lower panel, clone BAB281).

eventual control of the infection, both by NK cells themselves and by the development of a protective adaptive immune response.

The difference seen in cytotoxic activity of NK cells in HIV LTNP relative to viremic HIV patients lead us to investigate if NK cell surface phenotype correlated with functional outcome. Perturbation of NK cell receptor repertoire has been reported in HIV infection, affecting both inhibitory receptors [6,7] and activatory NCRs. We observed decreased expression of Nkp46 and Nkp30 in viremic HIV patients as previously reported [16], but this was seen equally in the HIV LTNP group. Increased levels of inhibition of NK cells have also been proposed as a reason for decreased NK cell activity in HIV. We did not observe any increase in KIR or NKG2A expression on NK cells during HIV, however levels of LILRB1-positive NK cells were significantly increased in both HIV samples in agreement with previous reports [7]. As LILRB1 is an inhibitory receptor (which binds to several HLA alleles) it is an unlikely candidate to explain an increase in cytotoxicity. The reduction in NCR expression and the lack of reduction (and in some cases increase) in expression of the inhibitory receptors examined, in both HIV groups, suggests that maintenance of NK cell killing in HIV LTNP cannot be attributed to these factors. In addition to cell surface receptor expression, NK cell cytotoxicity is also governed by a range of factors, including the expression of adhesion and costimulatory molecules, levels of granzyme and perforin and the cytokine environment, and differences seen in levels of killing in the HIV groups examined here may reflect alterations in any one of these regulatory factors.

Although our results are consistent with a model in which increased NK cell activity keeps HIV infection under control in LTNP, this model does not consider the effects that HIV infection itself may have on NK cells. NK cell parameters examined could potentially be altered both by the immune environment created during HIV infection and by direct interaction with HIV proteins such as gp120, which has been shown to suppress proliferation, activity and survival of NK cells [20]. It is difficult to conclude if our data from LTNP patients reflect differences in NK cell biology that lead to control of HIV, or a beneficial effect of low viral load on NK cell function. It has been reported that many of the alterations of the immune system, including those of NK cells are dependent on viremia and can be restored by suppression of viral load with HAART [6,7]. Other components of the immune system such as CD8⁺ T cells have also been shown to contribute to control of HIV progression in some cohorts [21,22] and indeed we observed a trend towards increased expression of KIR on T cells of both HIV patient groups, possibly due to the activation of T cells in the response to HIV viremia. KIR expression on HIV-specific CD8⁺ T cells has been shown to limit their cytolytic function and may contribute to disease progression by inhibiting the elimination of infected cells by cytotoxic T cells [23].

It is likely that more than one component of the immune system is required to mount an effective immune response to HIV and it is probably their synergistic cooperation that allows for control of viremia in LTNP. In this respect, it is possible that the role of NK cells may be one in which they act early in infection to limit viral spread, eliminate infected cells and help in the generation of an appropriate adaptive immune response.

Acknowledgment

This work was supported by an Irish Health Research Board Project grant.

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Functional Polymorphism of the KIR3DL1/S1 Receptor on Human NK Cells¹

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NK cells express both inhibitory and activatory receptors that allow them to recognize target cells through HLA class I Ag expression. KIR3DL1 is a receptor that recognizes the HLA-Bw4 public epitope of *HLA-B* alleles. We demonstrate that polymorphism within the KIR3DL1 receptor has functional consequences in terms of NK cell recognition of target. Inhibitory alleles of *KIR3DL1* differ in their ability to recognize HLA-Bw4 ligand, and a consistent hierarchy of ligand reactivity can be defined. *KIR3DS1*, which segregates as an allele of *KIR3DL1*, has a short cytoplasmic tail characteristic of activatory receptors. Because it is very similar to KIR3DL1 in the extracellular domains, it has been assumed that KIR3DS1 will recognize a HLA-Bw4 ligand. In this study, we demonstrate that KIR3DS1 is expressed as a protein at the cell surface of NK cells, where it is recognized by the Z27 Ab. Using this Ab, we found that KIR3DS1 is expressed on a higher percentage of NK cells in *KIR3DS1* homozygous compared with heterozygous donors. In contrast to the inhibitory KIR3DL1 allotypes, KIR3DS1 did not recognize HLA-Bw4 on EBV-transformed cell lines. *The Journal of Immunology*, 2007, 178: 235–241.

Natural killer cells are cytotoxic lymphocytes of the innate immune system that are particularly important in the control of viral infections (1, 2). NK cells express a range of activatory and inhibitory cell surface receptors, and their responses are controlled by the overall balance of positive and negative signals received (3, 4). Cell activation can result from either increased activatory or decreased inhibitory signaling inputs. An important source of inhibitory signals comes from HLA-specific receptors, including members of the killer cell Ig-like receptor (KIR)³ family and the CD94/NKG2a heterodimer (5, 6). When HLA expression is altered during viral infection or during transformation, this inhibitory signal is disrupted; NK cells thus become activated and can mediate cytotoxicity against the target cell.

HLA class I genes are highly polymorphic, and most of the variability resides in the Ag-binding groove comprised of the $\alpha 1$ and $\alpha 2$ domains of the H chain (6, 7). This variability affects the peptide that can bind and therefore influences recognition of Ag by the immune system (8). To cope with diversity inherent in HLA, NK cell receptors target relatively conserved epitopes within HLA. HLA-B alleles are characterized by having either a Bw4 or a Bw6 epitope at residues 77–84 of the $\alpha 1$ H chain (9). Although there is no known receptor for Bw6, approximately one-third of HLA-B

alleles have the Bw4 epitope that is recognized by the KIR3DL1 (3DL1) receptor on NK cells (10). Although highly conserved, there is some limited variability within the Bw4 epitope and at least four different amino acid sequences are known (11). This diversity has the potential to influence NK cell recognition through 3DL1.

KIR genes are characterized by diversity at many levels including polygeny, polymorphism, and expression characteristics (5). This is a reflection of their recent and rapid evolution (12). We hypothesized that given the strong pressure on KIR to diversify, polymorphism present in individual genes would have functional consequences within the human immune system. As our prototype gene we investigated *3DL1*, which we have previously shown to be highly polymorphic (13). Most of the alleles of the *3DL1* locus encode for inhibitory receptors, of which 3DL1*001 and 3DL1*002 are the most common (13). We therefore chose these receptors for investigation along with the distinctive *KIR3DS1* (3DS1) allele.

3DS1 is present in ~38% of the population (14). It is highly homologous to *3DL1* in its extracellular domains (ECD) but resembles activatory KIR in the intracellular portion of the molecule. Activatory KIR have no inherent signaling capacity because they have a truncated cytoplasmic tail (5). However, upon receptor ligation, they can recruit positive signaling adaptor molecules that result in NK cell activation (15–17). No serological reagents for 3DS1 have been described to date, and this has hampered biochemical and functional characterization of this receptor. 3DS1 is of particular interest because it has been reported that, in combination with HLA-Bw4 molecules with isoleucine at position 80 (Bw4–80Ile), its expression is associated with delayed progression to AIDS (17). This association was only seen in the presence of both *3DS1* and *Bw4–80Ile*, suggesting that in these patients, these receptors interact functionally. One attractive hypothesis is that 3DS1⁺ NK cells recognize and kill HLA Bw4–80Ile HIV-infected targets, allowing control of infection. Due to the very high degree of similarity to 3DL1 extracellularly, 3DS1 is expected to have similar ligand specificity, but neither this nor definitive cell surface expression have been demonstrated.

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Received for publication May 31, 2006. Accepted for publication October 4, 2006.

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¹ This work was supported by an Irish Health Research Board Project grant.

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³ Abbreviations used in this paper: KIR, killer cell Ig-like receptor; ECD, extracellular domain; LILR, leukocyte Ig-like receptor; F, forward; R, reverse.

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In this study, we demonstrate that polymorphism within 3DL1 influences recognition by the HLA-Bw4 epitope and, in turn, polymorphism within HLA-B contributes to altered recognition of 3DL1. In addition, we demonstrate that 3DS1 is expressed at the cell surface, where it is recognized by the Z27 Ab. However, despite a significant genetic association with delayed disease progression in HIV-infected patients (17), we did not detect any functional interaction between 3DS1 and HLA-Bw4.

Materials and Methods

Cell culture

The T cell line Jurkat, the MHC class I-deficient EBV-transformed B cell line 721.221, and its transfectants 721.221B*0702, 721.221B*2705, 721.221B*3801, 721.221B*5101, and 721.221B*5801 (10) were maintained in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS (PAA Laboratories). Transfectants with similar levels of protein expression were used in experiments.

Generation of NK cell cultures

Blood samples were drawn from normal healthy individuals from whom written consent was obtained. PBMCs were isolated using Lymphoprep (Axis-Shield) gradient. DNA was isolated using Wizard Genomic DNA isolation kit (Promega), and 3DL1 allelic typing analysis was performed as described previously (13). NK cells were isolated by magnetic bead isolation using NK Isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. NK cells were stained with anti-CD56FITC Ab (BD Biosciences), anti-3DL1PE Ab (Beckman Coulter), and anti-CD3PerCP Ab (BD Biosciences), and CD56-positive, CD3-negative, 3DL1-positive cells were sorted on a BD FACS Aria (BD Biosciences). Polyclonal cultures were maintained in CellGro medium (CellGenix) supplemented with 5% human AB serum (Sigma-Aldrich), 200 U/ml human recombinant IL-2 (Biological Resources Branch, National Cancer Institute), 50 ng/ml OKT3 (eBioscience), and 0.5×10^6 irradiated PBMCs/ml. NK cell clones were generated by limited dilution as described in Ref. 18. NK cells were characterized by FACS with anti-CD56FITC Ab, anti-3DL1PE (Z27) (Beckman Coulter), anti-CD3PerCP Ab, anti-leukocyte Ig-like receptor (LILRB1) Ab (Beckman Coulter), anti-CD94 Ab (DakoCytomation), and anti-NKG2A Ab (Beckman Coulter). LILRB1-negative clones were preferentially chosen for analysis.

RT-PCR

Total RNA was extracted from Z27-negative and Z27-dim-sorted NK cell populations with Tri Reagent (Molecular Research Centre). cDNA was generated with random hexamers using ImProm-II Reverse Transcription System (Promega). PCR was conducted on cDNA samples with the following primers: 2DL4RT (forward (F), CTGTCCCTGAGCTCTACAA and reverse (R), CACTGAGTACCTAATCACAG) to ensure quality of the cDNA; and 3SD1RT (F, GGCACCCAGCAACCCCA and R, AAGGGCACGCATCATGGA) for the presence of 3DS1 mRNA using TaqDNA polymerase (Invitrogen Life Technologies).

CD107a/CD69 activation

Purified NK cells were plated at 1×10^6 cell/ml and stimulated with 721.221 cell lines at a stimulator:responder ratio of 1:5 (CD107a) or 1:1 (CD69). Anti-CD107a FITC Ab and 10 μ M monensin (Sigma-Aldrich) were added at time of stimulation for the CD107a experiment. Cells were incubated at 37°C for 6 h (CD107a) or 18 h (CD69), and then stained with anti-3DL1PE (Z27) and anti-CD56PECy5 (BD Biosciences). CD69 samples were stained with anti-CD69 Ab (BD Biosciences).

^{51}Cr release cytotoxicity assay

Target cell lines (721.221 and transfectants) were labeled with 50 μ Ci of $\text{Na}^{51}\text{CrO}_4$ for 1 h at 37°C. Cells were then washed twice in complete medium and incubated with effector cells (NK cell clones, NK cell polyclonal cultures) at an E:T ratio of 6:1. After 4 h at 37°C, a sample of supernatant was counted on a Microbeta Trilux scintillation counter (PerkinElmer). Percentage cytotoxicity was calculated using the formula (experimental - spons)/(maximum - spons) $\times$ 100%, where spons = release from targets incubated with medium alone and maximum = release from targets by 5% Triton X-100 (Sigma-Aldrich). Blocking with anti-3DL1 DX9 Ab (BD Biosciences), Z27 (Immunotech), or with anti-CD94 Ab (Serotec) was conducted at 10 μ g/ml.

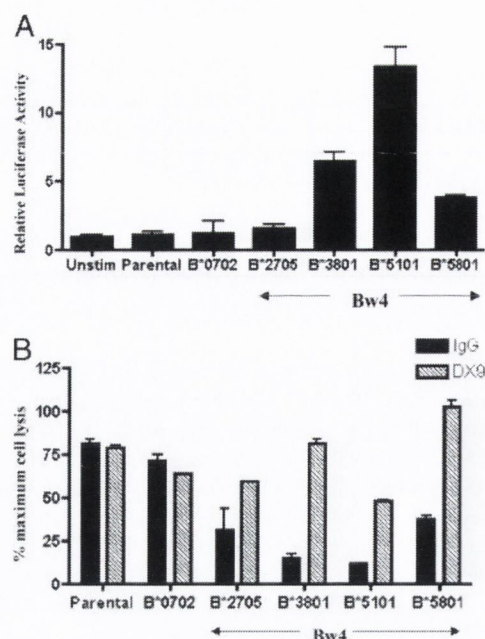


FIGURE 1. Variability in HLA-Bw4 affects recognition by 3DL1*001. **A**, 3DL1*001ECD Jurkat cells were transfected with a NFAT-luciferase reporter plasmid. Cells were then stimulated with the 721.221 cell line and a panel of its HLA transfectants at a stimulator:responder ratio of 1:5. Firefly luciferase activity was measured after 18 h as a readout of NFAT activation and is expressed relative to unstimulated control. CD3/CD28 Dynabeads (3 beads/cell) were used as a positive control. Data shown are representative of four independent experiments and error bars represent SDs. **B**, 3DL1*001-positive clones were used as effectors in a ^{51}Cr release cytotoxicity assay against a panel of 721.221 transfectant targets. Cells were incubated with anti-KIR3DL1 Ab DX9 at 10 μ g/ml. Data shown are representative of three independent experiments and error bars represent SD.

Generation of KIR3DS1 FLAG

Total RNA was isolated from PBMCs of a 3DS1-positive individual using Tri Reagent (Molecular Research Center). cDNA was generated with random hexamer primers using ImProm-II Reverse Transcription System (Promega). Full-length 3DS1 was amplified with primers that contained *NotI* and *EcoRI* restriction sites for subcloning (F, 5'-ccgaatcgccgcccac cggcagcaccatgt-3' and R, 5'-atcatgcatcttctctgtgaaacacagcttccaatta-3'). PCR products were cloned into TOPO cloning kit (Invitrogen Life Technologies) and sequenced. An error-free clone was digested and subcloned into the expression vector pcDEFIII. Leader-FLAG (F, 5'-ccgaatcgccg caccggcagcaccatgt-3' and R, 5'-cttatcgctgctgcatctctgtatct ggacggccctt ggaccaa-3') and FLAG-D0 (F, 5'-gattacaaggatgacgacgataagcaccatggg tggcaggacaa-3' and R, 5'-caccacagcgtggcagg-3') sequences were amplified by PCR. Recombinant PCR was used to generate a leader-FLAG-D0 construct. This construct and the full-length 3DS1 construct were digested using *NotI* and *EcoRI*, and the leader-FLAG-D0 sequence was ligated into the 3DS1 construct.

Generation of KIR3DL1ECD/CD3 ζ -expressing cell lines

Chimeric constructs consisting of the ECD of different 3DL1 linked with the intracellular CD3 ζ chain were generated by recombinant PCR using a similar strategy to that described previously (19). Briefly, error-free clones of 3DL1*001, 3DL1*002, and 3DS1 were used as templates for first round PCR, which generated a product containing a ζ -chain overlap. Similarly, first-round PCR from a ζ -chain-containing plasmid generated a ζ -chain with a 3DL1 overlap. Second-round PCR created a chimeric construct encoding 3DL1 in ECD and CD3 ζ in cytoplasmic domain. PCR products were cloned into a GFP-expressing plasmid such that GFP was in frame at the C terminus of the 3DL1/CD3 ζ chimeric protein. Subsequently, error-free clones of 3DL1/CD3 ζ /GFP chimeras were amplified using primers containing new restriction sites (*NotI* and *XbaI*) and subcloned into the pcDEF vector. Jurkat cells were then transfected by electroporation and cultured in G418 (Sigma-Aldrich) at

Table I. Polymorphism present in Bw4/Bw6 epitopes of HLA-B alleles

Allele	Serotype	Amino Acid Residue Number				
		77	80	81	82	83
HLA-B*0702	Bw6	S	N	L	R	G
HLA-B*2705	Bw4	D	T	L	L	R
HLA-B*3801	Bw4	N	I	A	L	R
HLA-B*5101	Bw4	N	I	A	L	R
HLA-B*5801	Bw4	N	I	A	L	R
HLA-B*1513	Bw4	N	I	A	L	R

2 mg/ml. After selection, GFP-expressing cells were sorted to generate stable cell lines expressing different allelic variants of 3DL1. These variants were named KIR3DL1*001ECD, KIR3DL1*002ECD, and KIR3DS1ECD for 3DL1*001, 3DL1*002, and 3DS1 alleles, respectively. They expressed protein at similar levels as measured by flow cytometry.

Transfection

Transfection of Jurkat cells was performed using a Bio-Rad Gene Pulser II electroporator. A total of 15 µg of plasmid DNA (KIR3DS1FLAG, KIR3DL1/CD3ζ, or NFAT-luciferase construct) was transfected into 10⁷ Jurkat cells in 250 µl of cytomix electroporation buffer (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM HEPES, 2 mM EGTA, 2 mM ATP, and 5 mM glutathione (pH 7.6)) in a 2-mm gap cuvette. The cuvette was then subjected to two pulses, each 240 V and 100 µF, 30 s apart.

Reporter assay

A total of 10⁷ KIR3DL1ECD/CD3ζ Jurkat cells was transfected with 15 µg of NFAT-luciferase plasmid. Transfected Jurkat cells were plated at a concentration of 1 × 10⁶ cells/ml, and stimulator cells (721.221 cell line and its transfectants) were added to give a stimulator:responder ratio of 1:5. CD3/CD28 Dynabeads (DynaL Biotech) (3 beads/cell) were added to pos-

itive wells. Cells were incubated for 18 h at 37°C with 5% CO₂, after which they were harvested, and the cells were lysed for 15 min on a shaker at room temperature with 50 µl of 1× Passive Lysis Buffer (Promega). Firefly luciferase activity was assayed by the addition of 40 µl of luciferase assay mix (20 mM tricine, 0.26 mM (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67 mM MgSO₄, 0.1 M EDTA, 33.3 mM DTT, 270 µM CoA, 470 mM luciferin, 530 µM ATP) to the sample; luminescence was read using the Reporter microplate luminometer (Turner Designs) and expressed relative to unstimulated control.

Results

Polymorphism of HLA-Bw4 alleles affects recognition by 3DL1*001

3DL1 recognizes the subgroup of HLA-B molecules that express the Bw4 serological epitope. We wished to investigate whether natural variability within the Bw4 epitope of *HLA-B* alleles affects recognition by 3DL1. To address this question, genetic constructs were generated that consisted of the ECD of different alleles of 3DL1 linked to the CD3 ζ-chain intracellularly. These were transfected into the Jurkat T cell line, which we know to be KIR negative, to create a panel of Jurkat cells expressing single 3DL1 alleles in isolation. 3DL1*001 (KIR3DL1*001ECD), 3DL1*002 (KIR3DL1*002ECD), and 3DS1 (KIR3DS1ECD) expressing cells were identified by GFP staining and cell sorted to generate stably transfected cell lines. Ligation of 3DL1 on the cell surface leads to positive signaling through NFAT, which can then be measured by a standard reporter assay. To identify receptor specificity, we used a panel of stimulator cells (721.221 cell line) transfected with individual HLA alleles. This reductionist system allows interactions between 3DL1 and HLA to be studied in isolation from the complexities of multiple receptor expression. Incubation of KIR3DL1*001ECD-transfected Jurkat cell line with stimulator 721.221 cell lines expressing Bw4, but not Bw6 HLA-B allotypes,

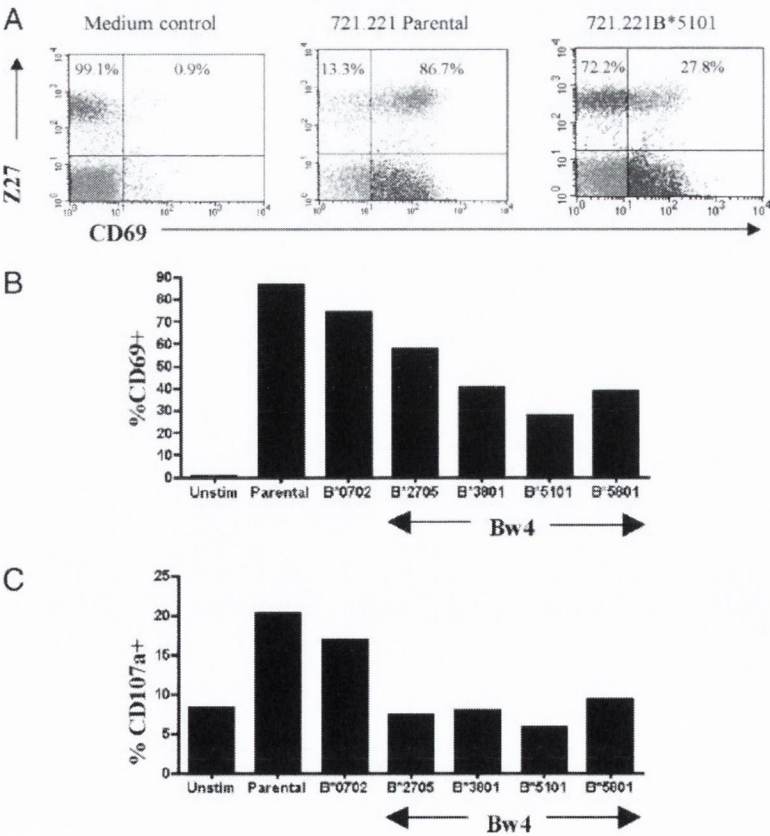


FIGURE 2. Primary NK cells from a donor expressing 3DL1*001 are differentially inhibited by HLA-B allotypes. Purified NK cells were incubated with a panel of 721.221 transfectants at a stimulator:responder ratio of 1:1 (CD69) or 1:5 (CD107a). Cells were coincubated at 37°C for 6 h (CD107a) or 18 h (CD69), and then stained with anti-KIR3DL1PE (Z27) and anti-CD56PECy5. Data for HLA-B*5101 is shown as a representative plot in A. B and C. Data for CD69 and CD107a, respectively.

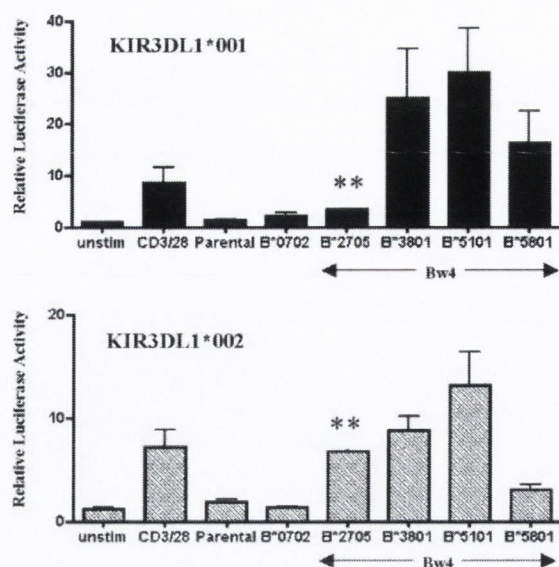


FIGURE 3. KIR3DL1 polymorphism affects HLA-B recognition. 3DL1*001ECD and 3DL1*002ECD Jurkat cell lines were transfected with NFAT-luciferase reporter plasmid. Cells were then stimulated with 721.221 cells and a panel of its HLA transfectants at a stimulator: responder ratio of 1:5. Firefly luciferase activity was measured after 18 h as a readout of NFAT activation and is expressed relative to unstimulated control. CD3/CD28 Dynabeads (3 beads/cell) were used as a positive control. Data shown are the average of four independent experiments. Error bars, SEM.

led to the generation of a NFAT signal as expected (Fig. 1A). Clear and consistent differences in the strength of signal generated from different HLA-B allotypes were observed, even when the amino acid sequence of the HLA-Bw4 epitopes were similar (see Table I). Interaction of 3DL1*001 with B*5101 consistently produced a stronger inhibitory signal than any other HLA-B allotype tested, whereas B*2705 generated only a very weak signal.

3DL1*001 specificity was also examined in NK cell clones. IL-2-activated NK clones that were positive for 3DL1*001 were used as effector cells against our panel of 721.221 transfectants in a ^{51}Cr release assay as described previously (20). As expected, cells expressing HLA-Bw4 allotypes were protected from lysis by 3DL1*001-positive clones, and this inhibition of killing was reversed in the presence of the anti-3DL1 Ab DX9 (Fig. 1B). The hierarchy of recognition was similar to that seen in Fig. 1A with B*5101 giving the greatest level of protection, followed by B*3801, B*5801, and B*2705.

Primary, bulk populations of peripheral blood NK cells were purified from two 3DL1*001 homozygous donors and incubated with the panel of 721.221 transfectants, after which activation (CD69) and degranulation (CD107a) markers were examined. Double staining with the anti-3DL1 Ab Z27 allowed for identification of the 3DL1-positive NK cells. Incubation of NK cells with the 721.221 parental cell line led to a dramatic increase in CD69 expression in both KIR-positive and KIR-negative NK cells (Fig. 2). Quantification of the reduction in the percentage of CD69 and CD107a-positive 3DL1*001-positive NK cells produced a hierarchy that is consistent with that obtained from the Jurkat transfectant and clonal cytotoxicity assays. B*5101 is consistently the strongest ligand for 3DL1*001 followed by B*3801 and B*5801. Although B*3801 appears to be significantly stronger than B*5801 in both the Jurkat and clone assays, the difference is less clear with freshly isolated NK cells. B*2705 is also capable of inhibiting

CD69 and particularly CD107a expression in this assay. Thus, using a number of approaches, it is clear that HLA-B Bw4 variability impacts on 3DL1 recognition.

KIR3DL1 polymorphism affects HLA-Bw4 recognition

We next examined whether differences between the 3DL1*001 and 3DL1*002 allotypes affected their interaction with the panel of HLA-B allotypes. Using the Jurkat assay, both 3DL1 alleles showed recognition of HLA-B Bw4 allotypes but not of the parental or Bw6-positive cell line as expected. Although both 3DL1 allotypes showed a preference for B*5101, their interactions with B*2705 and B*5801 were markedly different. Although B*2705 appears to be a weak ligand for 3DL1*001, with only a marginal increase over the Bw6 allotype HLA-B*0702, it is a much stronger ligand for 3DL1*002 (Fig. 3). The reverse is true for HLA-B*5801. Thus, polymorphism present in the 3DL1 receptor affects NK cell recognition of HLA-Bw4.

KIR3DS1 is expressed at the NK cell surface

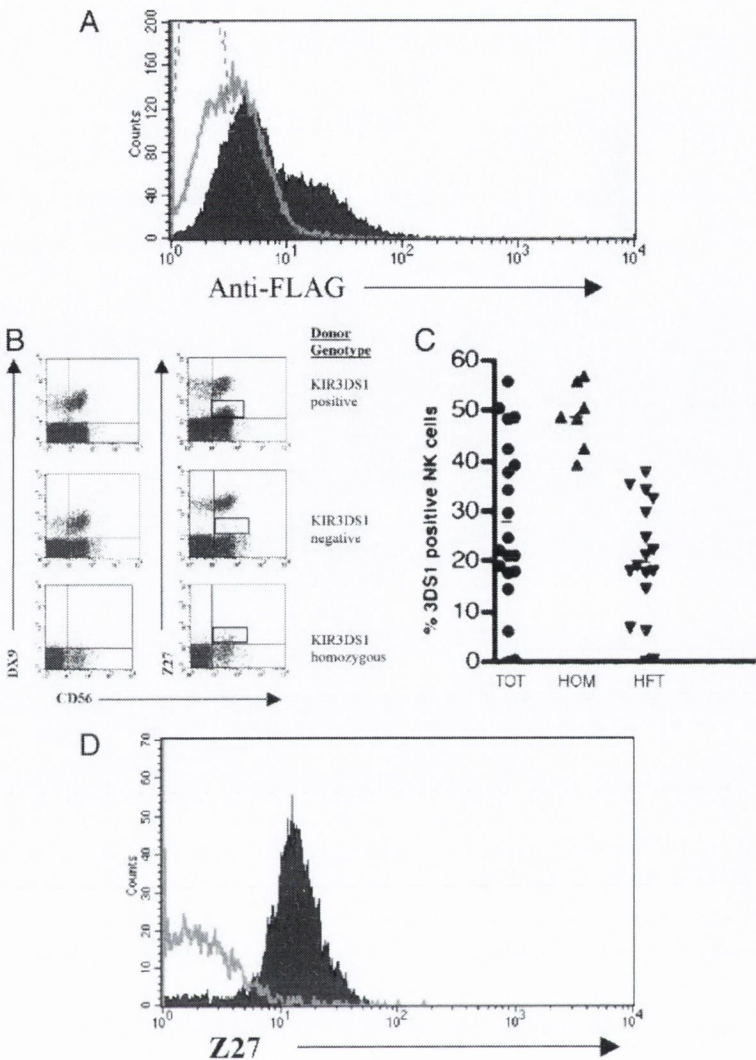
3DS1, a short-tailed allele of 3DL1 that is predicted to be activating, is highly homologous to other 3DL1 alleles extracellularly. Although 3DS1 mRNA is transcribed, 3DS1 does not bind to the anti-3DL1 Ab DX9 and cell surface expression of the 3DS1 protein has not been demonstrated. To address this question, we generated a full-length 3DS1 construct that contained a FLAG epitope at the N terminus by inserting the FLAG sequence between the leader sequence and the first coding exon. This construct was then transfected into the Jurkat cell line and stained extracellularly using an anti-FLAG Ab. As shown in Fig. 4A, cells that were transfected with the FLAG-tagged 3DS1 construct stained with the anti-FLAG Ab whereas the mock-transfected cells did not, indicating that 3DS1 is expressed on the cell surface where it may be functionally active.

NK cells from 60 3DL1-typed donors were analyzed for binding to the anti-3DL1 mAb DX9 and Z27. Although the two Abs had similar reactions with 3DL1, their reaction with 3DS1 was different (Fig. 4B). Whereas DX9 was clearly negative, Z27 gave a weak positive reaction that defined a subpopulation of Z27⁺ NK cells present only in donors who were heterozygous or homozygous for 3DS1. Interestingly, we found that a significantly higher percentage of NK cells expressed 3DS1 when donors were homozygous for this allele when compared with 3DS1 heterozygotes ($p < 0.0001$; Fig. 4C). A Z27 dim subpopulation of CD8, but not CD4, T cells was also detected in 3DS1-positive donors (data not shown). These results indicated that 3DS1 is expressed at cell surfaces. To investigate this question further, we transfected Jurkat cells with the 3DS1ECD/CD3 ζ construct. These transfected cells bound Z27 to a level that clearly distinguished them from untransfected cells (Fig. 4D). In summary, these results demonstrate that 3DS1 is expressed at cell surfaces and can be recognized by an anti-3DL1 Ab.

KIR3DS1 does not recognize HLA-B on EBV-transformed B cells

To investigate the interaction of cell surface 3DS1 with HLA-B, we used the KIR3DS1ECD Jurkat cell line in the reporter assay as described above. We first demonstrated that our 3DS1 chimera was capable of transducing signal by cross-linking the receptor with the Z27 Ab (Fig. 5A, left panel). However, no interaction between 3DS1 and any of the HLA-B alleles tested was detected (Fig. 5A). Confocal microscopy imaging (data not shown) and Z27 Ab staining (Fig. 4D) of the Jurkat transfectant show cell surface expression of the KIR3DS1ECD/CD3 ζ construct, suggesting that intracellular retention of the construct is not responsible for the lack of

FIGURE 4. KIR3DS1 is expressed at the cell surface and is recognized by Z27 Ab. *A*, Jurkat cells were transfected with a FLAG-tagged KIR3DS1 construct and stained with the anti-FLAG Ab (filled histogram) and isotype-matched control Ab (open histogram). Mock-transfected cells were stained as a control (dashed line). *B* and *C*, Donors were typed for *KIR3DL1* allelic expression, and PBMCs were stained with anti-CD56 and either two anti-KIR3DL1 Abs (DX9 and Z27) (*B*) or Z27 (*C*). In *C*, each dot represents an individual donor. TOT shows data for percentage of NK cells expressing 3DS1 in the full panel of donors ($n = 24$). These donors were then split based on 3DS1 homozygosity (HOM; $n = 7$) or heterozygosity (HET; $n = 17$), and the percentage of 3DS1-positive NK cells is shown. Using a Student's unpaired t test, 3DS1 expression in the HOM group differed significantly from the HET group with $p < 0.0001$. *D*, Jurkats cells stably expressing KIR3DS1ECD were stained with anti-KIR3DL1 Ab Z27.



interaction seen. Our finding that the Z27 dim population represented 3DS1-positive cells allowed us to generate a polyclonal culture of 3DS1-positive NK cells by sorting and expanding Z27 dim NK cells. RT-PCR analysis confirmed 3DS1 expression in the Z27 dim but not in Z27-negative population (Fig. 5*B*). Polyclonal cultures of 3DS1-positive NK cells were used as effectors against a panel of 721.221 transfectants in a cytotoxicity assay. If 3DS1 is activated by HLA-B allotypes, we would expect that 721.221 cells transfected with HLA-B alleles would be killed to a greater degree than HLA-negative cells by 3DS1-positive cultures and that this effect could be reversed with Z27 Ab blocking. Although some differences were seen between 3DS1-positive and -negative cultures, these were only very slight and, more importantly, blocking of 3DS1 with the Z27 Ab did not alter killing of any of the target cell lines examined (Fig. 5*C*). These data agree with the Jurkat transfectant data and further support that 3DS1-positive NK cells are not activated by HLA-B.

Discussion

KIR have been identified in recent years as an important family of receptors expressed by NK cells, which allow them to respond to presence and levels of HLA class I Ag on target cells during infection or transformation events (16). 3DL1 is a polymorphic KIR that interacts with the HLA-Bw4 public epitope (10). We hypoth-

esized that polymorphism present in both 3DL1 and HLA-B would have functional consequences and indeed this proved to be the case. As has been reported previously (21, 22), we have observed that polymorphism within HLA-Bw4 alleles affected recognition by 3DL1. Using a number of experimental approaches, a consistent hierarchy of HLA-Bw4 reactivity was identified for the 3DL1*001 allotype. B*5101 gave the strongest response in all assays, whereas B*2705 was generally the weakest. Because B*2705 is characterized by having a different Bw4 amino acid sequence compared with the other Bw4 alleles tested, this may in part contribute to the results seen (see Table I). In addition, different HLA alleles have different peptide binding specificities that are influenced in part, but not exclusively, by the Bw4 epitope present. Divergent peptide repertoires presented by alleles with similar Bw4 epitopes will also contribute to the patterns of reactivity observed. The data presented in this study demonstrate that 3DL1 is influenced by different Bw4 allotypes and that this results in altered functional responses. Similarly, we found that polymorphism present within 3DL1 affected functional responses of NK cells because different allotypes were inhibited to a different degree by a single HLA-B allotype. Although B*2705 was recognized by both 3DL1*001 and 3DL1*002 allotypes, inhibition through the 3DL1*002 allotype was more pronounced. Khakoo et al. (19) previously reported a limited interaction between 3DL1*002 and

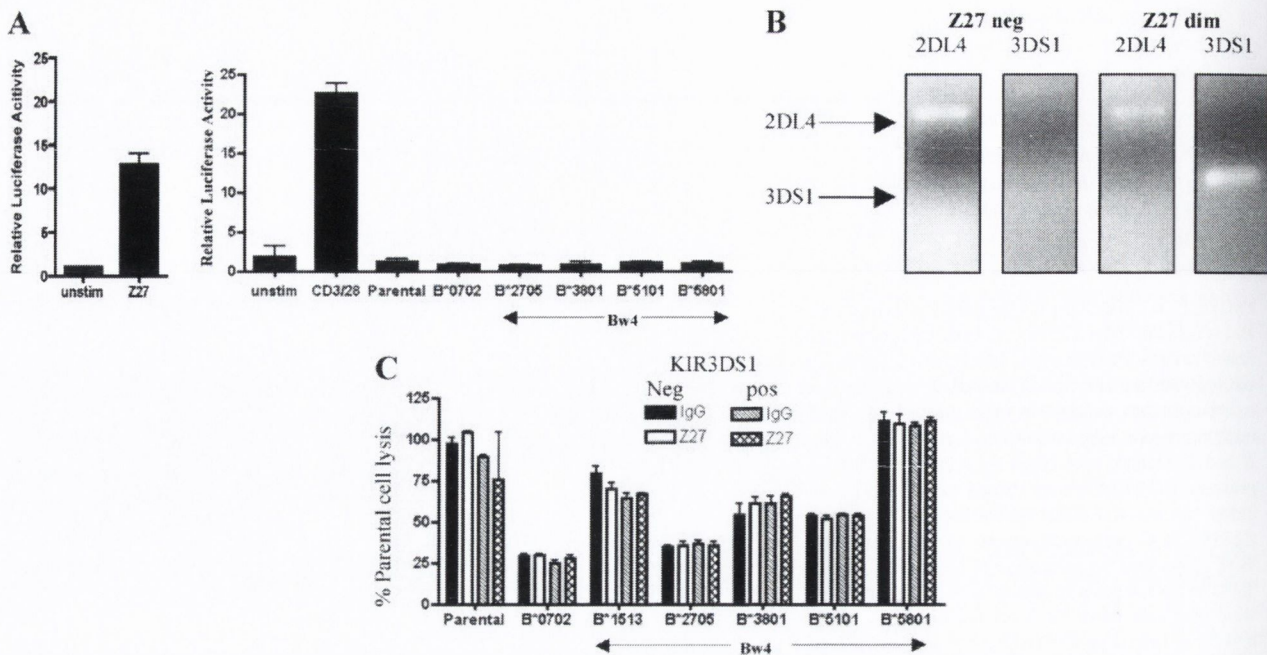


FIGURE 5. KIR3DS1 does not recognize HLA-Bw4. **A**, KIR3DS1ECD construct is functional because cross-linking with the Z27 Ab at 2 μ g/ml transduced a positive signal (left panel). KIR3DS1/CD3 ζ Jurkats were stimulated with 721.221 cells and a panel of its HLA transfectants at a stimulator:responder ratio of 1:5 (right panel). Firefly luciferase activity was measured after 18 h as a readout of NFAT activation and is expressed relative to unstimulated control. Data shown are the average of four independent experiments and error bars show the SEM. **B**, 3DS1 mRNA expression is restricted to cells reactive with the Z27 Ab. cDNA was generated from sorted Z27-negative and Z27 dim staining NK cell populations. RT-PCR was used to measure expression of KIR2DL4 (positive control for quality of cDNA) and KIR3DS1. **C**, KIR3DS1-positive cells from a homozygous donor were sorted, expanded, and used as effectors against a panel of 721.221 HLA transfectants in a 4-h 51 Cr release cytotoxicity assay. Cytotoxicity is presented as percentage killing of the 721.221 parental cell line. Data shown are representative of three independent donors and error bars show SD.

B*2705. Our results contrast with this and support the recent report by Carr et al. (23) in which 3DL1*002 was inhibited by B*2705. The study by Carr et al. (23) also found functional differences between 3DL1 alleles; in contrast to 3DL1*002 and similar to our findings for 3DL1*001, the 3DL1*007 allele was not inhibited by B*2705. Similar to Yawata et al. (24), the B*5801 HLA-Bw4 allele was informative in demonstrating functional polymorphisms of 3DL1. It provided a relatively strong ligand for the *001 allele in contrast to *002, which it barely triggered. Although both 3DL1*001 and *002 alleles were activated by B*3801 and B*5801, the signal activated through 3DL1*001 was much stronger, confirming it as a sensitive KIR receptor (24). The most informative differences between our 3DL1 alleles was apparent when we examined data for both B*2705 and B*5801 HLA types. Thus, 3DL1 alleles contribute to a qualitative spectrum of NK cell functional responses in terms of specific HLA-Bw4 recognition. This functional polymorphism expands the pool of pathogens to which NK cells can respond, and individual people will differ in their abilities to respond to pathogen depending on their combination of KIR genotype, phenotype, and HLA type.

3DL1 is highly polymorphic, and we have previously demonstrated that heterogeneity in flow cytometry staining patterns reflected allelic diversity at the *3DL1* locus (13). Particular alleles of 3DL1 (3DL1*001 and *002) had a bright peak when stained with the DX9 Ab, whereas others (3DL1*005) had a dim staining profile. Individuals who were heterozygous for combinations of these patterns had a characteristic bimodal pattern of staining. There were two allotypes identified in our study that did not react with the DX9 Ab: 3DL1*004 and 3DS1. It was subsequently demonstrated that 3DL1*004 was found to be poorly expressed at the cell surface, which explained its staining pattern (25). In this study, we

have definitively demonstrated that 3DS1 protein is expressed at the NK cell surface. It is not recognized by DX9 and in support of the suggestion in Ref. 26, we have defined Z27 as an Ab that recognizes 3DS1 with a characteristic very dim staining profile by flow cytometry. The low intensity of Z27 Ab staining may be due to a lower Ab affinity for 3DS1 relative to other alleles of 3DL1 or possibly a lower level of cell surface expression. Although Z27 and DX9 Abs have very similar staining profiles of 3DL1, their exact epitopes differ (19). Tyr²⁰⁰ has been defined as an amino acid important for DX9 but not Z27 recognition of 3DL1. This residue is common to all 3DL1/S1 alleles and is therefore not directly involved in Z27 discrimination of 3DS1. It is possible that amino acid residue 199, in close proximity, may contribute to differential Ab recognition because Pro is present in all 3DL1 alleles whereas Leu is present in 3DS1. In general, the intensity of Z27 Ab staining is consistently higher than that seen with DX9 staining of 3DL1. Using the Z27 Ab to stain NK cells of a panel of normal donors, we have found that individuals homozygous for 3DS1 express this receptor on a higher proportion of NK cells compared with 3DS1 heterozygotes. Although this may have an as yet unidentified functional significance, it may simply reflect a gene dose effect for inhibitory alleles of *3DL1*, as recently observed by Yawata et al. (24).

Although 3DS1 appears to segregate as an allele of *3DL1*, it differs from other inhibitory *3DL1* alleles in a number of respects. Most striking, in terms of its structure, is its similarity in the transmembrane and cytoplasmic tail regions to activating KIR receptors (13). 3DS1 is very similar to 3DL1 in its extracellular region, prompting the assumption that it would also recognize HLA-Bw4 ligand. We have shown that 3DS1, either on polyclonal NK cell cultures or present on the Jurkat transfectants, did not recognize any HLA-Bw4 allotype tested. Although 3DS1 is very similar to

3DL1 extracellularly, the patterns of amino acid substitutions differ from other alleles. Although inhibitory alleles of 3DL1 tend to share polymorphic substitutions in a “patchwork pattern” of substitutions, 3DS1 is unusual because it has six unique amino acid substitutions in its ECD (13). Our data suggest that these changes may result in differences in ligand specificity compared with other 3DL1 alleles.

It is also possible that peptide may play a role in restricting recognition of HLA-Bw4 by 3DS1. Presentation of peptide Ag by HLA class I to TCR-restricted T lymphocytes has been well characterized. However, data are beginning to emerge to support a possible role for the presentation of peptide to NK cells. Under normal conditions, NK cells recognize HLA class I Ag through inhibitory receptors on the cell surface. During a viral infection, removal of the inhibitory signal through down-regulation or absence of HLA class I Ag, provides a mechanism of NK cell activation (16). In addition, the presence of foreign peptide presented by HLA class I Ag can perturb inhibitory receptor ligation on the NK cell, which can result in its activation. This has previously been demonstrated in a gene therapy setting where T cells transduced with a retroviral vector became susceptible to autologous NK cell lysis (27). T cells presented foreign peptide on a HLA-Bw4 background, perturbing 3DL1 inhibitory signals to autologous NK cells that caused recognition and cytotoxicity of the T cells.

In terms of activatory KIR, it is not surprising that 3DS1 does not recognize HLA-Bw4 because NK cells should not be activated during normal homeostasis. However, we predict that 3DS1 will recognize HLA-Bw4 in a peptide-specific manner. A precedent for this comes from KIR3DL2, where the interaction between KIR3DL2 and HLA-A3 or HLA-A11 has been shown to be highly peptide specific; HLA-A3 or HLA-A11 tetramers folded in the presence of an EBV, but not self or HIV peptides, allowing binding (28). It is possible that there is a similar specificity with 3DS1 (although because we tested 3DS1 using EBV-transformed cell lines, it is unlikely to be EBV specific) with the presentation of specific, perhaps infection-associated, peptides by HLA-Bw4 required for recognition. Recognition of HIV-specific peptide by 3DS1 may provide the molecular basis for the genetic association between coexpression of 3DS1 and HLA-Bw4, and long-term nonprogression in HIV (17). Our understanding of the functional specificities of KIR is in its relative infancy compared with our knowledge of their genetics. Our findings in this study expand our knowledge of the functional consequences of KIR allelic diversity and adds another level of complexity that must be considered when investigating interactions between KIR and their HLA ligands.

Acknowledgment

We thank Dr. Verónica Athié-Morales for help in completing this work.

Disclosures

The authors have no financial conflict of interest.

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Putting the natural killer cell in its place

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Summary

Natural killer (NK) cells were originally described as 'null' lymphocytes, but we have increasing evidence of their role in recognizing pathogen, and our knowledge of NK cell receptors continues to expand exponentially. Human NK cells have many receptors for human leucocyte antigen (HLA) class I. These killer immunoglobulin-like receptors (KIRs) and CD94/NKG2 receptors can signal in both positive and negative ways to regulate NK cell functions. The inhibitory receptors are the best characterized, but even in these cases much of their functional biology remains elusive. In this review, some recent advances in terms of the three-immunoglobulin (3Ig)-domain KIRs are discussed. Natural cytotoxicity receptors (NCRs) are among the activatory receptors found on NK cells. While pathogen ligands for these receptors have been described, endogenous ligands remain elusive. NCRs and NKG2D, a receptor for stress-induced antigens, appear to play complementary functional roles in terms of NK cell activation. More recently described on NK cells are the Toll-like receptors. In particular, these receptors of the innate immune system allow NK cells to directly sense pathogen, and their ligation on accessory cells indirectly activates NK cells through cytokine production. It is becoming clear that none of these receptor systems functions in isolation and that it is the sum of the signals (which will reflect the pathogenic situation), in addition to the cytokine milieu, that will direct NK cell activation. The resulting cytotoxicity, cytokine production and direct cell–cell regulatory interactions with other cells of the immune system, for example dendritic cells, ultimately determine the role of the NK cell in the context of an overall immune response.

Keywords: natural killer; killer immunoglobulin-like receptor; Toll-like receptor; receptors; innate immunity

doi:10.1111/j.1365-2567.2005.02256.x

Received 6 July 2005; accepted 10 August 2005.

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Introduction

Natural killer (NK) cells were first identified in the 1970s by their ability to kill cancer cells and virally infected cells without prior sensitization.^{1–3} As such, they were considered 'non-specific' lymphocytes. Furthermore, as they did not express cognate receptors generated by recombination,^{4,5} T and B lymphocytes triumphed and dominated the field of immunology for many years as their complex receptor systems and biology were elucidated. However, the discovery of NK cell receptor systems complex enough to satisfy even the most avid T-cell receptor zealot has refocused energy on these cells. Indeed, we no

longer think of NK cells as 'non-specific', but rather as having very defined, and sometimes very subtle, specificities.

Cytotoxicity is perhaps the best characterized effector function of NK cells, and targets include tumour cells,⁶ virally infected cells,^{7,8} cells infected with intracellular bacterial pathogens^{9,10} and, as more recently reported, immature dendritic cells (iDCs).^{11–13} NK cells also produce a range of cytokines, including haematopoietic factors such as interleukin (IL)-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)- α and regulatory cytokines such as transforming growth factor (TGF)- β and interferon

(IFN)- γ .¹⁴ In both viral and bacterial models of infection, IFN- γ production by NK cells has been shown to be a key event in successful resolution of infection^{15–17}. As a general rule, IL-12 produced very early in infection is responsible for driving NK cells to produce IFN- γ .^{17,18} Thus, accessory cells are important in the NK cell response to pathogen, and NK cell recognition of pathogen is indirect. However, it has become apparent that direct recognition of pathogen by NK cells can also occur with corresponding activation of effector functions (see below). In terms of cytotoxicity, recognition of appropriate targets is the fundamental first step in the process, and most recent progress has been made in this area. NK cells have conventional activatory receptors but, more unusually, they also express constitutively active inhibitory receptors. It appears that the net sum of inhibition and activation signalling ultimately controls NK cell function. NK cell receptors broadly fall into two categories based on their ability (or inability) to recognize human leucocyte antigen (HLA) class I. In this review, we present a synopsis of new information on HLA class I receptors but also describe more recently characterized receptors, including natural cytotoxicity receptors (NCRs) and Toll-like receptors (TLRs), and address how these receptors function in concert to regulate NK cell function.

HLA class I specific inhibitory receptors

NK cells are programmed to kill and require inhibitory signals from normal autologous cells to prevent unwanted cell death. The best characterized inhibitory signals are those transduced by HLA-specific receptors, including members of the killer immunoglobulin-like receptor (KIR) family of receptors and the CD94/NKG2A heterodimer that belongs to a lectin-like family of receptors.¹⁹ Under normal circumstances, HLA class I antigen expression maintains NK cell tolerance. However, in certain pathological conditions, for example viral infections or cancer, HLA class I expression is commonly altered, thereby removing normal inhibition. The resulting NK cell activation, including cytotoxicity and cytokine production, is a fundamental component of the early immune response. Encoded on chromosome 19, KIRs comprise a family of 15 receptors that include both inhibitory and activatory members. Inhibitory receptors are characterized by immunotyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails, while activatory receptors recruit positive signalling adapter molecules. Expression is restricted to NK cells and a small subset of T cells (for a detailed description, see References 20 and 21). These receptors bind to subgroups of HLA class I molecules (see Table 1). Ligation of inhibitory KIRs by their HLA ligands on healthy cells results in the inhibition of NK cell activation and protection of the HLA⁺ cell from NK cell lysis. A detailed discussion of all KIRs is beyond

the scope of this review. Current information on three-immunoglobulin (3Ig)-domain KIRs, including KIR3DL1, a focus of study in our laboratory, is discussed.

3Ig KIR

KIR3DL1 is a well-characterized receptor that recognizes a subgroup of HLA-B molecules expressing the serological Bw4 epitope, which is defined by positions 77–83 on the $\alpha 1$ helix of the heavy chain.²² The Bw4 epitope is present on approximately one-third of all HLA-B alleles and is defined by the presence of leucine at position 82 and arginine at position 83, while other residues show variation among alleles. A 70-kDa protein, KIR3DL1 contains three extracellular immunoglobulin (Ig) domains (3D) and a long (L) intracellular domain that contains two ITIMs. Ligation of KIR3DL1 by its cellular ligand results in phosphorylation of these ITIMs and the recruitment of phosphatases, including SHP-1 and SHP-2, that inhibit activation pathways.^{23,24} KIR3DL1 is present in approximately 95% of the population and, like most KIRs, is highly polymorphic, with approximately 35 known alleles (P. Norman and P. Parham, Stanford University, Stanford, CA, personal communication). Polymorphism has been shown to affect antibody binding²⁵ and may have functional consequences. Indeed, we have preliminary evidence that different alleles vary in their ability to recognize an identical Bw4 target antigen (manuscript in preparation, G. O'Connor and C. Gardiner).

In addition to monitoring levels of HLA expression (frequently altered in viral and transformation events), KIR3DL1 is sensitive to the peptide presented by the HLA molecule.^{26,27} Sensitivity to peptide presented by HLA class I has also been found for KIR2DL1 and KIR3DL2^{28,29} (see also below). Unlike T-cell receptors, which display very fine peptide specificity, KIR3DL1 shows a broader specificity, with several unrelated peptides capable of preventing KIR3DL1 recognition, thus leading to KIR3DL1⁺ NK cell activation. This allows the possibility of NK cell detection of viral infection or transformation as a result of a perturbation in the inhibitory signal to KIR upon presentation of viral or tumour-derived peptide. This possibility was nicely illustrated by the discovery that retrovirally transduced T cells became a target for autologous NK cell lysis as a result of the presentation of a resistance gene-derived peptide that prevented KIR3DL1 recognition.³⁰

KIR3DS1, an allele of KIR3DL1, is highly homologous to KIR3DL1 extracellularly but resembles activatory receptors cytoplasmically. A genetic study has found that KIR3DS1, when expressed in combination with HLA-Bw4 molecules with isoleucine at position 80 (Bw4-80Ile), is associated with delayed progression to acquired immune deficiency syndrome (AIDS).³¹ One attractive hypothesis is that KIR3DS1⁺ NK cells recognize and kill HLA

Table 1. Natural killer (NK) cell receptors

Receptor	Ligand	Function	References
Immunoglobulin-like receptors			
KIR2DL1,	HLA-C, C2	Inhibitory	72, 73
KIR2DS1	HLA-C, C2	Activatory	74
KIR2DL2/3	HLA-C, C1	Inhibitory	72, 73
KIR2DS2	HLA-C, C1	Activatory	74
KIR3DL1	HLA-B Bw4	Inhibitory	22
KIR3DS1	HLA-B Bw4?	Activatory?	
KIR2DL4	HLA-G	Activatory?	75
KIR3DL2	HLA-A A3, A11	Inhibitory	29
LILR family (ILT)	HLA	Some inhibitory, some activatory	See 76
Lectin-like receptors			
CD94/NKG2A	HLA-E	Inhibitory	34
CD94/NKG2C	HLA-E	Activatory	77
Cytotoxicity receptors			
NKG2D	MICA, MICB, ULBP	Activatory	40
NKp30	Unknown	Activatory	49
NKp44	Unknown cellular ligand, viral HA	Activatory	50
NKp46	Unknown cellular ligand, viral HA	Activatory	78
CD16	IgG	Activatory	79
DNAM-1	PVR (CD155), nectin-2 (CD112)	Activatory,	80
NKp80	Unknown	Activatory, coreceptor	81
CD59	Unknown	Activatory, coreceptor	82
NTB-A	NTB-A	Activatory, coreceptor	83
2B4 (CD244)	CD48	Activatory, coreceptor	84, 85
CD2	LFA-2	Activatory, coreceptor	86
LFA-1	ICAM	Activatory	87
TLR	PAMPs, e.g. dsRNA	Activatory	41–43, 58

KIR, killer immunoglobulin-like receptor; LILR, leukocyte immunoglobulin-like receptor; DNAM, DNAX accessory molecule 1; NTB-A, NK-, T- and B-cell antigen; LFA, lymphocyte function-associated antigen; TLR, Toll-like receptor; HLA, human leucocyte antigen; MIC, MHC class I chain related; HA, haemagglutinin; IgG, immunoglobulin G; PVR, poliovirus receptor; ICAM, intercellular adhesion molecule; PAMP, pathogen-associated molecular pattern; ds, double-stranded.

Bw4-80Ile⁺ human immunodeficiency virus (HIV)-infected targets, allowing control of infection. Despite wide interest in this receptor, there is a lack of information on the expression and function of this important allele. Lack of antibody reagents against specific KIR molecules has hampered detailed analysis, and indeed KIR3DS1 cell surface expression has not been definitively established. However, we have recently generated some experimental evidence to suggest that KIR3DS1 is indeed present on the cell surface (manuscript in preparation, G. O'Connor and C. Gardiner). The ligand specificity of KIR3DS1 and its activatory nature are also inferred from its similarity to KIR3DL1 extracellularly and activatory receptors intracellularly, but remain to be proved by formal demonstration.

KIR3DL2, which is structurally similar to KIR3DL1, is one of the three framework KIR genes (along with KIR3DL3 and KIR2DL4) present on all haplotypes. The ligand of KIR3DL2 has been an issue of dispute, with difficulty experienced in repeating initial observations that it bound to HLA-A3 and HLA-A11.^{32,33} This discrepancy has

been recently resolved by the finding that the interaction between KIR3DL2 and these HLA-A alleles is highly peptide dependent.²⁹ Indeed, of a panel of eight peptides tested in tetramer binding assays, only one, an Epstein Barr virus (EBV)-derived peptide, allowed recognition. In support of this finding, initial experiments that observed protection of HLA-A3 or HLA-A11 positive cells from lysis by KIR3DL2⁺ NK cells used EBV-transformed B-cell lines as target. The functional significance of this finding is still unclear, as the existence of an inhibitory pathogen-derived peptide-specific receptor is counterintuitive.

CD94/NKG2

In parallel to the KIRs, NK cells express CD94 and NKG2 heterodimer receptors, members of the C-type lectin-like family, which recognize the non-classical class I HLA-E molecule.³⁴ HLA-E binds and presents the leader peptide of most HLA-A, -B and -C molecules as well as the non-classical HLA-G molecule.³⁵ Therefore, while KIRs

are sensitive to individual HLA allele changes, they are complemented functionally by CD94/NKG2A, which responds to changes in global HLA expression. This allows NK cells to respond to both subtle and major alterations in HLA expression caused by different pathological situations.

CD94 is expressed as a disulphide-linked heterodimer with members of the NKG2 family, including NKG2A, which transduces inhibitory signals, and NKG2C, which transduces activatory signals. As with KIR molecules, the inhibitory receptor has a higher binding affinity and inhibition dominates.³⁶ As CD94/NKG2 complements KIR functionally, its expression and activity must be taken into account in any experiments involving human NK cells. The converse also holds in that KIR expression must also be considered when investigating CD94/NKG2 in functional experiments. Herein lies a major limitation in terms of performing experimental work. In order to dissect out receptor specificities definitively, NK cells must be cloned, characterized and screened for receptor expression and then used in functional experiments. This is not a trivial task and is both time-consuming and technically demanding. Given the clonal distribution of NK cell receptors, the efficiency of isolating clones expressing a relevant repertoire of receptors is low.

Activatory receptors and their ligands

Our appreciation of and interest in activatory receptors on NK cells has ebbed and flowed over the years as initial discoveries failed to account for all the experimental evidence and to demonstrate how NK cells function. Indeed, it has only been since the discovery of the importance of inhibitory receptors in NK cell biology that activatory receptors have again become a hot research topic. NK cells express many activatory receptors including CD2, CD16, lymphocyte function-associated antigen (LFA)-1³⁷ and, as more recently described, the activatory KIRs,^{19,21} NKG2C,³⁸ NCRs,³⁹ NKG2D⁴⁰ and TLRs.^{41–43}

HLA-specific receptors

Among the KIR family are members that do not contain an inhibitory signalling motif and couple to signalling molecules that transduce activatory signals.^{21,44} Extracellularly, many of the activatory KIRs are highly homologous to inhibitory members of the KIR family (2SD1 and 2DL1) and bind to the same HLA ligands (Table 1). The activatory KIRs bind with lower affinity than their inhibitory counterparts,^{36,45} and non-major histocompatibility complex (MHC) ligands have been identified for some KIRs. KIR haplotypes vary in the number of activatory KIRs present, which ranges from one to six.¹⁹ In people with an AA haplotype (the simplest haplotype known), the only activatory KIR present in the genome is KIR2DS4, a common allele of which is not expressed.⁴⁶ Thus, in these

individuals, there is no activatory KIR present on NK cells, raising a question over the role played by activatory KIR. Perhaps, in these individuals, the CD94/NKG2C receptor is sufficient, which suggests redundancy in HLA-associated activatory receptor usage. This idea is supported by a detailed study of NK cell receptor usage in two individuals,⁴⁷ which appeared to show a differential dependence on either KIR or the CD94/NKG2 receptor system, depending on the HLA background of the donors.

NCR and NKG2D receptors do not recognize HLA class I ligands

The activatory NCRs and NKG2D receptors have received most recent attention (for a detailed review, see reference 39). Their expression is predominantly restricted to NK cells, and they have the ability to activate NK cells in the absence of additional stimuli. NKp46⁴⁸ and NKp30⁴⁹ are expressed by all NK cells, while NKp44⁵⁰ expression is restricted to activated NK cells. Although not an absolute phenomenon, NK cell clones can be identified as NCR^{bright} or NCR^{dull} based on the surface receptor densities and high levels of NCR correlated with high natural cytotoxicity against many target cells.^{48,49} Indeed, a complementary relationship between NCR and NKG2D appears to exist, and NCR^{dull} clones kill tumour cells in an NKG2D-dependent manner.⁵¹ Together, NCRs and NKG2D accounted for virtually all cytotoxicity mediated by NK cells against a wide variety of tumour target cell types. While the ligands for NKG2D are known to be the stress-induced antigens MHC class I chain related (MIC)A and MICB,⁴⁰ the endogenous ligands for the NCRs remain to be identified.⁵¹ It appears, however, that NCRs may have been co-opted by NK cells to recognize pathogen-specific moieties, as both NKp46 and NKp44 recognize virus-specific haemagglutinins and facilitate NK cell lysis of virally infected cells.^{52,53} In contrast, NKp30 appears to have been targeted by the pp65 protein of human cytomegalovirus (HCMV), which binds NKp30 and inhibits NK cell cytotoxicity.⁵⁴ In the mouse, the activatory Ly49H receptor (a KIR functional homologue involved in recognition of H-2 antigen) specifically recognizes the m157 protein of mouse cytomegalovirus (MCMV).⁵⁵ The selection of NK cell receptors by the immune system for specific recognition of pathogen and their targeting by specific viral proteins highlight their importance in the immune response to these infections.

TLRs: direct and indirect pathogen recognition by NK cells

TLRs are a recently described family of innate immune receptors which recognize conserved pathogen-associated molecular patterns (PAMPs); for example, lipopolysaccharide (LPS), a component of the Gram-negative bacterial

cell wall, is recognized by TLR4.⁵⁶ Ten mammalian TLRs have been identified to date, and there has been a huge expansion in terms of elucidating TLR ligand recognition and signalling pathways (for comprehensive reviews, see references 56 and 57). Given that NK cells play an important role in the early innate immune response to pathogen, and the fact that they lack cognate receptors for the generation of a specific adaptive immune response, it is attractive to speculate that NK cells express TLRs and that these would function in the early sensing of infection. Indeed, this has proved to be the case, and reports are beginning to emerge detailing a role for TLR2,⁴³ TLR3,^{41,42,58} TLR9⁴¹ and, more recently, TLR7 and TLR8 in NK cell biology.

It is interesting that, despite the obvious importance of TLRs in viral infection, the first evidence for TLR function in NK cells came from models of parasitic and bacterial infections. Lipophosphoglycan from *Leishmania major* stimulated NK cells to produce the proinflammatory cytokines IFN- γ and TNF- α through cell surface ligation of TLR2.⁴³ Subsequently, NK cells were shown to respond to the outer membrane protein A from *Klebsiella pneumoniae* and flagellin from *Escherichia coli* through TLR2 and TLR5, respectively. These TLR agonists acted directly and in synergy with proinflammatory cytokines, to induce IFN- γ production. However, the role of TLR3 in NK cells has been the best characterized to date, with complimentary, and occasionally conflicting, data emerging from different laboratories.^{41,42,58} Despite an early report to the contrary,⁵⁹ there is consensus that NK cells express TLR3, and, as it has been known for many years that NK cells respond to polyinosinic-polycytidylic acid [poly I:C, a synthetic analogue of double-stranded RNA (dsRNA)], data soon emerged to support TLR3 as the receptor mediating this effect. While the original study reported direct activation of human NK cells by poly I:C,⁴² a second study reported that IL-12 was required for this to occur, i.e. there was a requirement for accessory cell-derived cytokine.⁴¹ Our findings support both a direct and an indirect role of poly I:C activation through TLR3. While highly purified NK cells can be directly activated, this effect is significantly enhanced by accessory cells, as demonstrated by cytokine blocking and cell reconstitution experiments. In fact, even very low numbers of monocytes can have potent effects on NK cell activation.⁶⁰

TLR9 is activated by double-stranded unmethylated CpG motifs which are present in bacterial and, occasionally, viral genomes.⁵⁷ CpG has been reported to activate human NK cells *in vitro*, although this was accessory cell dependent as IL-12 was required for this effect.⁴¹ When the immune response to MCMV was examined *in vivo*, it was surprising to find that infections were more severe in TLR9^{-/-} than TLR3^{-/-} mice.^{61,62} In fact, although TLR3^{-/-} mice did have an impaired immune response to MCMV, IFN- γ production by NK cells was relatively normal,

while it was almost completely absent in TLR9^{-/-} animals.⁶² In addition, type 1 IFN production was also more profoundly affected in TLR9^{-/-} mice.⁶² It has also been shown that activation of NK cells in MCMV is indirect and mediated by dendritic cells (DCs) stimulated through TLR9.⁶¹ Differences in TLR expression and function have been reported between humans and mice, and such differences appear to exist for TLR9 as, in contrast to human cells, murine NK cells do not express TLR9 or respond to TLR9 agonists *in vitro*.⁶¹ Further studies are required to confirm these findings.

We have recently reported that TLR7 and TLR8 are also expressed by NK cells.⁶⁰ The physiological ligands for TLR7 and TLR8 are viral single-stranded RNA (ssRNA).^{63–65} NK cells respond to the TLR7/8 agonist R848 and demonstrate increased cytotoxicity and cytokine production. While NK cells required priming with cytokine to directly transduce signal in response to R848, they were potently activated by accessory cell-derived cytokines. In fact, IFN- γ production by NK cells in response to R848 was entirely IL-12, and therefore accessory cell dependent. The increased cytotoxicity observed was also partly attributable to the accessory cell involvement.⁶⁰

Finally, in contrast to expression on other lymphocyte subsets, we also have observed that TLR4 is expressed at the surface of human NK cells (Fig. 1). In fact, almost all CD56^{dim} NK cells expressed TLR4, while TLR4 was not expressed by the functionally distinct subset of CD56^{bright} NK cells. However, NK cells did not respond to LPS stimulation (unpublished observations, O. Hart), which may reflect lack of required costimulatory signals such as CD14 or MD2. Therefore, although NK cells express virtually all TLRs, not all are inherently functional, and indirect activation of NK cells by TLR ligand on accessory cells is likely to play a significant role in the NK cell response to microbial pathogens.

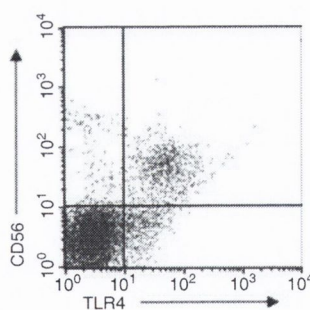


Figure 1. Toll-like receptor 4 (TLR4) is expressed on the surface of human natural killer (NK) cells. All CD56^{dim} NK cells expressed TLR4 on their cell surface, in contrast to the CD56^{bright} subset of NK cells. Cells were stained with anti-CD56 (BD Biosciences, Oxford, UK) and anti-TLR4 antibodies (E-biosciences, San Diego, CA), and analysed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

Balance of signals is context dependent

A recurring theme in NK cell biology is the integration and balancing of positive and negative signals. Activation can occur as a result of either a decrease in inhibitory signalling or an increase in the ligation of activatory receptors. The importance of the role played by different receptors is dependent on the challenge (viral infection, transformation, etc.) faced by the NK cell.

During a transformation event, a number of changes occur that can allow NK cell activation. One of these is the down-regulation by tumour cells of HLA class I molecules, which provide an inhibitory ligand for NK cells. Loss of individual HLA alleles can cause the activation of a subset of NK cells that rely on inhibitory KIR signals generated by ligation of these ligands. Stress-induced expression of heat-shock protein (hsp) 60 may also lead to activation of CD94/NKG2A⁺ NK cells, as a peptide derived from hsp 60 binds to HLA-E but is not recognized by CD94/NKG2A.⁶⁶ Stress proteins such as MICA, MICB and UL16 binding protein (ULBPs) not generally expressed on normal cells act as ligands for the activatory receptor NKG2D.³⁰ Ligation of NKG2D allows killing of transformed cells that express normal levels of HLA by overcoming inhibitory signals. Thus, transformed cells may be targeted for lysis by NK cells as a result of both a decrease in inhibitory signals and an increase in activatory signals.

A viral infection may lead to the activation of NK cells in a number of different ways. The expression of TLR3 by NK cells allows them to detect dsRNA, a common feature of viral replication. Cytokines produced by virally activated antigen-presenting cells (APCs), such as IL-12 and IFN- α , are potent activators of NK cell activity. NK cells may also be capable of responding to more subtle signs of viral infection. KIR-mediated inhibition of NK cells is sensitive to presented peptide, and evidence suggests that the skewing of cellular peptides that accompanies viral infection may prevent KIR recognition and render infected cells sensitive to lysis by a subset of NK cells.³⁰ The importance of the balance of receptors is highlighted by the numerous strategies devised by viruses to subvert NK cell responses (reviewed in reference 67), which include the expression of decoy HLA-like molecules to inhibit NK cell activity and molecules such as the HCMV protein UL16, which prevents ULBP/NKG2D-mediated activation of NK cells.

NK cells direct DCs in the generation of an adaptive immune response

NK cells have long been regarded as 'crude' effectors of the innate immune system, but there is now mounting evidence that they are important players in setting the stage for an appropriate adaptive immune response through their interaction with DCs.^{11,12} DCs are crucial regulators of the adaptive T-cell response to pathogen,

acting as the main APC to prime naïve T cells to mount a suitable adaptive response to pathogen. iDCs, which patrol the periphery, are specialized for antigen uptake and processing. Upon antigen capture these cells switch to an antigen-presenting mode and migrate to the lymph node for interaction with T cells. This switch is characterized by the up-regulation of costimulation markers, including HLA molecules. Evidence of interactions between these two cell types suggests that bidirectional cross-talk regulates the response of both cell types.

Activated NK cells are capable of killing iDCs, despite normal levels of HLA class I expression by these cells. Upon maturation, these cells up-regulate HLA and become resistant to NK cell lysis. Killing of iDC by NK cells is mediated almost exclusively by the activatory receptor Nkp30, with little if any role for Nkp44 or Nkp46.¹³ Nkp30 (and in part NKG2D) are down-regulated on the surface of NK cells in response to TGF- β 1, which may provide resistance to TGF- β 1-producing DCs.⁶⁸ This killing of DCs that have failed to become fully activated has been proposed to form part of a 'quality control' system, where the quantity and quality of antigen presentation during an infection are controlled in part by NK cells.

Interaction between NK cells and DCs can also induce DC maturation, mediated by NK cell-derived cytokines. Ligation of Nkp30 on NK cells by DCs promotes the secretion of TNF- α and IFN- γ , which leads to DC maturation.⁶⁹ Indeed, supernatant from Nkp30-triggered NK cells is sufficient to cause DC maturation and production of IL-12. Mature DCs have recently been shown to induce the activation (including cytotoxicity and IFN- γ production) and proliferation of resting NK cells. Proliferation of NK cells and production of IFN- γ appear to be confined to the CD56^{bright} subset of NK cells.⁷⁰ This subset of NK cells is characterized by a distinct chemokine receptor profile (including CD62L, CCR7 and CXCR3), perhaps allowing these cells to traffic to secondary lymphoid organs for interaction with DCs during an immune response. Different DC-derived cytokines are responsible for NK cell activation, with IL-12 promoting NK cell IFN- γ production and IL-15 inducing proliferation.⁷¹

During an immune response, NK cells are likely to encounter DCs in inflamed tissue where DC-mediated increases in NK cell cytolytic activity and IFN- γ production promote both killing of infected cells and generation of a T helper type 1 (Th1) response. In turn, NK cells can optimize the DC response by editing unsuitable iDC or promoting maturation. Thus, these two cell types can work in concert to generate a maximal adaptive response.

Back to basics

Our basic knowledge of NK cells has greatly expanded recently, and we can now explain at a molecular level

many of the responses observed. Indeed, new roles for NK cells in directing both innate and adaptive immune responses have also been identified. It is somewhat ironic, perhaps, that the complexity of NK cell receptors, while initially providing a watershed for identification and molecular characterization of novel receptors, has now proved to be a rate-limiting step in terms of truly understanding how NK cells function. We have gone from having a general and global overview of the importance of NK cells in an immune response to a reductionist extreme of dissecting the minutiae of individual receptors at the molecular level. It seems an appropriate time to stand back and reflect on NK cells in the light of all this new information.

Thus, when thinking about NK cells as effector cells in the immune system, it is important to consider the full range of receptors that they express (with both HLA and non-HLA ligands), which may be differentially activated or indeed activated simultaneously to different extents, depending on the pathological environment in which they

find themselves; for example, infection versus cancer, as discussed above. This should be considered when interpreting data pertinent to an individual receptor system.

It is also worth restating the obvious, that an immune system functions as a biological 'system' and that any system is designed to operate optimally as a whole. Therefore, while definition of events at a molecular and biochemical level is fundamental to both our understanding and our exploitation of individual events, they must be examined in the context of the global immune response. During infection, an NK cell is directly affected by the infection itself, by direct sensing of pathogen, but is also subject to indirect effects of infection through pathogen activation of other cells of the immune system, which in turn lead to downstream effects (both receptor and soluble factor mediated) on the NK cell. It is the sum of these direct and indirect interactions that will determine the extent of NK cell activation and function (Fig. 2). In addition, the NK cell itself contributes to the effector status of other cells of the immune system, for

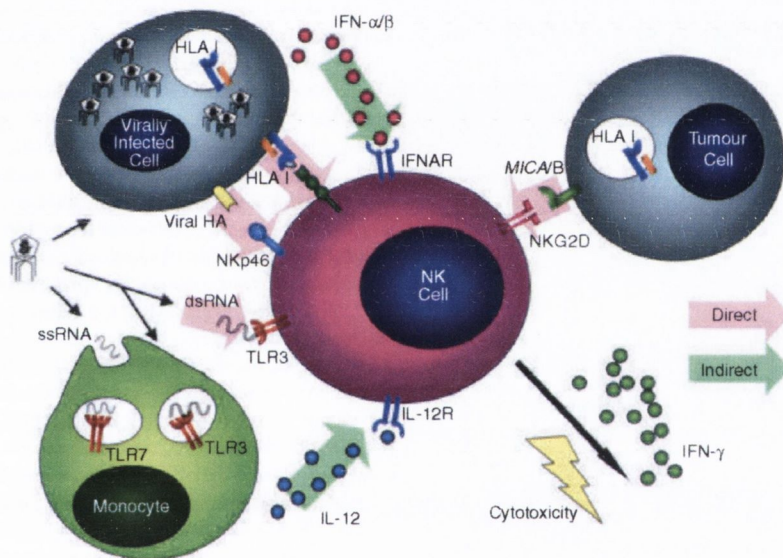


Figure 2. Direct and indirect activation of human natural killer (NK) cells. NK cells receive activation signals via a variety of direct and indirect mechanisms. A number of events occur in a virally infected cell which affect NK cells. Viral protein is processed by the antigen-processing pathway and viral peptide is presented on the surface of the infected cell in the context of human leucocyte antigen (HLA) class I. The altered peptide presented may result in perturbation of an inhibitory signal through a killer immunoglobulin-like receptor (KIR) to the NK cell, resulting in its activation. KIRs are also sensitive to the loss of HLA class I from the cell surface, a mechanism used by some viruses to escape cytotoxic T lymphocyte (CTL) recognition. Type 1 interferon (IFN) is produced by virally infected cells, and this is a potent cytokine in terms of activating NK cell effector functions such as cytotoxicity. Viral antigen, for example haemagglutinin (HA), expressed at the infected cell surface can directly engage NK cell activatory receptors such as NKp46. Direct recognition of viral pathogen can also occur through sensing of viral replication products, for example double-stranded (ds) RNA, through cell surface Toll-like receptor 3 (TLR3). Viral replication products such as single-stranded (ss) and dsRNA can also indirectly activate NK cells by interaction with appropriate TLRs on accessory cells, with resultant production of NK cell stimulatory cytokines such as interleukin (IL)-12. Some similar mechanisms exist in tumour cell recognition, including down-regulation of HLA class I to escape from CTLs which can then result in NK cell activation. Different mechanisms involved include induction of stress antigens, for example MICA and MICB, which activate NK cell effector functions through NKG2D. NK cells thus receive a number of signals dependent on the immunological challenge, which can be both direct and indirect (see above), and it is the balance of these that culminates in the final NK cell response.

example monocytes and DCs, through both direct and indirect mechanisms. In summary, the NK cell is a complex lymphocyte that deserves increased recognition for its contribution to overall host immunity.

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