The Role of KIR genes and NK Alloreactivity on Allogeneic Haematopoietic Stem Cell Transplantation

Dianne De Santis
BSc

THE UNIVERSITY OF WESTERN AUSTRALIA

This thesis is presented for the degree of Doctor of Philosophy of the University of Western Australia

2010

School of Pathology and Laboratory Medicine

Statement of Candidate Contribution

All work was performed by the author unless otherwise stated in the thesis

____

Dianne De Santis
Student

____

Frank T Christiansen
Coordinating Supervisor

____

Campbell S Witt
Supervisor

The data presented in Chapter 3 has been published. The bibliographic details and percentage contribution of each author to the work are set out below.

De Santis D, Witt C, Gomez-Lozano N, Vilches C, Garcia CA, Marsh SGE, Williams F, Middleton D, Hsu K, Dupont B and Christiansen F. A MULTI-LABORATORY EVALUATION OF REFERENCE CELLS FOR KIR TYPING. *Immunobiology of the Human MHC-Proceedings of the 13th International Histocompatibility Workshop and Conference, (Ed: Hansen JA.), vol 1 p1233-1236, International Histocompatibilitiy Working Group Press.*

Author Contribution

De Santis D (80%)

Witt CS (2%)

Gomez-Lozano N (2%)

Vilches C (2%)

Garcia CA (2%)

Marsh SGE (2%)

Williams F (2%)

Middleton D (2%)

Hsu K (2%)

Dupont B (2%)

Christiansen FT (2%)

____

Coordinating Supervisor Signature:

Professor Frank T Christiansen

The data presented in chapter 4 has been published. The bibliographic details and percentage contribution of each author to the work are set out below.

Bishara A, De Santis D, Witt CS, Brautbar C, Christiansen FT, Or R, Nagler A, Slavin S. THE BENEFICIAL ROLE OF INHIBITORY KIR GENES OF HLA CLASS I NK EPITOPES IN HAPLOIDENTICALLY MISMATCHED STEM CELL ALLOGRAFTS MAY BE MASKED BY RESIDUAL DONOR-ALLOREACTIVE T CELLS CAUSING GVHD. *Tissue Antigens. 2004 Mar;63(3):204-11.*

Author Contribution

Bishara A (20%)

De Santis D (70%)

Witt CS (2%)

Brautbar C (2%)

Christiansen FT (2%)

Or R (2%)

Nagler A (2%)

____

Coordinating Supervisor Signature:

Professor Frank T Christiansen

The data presented in chapter 5 has been published. The bibliographic details and percentage contribution of each author to the work are set out below.

De Santis D, Bishara A, Witt CS, Nagler A, Brautbar C, Slavin S, Christiansen FT. NATURAL KILLER CELL HLA-C EPITOPES AND KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS BOTH INFLUENCE OUTCOME OF MISMATCHED UNRELATED DONOR BONE MARROW TRANSPLANTS. *Tissue Antigens. 2005 Jun;65(6):519-28.*

Author Contribution

De Santis D (85%)

Bishara A (8%)

Witt CS (3%)

Nagler A (1%)

Brautbar C (1%)

Slavin S (1%)

Christiansen FT (1%)

____

Coordinating Supervisor Signature:

Professor Frank T Christiansen

The data presented in chapter 6 has been published. The bibliographic details and percentage contribution of each author to the work are set out below.

De Santis D, Foley BA, John E, Senitzer D, Christiansen FT, Witt CS. RAPID, FLOW CYTOMETRIC ASSAY FOR NK ALLOREACTIVITY REVEALS EXCEPTIONS TO RULES GOVERNING ALLOREACTIVITY. *Biol Blood Marrow Transplant. 2010 Feb;16(2):179-91.*

Author Contribution

De Santis D (85%)

Foley BA (4%)

John E (2%)

Senitzer D (1%)

Christiansen FT (2%)

Witt CS (6%)

____

Coordinating Supervisor Signature:

Professor Frank T Christiansen

The data presented in chapter 7 has been published. The bibliographic details and percentage contribution of each author to the work are set out below.

Foley BA, De Santis D, Lathbury LJ, Christiansen FT, Witt CS. KIR2DS1-MEDIATED ACTIVATION OVERRIDES NKG2A MEDIATED INHIBITION IN HLA-C C2-NEGATIVE INDIVIDUALS. *International Immunology, Vol.20(4), 555-563.*

Author Contribution

Foley BA (65%)

De Santis D (20%)

Lathbury LJ (3%)

Christiansen FT (2%)

Witt CS (10%)

____

Coordinating Supervisor Signature:

Professor Frank T Christiansen

The data presented in chapter 8 has been published. The bibliographic details and percentage contribution of each author to the work are set out below.

Foley BA, De Santis D, Van Beelen E, Lathbury LJ, Christiansen FT, Witt CS. THE REACTIVITY OF BW4^{+} HLA-B AND HLA-A ALLELES WITH KIR3DL1: IMPLICATIONS FOR PATIENT AND DONOR SUITABILITY FOR HAPLOIDENTICAL STEM CELL TRANSPLANTATIONS. *Blood. 2008 Jul 15;112(2):435-43*

Authors Contributions

Foley BA (65%)

De Santis D (20%)

Van Beelen E (4%)

Lathbury LJ (2%)

Christiansen FT (2%)

Witt CS (7%)

Coordinating Supervisor Signature:

Professor Frank T Christiansen

ACKNOWLEDGEMENTS

I would like to firstly thank my supervisors, Professor Frank Christiansen and Associate Professor Campbell Witt, for giving me the opportunity to be part of their research team. I would like to thank them for all the support, knowledge, enthusiasm and encouragement over the many years. Thank you for providing me with the inspiration to keep at it, at times I didn't think I would get there! Your time and dedication will always be greatly appreciated.

Thank you to my fellow NK researchers Bree Foley, Jodie Goodridge, Louise Lathbury and Elizabeth John for all the technical help, encouragement and support over the many years. Bree Foley thank you for helping me master NK cloning and chromium testing, and for your help in the cell and flow labs.

Thank you to all the past and present staff at the Department of Clinical Immunology for all their assistance and support over the years. A special thank you to David Sayer who provided me with the initial inspiration and whose words of encouragement will always be appreciated, Catena Causerano for the technical help in the cell culture lab, the DCI sequencing and PCR teams who helped me along the way with the 1000's of PCR and sequencing reactions and Natalie Zurzolo for your help in compiling my written thesis.

To Laila Gizzarelli, Rebecca Whidbourne, Allison Castley, Linda Smith and Leanne Langan for your encouraging words and support.

Thank you to the staff at the Flow Cytometry Unit (RPH) especially Rom Krueger for teaching me the flow basics and for answering my many questions.

Thank you to our collaborators at the Tissue Typing Laboratory at the Hadassah University, Jerusalem, Israel, especially Amal Bishara whose hard work helped with the publication of two of the chapters presented in this thesis.

I would also like to acknowledge the support of the Ray and Dobney Foundation and Western Australian Institute for Medical Research.

To my family and friends, especially my husband Carlo and my children, Jacob and Benjamin, my Mum and my sisters, thank you for your love and support. Thank you for giving me the time I needed to finish my PhD. I know there were many hours and countless weekends away from home. Thank you for your continued words of encouragement, I would not have succeeded without you.

Lastly, I dedicate this work to my dad who no doubt would have been very proud. His words of encouragement and the years of watching him work tirelessly have taught me with hard work and dedication comes success. Thank you.

PUBLICATIONS ARISING FROM THIS THESIS

De Santis D, Witt CS, Gomez-Lozano N et al. A Multi-Laboratory Evaluation of Reference Cells for KIR Typing. *Immunobiology of the Human MHC: Proceedings of the 13th International Histocompatability Workshop and Conference* 2006: 1: 1233-1236. (Chapter 3)

Bishara A, De Santis D, Witt CS et al. The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. *Tissue Antigens* 2004: 63: 204-211. (Chapter 4)

De Santis D, Bishara A, Witt CS et al. HLA natural killer cell HLA-C epitopes and killer cell immunoglobulin-like receptors both influence outcome of mismatched unrelated donor bone marrow transplants. *Tissue Antigens* 2005: 65: 519-528. (Chapter 5)

De Santis D, Foley BA, John E, Senitzer D, Christiansen FT, and Witt CS. Rapid, flow cytometric assay for NK alloreactivity reveals exceptions to rules governing alloreactivity. *Biol Blood Marrow Transplant* 2010: 16: 179-191. (Chapter 6)

Foley BA, De Santis D, Lathbury L, Christiansen FT, and Witt CS. KIR2DS1-mediated activation overrides NKG2A-mediated inhibition in HLA-C C2-negative individuals. *Int Immunol* 2008: 20: 555-563. (Chapter 7)

Foley BA, De Santis D, Van Beelen E, Lathbury LJ, Christiansen FT, and Witt CS. The reactivity of Bw4+ HLA-B and HLA-A alleles with KIR3DL1: implications for patient and donor suitability for haploidentical stem cell transplantations. *Blood* 2008: 112: 435-443. (Chapter 8)

Other publications completed during my candidature but not presented in this thesis.

Gaudieri S, De Santis D, McKinnon E et al. Killer immunoglobulin-like receptors and HLA act both independently and synergistically to modify HIV disease progression. *Genes Immun* 2005: 6: 683-690.

Price P, Witt C, De Santis D, and French MA. Killer Immunoglobulin-Like Receptor Genotype May Distinguish Immunodeficient HIV-Infected Patients Resistant to Immune Restoration Diseases Associated With Herpes Virus Infections. *J Acquir Immune Defic Syndr* 2007: 45: 359-361.

ABSTRACT

NK cell cytotoxicity is regulated by a balance of inhibitory and activating signals from receptors expressed on the cell surface. NK cell cytotoxicity is inhibited when inhibitory receptors such as Killer immunoglobulin-like receptors (KIR) interact with self class I MHC molecules on potential targets. Class I HLA incompatibility in haematopoietic stem transplantation (HSCT) may therefore result in the potential for NK alloreactivity. The interaction of KIR and class I MHC molecules, and the role they play in NK alloreactivity in HSCT is investigated in this thesis.

In related haploidentical transplants, donor and recipient pairs are mismatched for one haplotype, while in matched unrelated donor (MUD) transplants traditionally typed for HLA-A, -B and –DRB1 only, HLA-C is often mismatched. In both these transplant settings, there is potential for NK alloreactivity. Ruggeri et al (Ruggeri et al. 1999 and 2002) demonstrated in AML patients receiving haploidentical HSCT, NK alloreactivity in the graft-versus-host (GVH) direction resulted in less relapse, rejection and aGVHD. Based on these results I investigated whether these benefits could be observed in other allo-transplant protocols. I also investigated the interaction of KIR receptors with their ligands in a more systematic way than previously published.

I firstly developed a robust PCR-SSP KIR genotyping method which incorporated, (i) an internal PCR control (amplification of human growth hormone) to allow detection of false negatives and (ii) new primers to amplify

KIR alleles not amplified by the primers described by Uhrberg et al (Uhrberg et al. 1997). This method was then validated in a multi-laboratory evaluation.

Using this KIR genotyping method, I retrospectively KIR genotyped donors of haploidentical HSCT and HLA-C mismatched MUD transplants performed in Israel. In haploidentical donor HSCT (chapter 4), my results contrasted with those of Ruggeri et al (Ruggeri et al. 1999 and 2002) in that ligand incompatibility in the GVH direction was associated with higher incidence of GVHD, rejection and significant decrease in overall survival. Similar results were obtained in the MUD transplants. In haploidentical HSCT, high number of KIR receptors (both inhibitory and activating) in the donor was associated with a significantly higher incidence and more severe aGVHD (grade III-IV aGVHD) and a trend towards improved survival (chapter 4). Surprisingly, the opposite effect was observed in the MUD transplants, where high a number of donor KIR receptors was associated with significantly decreased aGVHD and improved survival (chapter 5).

If NK cell alloreactivity is to be exploited in HSCT, it will be important to be able to reliably select donors who have NK alloreactivity towards the patient. Donor NK alloreactivity towards the patient has traditionally been evaluated by NK cloning and ^{51}Cr-release assay. This approach is laborious and time consuming with results taking up to 6 weeks. In chapter 6, I describe the development of a rapid flow cytometric cytotoxicity assay utilising CD107a expression on 12-day polyclonally expanded NK cells. I demonstrated that NK alloreactivity mediated by inhibitory or activating KIR (KIR2DS1) can be detected by measuring CD107a expression following incubation with BLCL targets lacking the appropriate KIR ligand.

Using the CD107a functional assay, a systematic analysis of the ability of common HLA-B alleles and $Bw4^+$ HLA-A alleles to inhibit KIR3DL1 was performed. This study showed that most $Bw4^+$ HLA-B alleles tested, but not B*1301 and B*1302 are ligands for KIR3DL1. HLA-A*2402 and HLA-A*3201 were also shown to be ligands for KIR3DL1 but HLA-A*2501 and HLA-A*2301 provided target cells with very weak protection from lysis. KIR3DL1-dependent NK alloreactive clones were generated in donors who expressed HLA-A*2402 alleles but not in donors who expressed the $Bw4^+$HLA-B*1301 or -1302 alleles.
There is some evidence that activating KIR (KIR2DS1) can confer NK cell alloreactivity. Therefore the ability of NK clones expressing the activating KIR2DS1 receptor to lyse allogeneic target cells was investigated. These studies showed that KIR2DS1-positive NK clones are activated by recognition of the C2 epitope on BLCL targets and non-transformed cells (PHA blasts) and that the activation signal generated by KIR2DS1-C2 overrides the inhibitory signal generated by CD94/NKG2A on these clones.

The findings presented in this thesis have potentially important implications in donor selection and the clinical outcomes of haematopoietic stem cell transplantation.

Table of Contents

STATEMENT OF CANDIDATE CONTRIBUTION....................II
ACKNOWLEDGMENTS....................IX
LIST OF PUBLICATIONS....................XI
ABSTRACT....................XIII
TABLE OF CONTENTS....................XVI
LIST OF TABLES....................XIX
LIST OF FIGURES....................XXI

CHAPTER 1: LITERATURE REVIEW....................1
1.1 RECOGNITION OF NON-SELF BY THE ADAPTIVE AND INNATE IMMUNE SYSTEMS....................2
1.2 NATURAL KILLER CELLS....................5
1.3 "MISSING SELF" HYPOTHESIS & HYBRID RESISTANCE....................6
1.4 NATURAL KILLER CELLS RECEPTORS....................7
1.5 KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS (KIR)....................9
1.5.1 KIR structure and nomenclature....................9
1.5.2 KIR Genomic Organisation....................12
1.5.3 KIR Haplotypes....................14
1.5.4 KIR Ligands....................16
1.5.4.1 Activating KIR receptors with class I HLA specificity....................19
1.5.5 KIR Receptor Expression....................20
1.5.6 NK Cell Allorecognition....................21

1.6 KIR AND HAEMATOPOIETIC STEM CELL TRANSPLANTATION....................23
1.6.1 Graft-versus-Leukemia (GVL) effect....................23
1.6.2 Graft-versus-Host Disease (GVHD)....................24
1.6.3 Donor NK Alloreactivity in HSCT....................24
1.6.3.1 GVL effects mediated by NK cells....................25
1.6.3.2 Alloreactive NK cells prevent GVHD....................27
1.6.3.3 NK Alloreactivity and Allograft Rejection....................28
1.6.3.4 Predicting Donor NK Alloreactivity using HLA and KIR Genotype....................29
1.7 HYPOTHESES AND AIMS....................31

CHAPTER 2: MATERIALS AND METHODS....................34
2.1 DNA....................35
2.1.1 PCR optimisation and standardisation of PCR-SSP reaction for KIR genes....................35
2.1.2 Haematopoietic Stem Cell Transplantation....................35

2.1.2.1 Haploidentical transplant donor-recipient pairs......................35
2.1.2.2 Matched unrelated donor (MUD) transplant donor-recipient pairs...36
2.2 SPECIFIC PRIMING (PCR-SSP) ASSAY FOR KIR GENOTYPING.......................37
2.2.1 Oligonucleotide Primers..37
2.2.2 PCR-SSP Optimisation..38
2.3 STATISTICAL ANALYSIS..40
2.4 TARGET CELL LINES FOR NK MEDIATED CYTOTOXICITY...............................40
2.4.1 Cell Lines..40
2.4.2 Feeder Cells for In Vitro Expansion of NK cells.....................................41
2.4.2.1 Preparation Of Peripheral Blood Lymphocyte (PBMC) Feeder Cells...41
2.4.2.2 Preparation of RPMI-8866 feeder cells.......................................42
2.4.2.3 Irradiation of allogeneic PBMC and RPMI-8866 cells..................42
2.5 NATURAL KILLER CELL CLONING...42
2.5.1 Enrichment of Natural Killer Cells...42
2.5.2 Natural Killer Cell Cloning...43
2.5.3 Natural Killer Cell Polyclonal Cultures...44
2.6 NATURAL KILLER CYTOTOXICITY ASSAYS...45
2.6.1 ^{51}Cr-Release Cytotoxicity Assay..45
2.6.1.1 Preparation of EBV Target Cells...45
2.6.1.2 Preparation of Effector NK Cells...46
2.6.1.3 ^{51}Cr-Release Assay...46
2.6.2 CD107a Flow Cytometric Cytotoxicity Assay...47
2.7 IMMUNOPHENOTYPING OF NK CELLS..50

CHAPTER 3: A MULTI-LABORATORY EVALUATION OF REFERENCE CELLS FOR KIR TYPING...51

Immunobiology of the Human MHC-Proceedings of the 13th International Histocompatibility Workshop and Conference, (Ed: Hansen JA.), vol 1 p1233-1236, International Histocompatibilitiy Working Group Press.

CHAPTER 4: THE BENEFICIAL ROLE OF INHIBITORY KIR GENES OF HLA CLASS I NK EPITOPES IN HAPLOIDENTICALLY MISMATCHED STEM CELL ALLOGRAFTS MAY BE MASKED BY RESIDUAL DONOR-ALLOREACTIVE T CELLS CAUSING GVHD...................61

Tissue Antigens. 2004 Mar;63(3):204-11.

CHAPTER 5: NATURAL KILLER CELL HLA-C EPITOPES AND KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS BOTH INFLUENCE OUTCOME OF MISMATCHED UNRELATED DONOR BONE MARROW TRANSPLANTS..71

Tissue Antigens. 2005 Jun;65(6):519-28.

CHAPTER 6: RAPID, FLOW CYTOMETRIC ASSAY FOR NK ALLOREACTIVITY REVEALS EXCEPTIONS TO RULES GOVERNING ALLOREACTIVITY..83

Biol Blood Marrow Transplant. 2010 Feb;16(2):179-91.

CHAPTER 7: KIR2DS1-MEDIATED ACTIVATION OVERRIDES NKG2A MEDIATED INHIBITION IN HLA-C C2-NEGATIVE INDIVIDUALS...98

International Immunology, Vol. 20 (4), 555-563.

CHAPTER 8: THE REACTIVITY OF BW4^{+} HLA-B AND HLA-AALLELES WITH KIR3DL1: IMPLICATIONS FOR PATIENT AND DONOR SUITABILITY FOR HAPLOIDENTICAL STEM CELL TRANSPLANTATIONS...109

Blood. 2008 Jul 15;112(2):435-43

CHAPTER 9: FINAL DISCUSSIONS AND CONCLUSIONS...121

CHAPTER 10: REFERENCES...150

APPENDICES...161

- **APPENDIX I:** PRIMER SEQUENCES..162
- **APPENDIX II:** SOLUTIONS AND BUFFERS...164
- **APPENDIX III:** ANTIBODIES...169
- **APPENDIX IV:** SUPPLEMENTARY DATA FOR CHAPTER 6: RAPID, FLOW CYTOMETRIC ASSAY FOR NK ALLOREACTIVITY REVEALS EXCEPTIONS TO RULES GOVERNING ALLOREACTIVITY……………………………………………..170

LIST OF TABLES

Chapter 1:

Table 1 Clinical and transplant outcome data in HLA haploidentical transplants with and without ligand mismatching in the GVH direction {7388}..........26

Chapter 2:

Table 1 HLA types and KIR epitopes of BLCL used as target cells used in the CD107a assay.40

Table 2 The specificity of NK clones by the ^{51}Cr-Release Assay was determined by lyse of specific target cells..........47

Chapter 3:

Table 1 KIR2DS2 results provided by each of the laboratories for cell lines PITOUT and WT100BIS..........59

Chapter 4:

Table 1 Analysis of engraftment and relapse in relation to potential natural killer (NK) alloreactivity..........66

Chapter 5:

Table 1 Frequency of engraftment and rejection in transplants with NK epitope mismatching in the GVH direction, rejection direction, and either direction..........76

Table 2 The incidence of severe acute GVHD in transplants with NK epitope mismatching in the GVH direction, rejection direction, and either direction..........76

Table 3 Relative hazards and P-values for grades II–IV acute GVHD associated with the presence of each KIR gene in the donor..........78

Table 4 TRM in transplants with NK epitope mismatching in the GVH, rejection, and either direction..........78

Table 5 Relative hazards and Kaplan–Meier P-values for the null hypothesis that overall survival (OAS) and disease-free survival (DFS) are not associated with the presence of individual killer cell immunoglobulin-like receptor (KIR) genes..........80

Chapter 6:

Table 1 HLA types and KIR epitopes of BLCL used as target cells used in the CD107a assay.86

Table 2 Antibodies Used to Identify Potentially Alloreactive NK Cells..........86

Chapter 7:

Table 1 Class I HLA typing and NK epitopes of BLCL target cell panel..........101

Table 2 HLA typing NK epitopes and KIR typing of NK cell donors..102

Chapter 8:

Table 1 HLA class I and KIR3DL1 allele typing of NK-cell donors..113

Table 2 HLA class I typing of BLCL target cell panel...114

LIST OF FIGURES

Chapter 1:

Figure 1 KIR protein and gene organisation..10

Figure 2 Inhibitory and activating KIR receptor structure..12

Chapter 3:

Figure 1 KIR genotyping results received by each of the participating laboratories....................58

Figure 2 Consensus KIR results for 20 cell lines with results from 3 or more laboratories.........58

Chapter 4:

Figure 1 Potential natural killer (NK) alloreactivity in the graft vs host (GVH) direction was associated with grades III–IV GVHD..67

Figure 2 A higher number of activating killer immunoglobulin-like receptors (KIR) in donors is associated with grade II–IV graft vs host disease (GVHD)...67

Figure 3 Patients matched with their donors in both graft vs host (GVH) and host vs graft (HVG) directions had significantly better survival..68

Chapter 5:

Figure 1 Severity of acute graft-vs-host disease (aGVHD) is inversely related to the number of donor killer cell immunoglobulin-like receptor (KIR) genes...77

Figure 2 Natural killer (NK) epitope mismatching is associated with poorer disease-free survival...79

Figure 3 Increasing number of donor killer cell immunoglobulin-like receptor (KIR) genes is associated with improved disease-free survival..79

Chapter 6:

Figure 1 NK cells identified as potentially alloreactive on the basis of KIR receptor expression, express CD107a in response to target cells lacking the appropriate epitope.............................88

Figure 2 CD107a expression in response to epitope negative targets is dependent on the HLA type of the NK cell donor..90

Figure 3 Appropriate analysis of CD107a data reveals alloreactivity that correlates well with alloreactivity predicted by HLA and KIR genetics...92

Figure 4 Appropriate analysis of CD107a data results in good correlation with KIR2DS1-mediated alloreactivity predicted by HLA and KIR genetics..93

Figure 5 NKG2A1 cells do not contribute significantly to the alloreactive subsets....................94

Figure 6 Twelve-day culture expands the alloreactive subset of NK cells.................................95

Chapter 7:

Figure 1 Receptor expression and cytotoxicity pattern of CD158b-negative clones showing specificity for C2-positive targets...103

Figure 2 Despite expression of KIR3DL2, clone C9 was inhibited equally well by HLA-A*0301 and HLA-A*0201 homozygous BLCL target...104

Figure 3 NK clone C9 only lyses C2-positive BLCL targets and shows a C2 dose effect.........104

Figure 4 NK clone C9 transcribes KIR2DS1*002...104

Figure 5 Target cell lysis by NK clone C9 is enhanced by anti-NKG2A antibody and inhibited by anti-CD158a (anti-KIR2DL1/KIR2DS1)..104

Figure 6 NK cell activation through KIR2DS1 (CD158a) only occurs in C2 negative, KIR2DS1-positive individuals...105

Figure 7 C2-positive EBV-transformed and PHA blast cells both activate CD158a-positive NK cells..106

Chapter 8:

Figure 1 Low-level KIR3DL1 expression differs in 2 donors due to a nonsynonymous mutation at nucleotide position 115 in exon 3 encoding the D0 domain of the KIR3DL1*005 allele...114

Figure 2 CD107a expression by KIR3DL1$^+$ polyclonal NK cells is inhibited by most Bw4$^+$ targets...115

Figure 3 Level of Bw4 expression does not correlate with percentage of CD107a expression induced on KIR3DL1$^+$ NK cells...116

Figure 4 HLA-B*1302 and HLA-B*1301 do not inhibit cytotoxicity of KIR3DL1-dependent NK cells..116

Figure 5 Inhibition through Bw4-expressing HLA-A alleles can be reversed by addition of anti-KIR3DL1...117

Figure 6 Donors who express HLA-B*1302 or HLAB*1301 make very few KIR3DL1-dependent NK cells..118

CHAPTER 1

LITERATURE REVIEW

CHAPTER 1: LITERATURE REVIEW

The literature review will cover literature published prior to the commencement of this thesis (2002). Literature published after this time will be discussed in the relevant chapters and in the final discussion chapter.

1.1 RECOGNITION OF NON-SELF BY THE ADAPTIVE AND INNATE IMMUNE SYSTEMS

The immune system can be divided into two subsystems: the adaptive and the innate immune systems. The adaptive immune response is antigen specific. The lymphocytes involved in the adaptive immune system require a sensitising event to a pathogen and the response is improved with subsequent exposure to the same pathogen. In contrast, the response of the innate immune system is rapid, has no memory and does not discriminate among different microbes; that is, the response is antigen non-specific (Abbas et al. 1997). The innate immune system represents the first line of defence against tumour cells, bacterial and viral infections.

The cells involved in the adaptive immune response are T and B cells. There are two different kinds of T cells; CD4+ T helper cells which provide help for B cell growth and differentiation, and CD8+ T cytotoxic cells which recognise and kill viral antigens on infected cells. B cells differentiate into plasma cells which help destroy extracellular pathogens and their products by releasing antibodies that react with them. Both T and B cells express highly specific antigen receptors on their surface. However individual clones of T and B cells express only one such receptor which allows each cell to recognise a particular antigen. The diversity of antigen receptors enables T and B cells to recognise a diverse range of antigens. The majority of peripheral T cells express a T cell receptor

(TCR) for antigen, composed of an α and β polypeptide chain. The TCR forms a complex in the T cell membrane with the signalling molecule CD3 and either CD4 or CD8. When interacting with other cells, the CD4 molecule on helper T cells binds to a non-polymorphic region of class II MHC molecules while CD8 on T cytotoxic cells binds to a non-polymorphic region of class I MHC molecules. The TCR does not recognise foreign antigens directly. Rather, it recognises short antigenic peptides bound in the peptide binding groove of MHC molecules on the surface of antigen presenting cells or target cells (Janeway Jr et al. 1997).

The B cell antigen receptors (BCR) are membrane bound immunoglobulins (Ig) that, when bound to a specific antigen, initiate B cell division and differentiation into plasma cells which produce antibody. The BCR captures antigen which is then internalised and a fragment of the antigen is presented on the cell surface bound in the peptide binding groove of a class II MHC molecule. Helper T cells recognise the peptide-MHC complex and deliver activating signals to the B cell which enable it to proliferate and differentiate into antibody-secreting cells (Janeway Jr et al. 1997).

A third group of lymphocytes, natural killer (NK) cells, are cells of the innate immune system. NK cells, without prior sensitisation, lyse a variety of target cells including tumour cells and cells infected with viruses. NK cells also produce a range of immunoregulatory cytokines (eg., interferon, tumour necrosis factor-α, granulocyte macrophage colony-stimulating factor) and chemokines (MIP-1, RANTES) that are involved in the destruction of intracellular pathogens or recruitment of other effector cells (reviewed in Biron

et al. 1999). NK cell cytotoxicity is regulated by a balance of signals from inhibitory and activating receptors expressed on the cell surface (reviewed in Borrego et al. 2002). NK cell inhibitory receptors (Killer cell immunoglobulin-like receptors (KIR) and CD94/NKG2A) interact with class I MHC molecules to inhibit NK cell-mediated lysis (reviewed in Borrego et al. 2002). If target cells lose class I MHC expression, inhibitory receptors on NK cells fail to engage their ligands resulting in NK cell lysis. NK cell cytotoxicity is promoted when class I MHC-specific activating receptors (KIR, CD94/NKG2C/E/H) or other activating receptors (Natural Cytotoxicity Receptors (NCR) and NKG2D) interact with their ligands. The ligands for the NCRs (NKp46, NKp44, and NKp30) were unknown at the commencement of this thesis. The activating receptor NKG2D interacts with the stress-inducible ligand including the MIC-A and MIC-B antigens, the UL16 binding proteins (ULBPs) and RAET antigens (Bauer et al. 1999, Cosman et al. 2001). Both the NCRs and NKG2D have been shown to be important in mediating NK cell recognition and lysis of tumour cells. Pende et al (Pende et al. 2001) showed that NKG2D triggering of NK cells was solely responsible for killing of some tumour cell lines while for other tumours, triggering from both NCR and NKG2D activating receptors was required. NK cells also express the activating receptor CD16 which is specific for the Fc portion of IgG molecules that have bound to their antigen (reviewed in Borrego et al. 2002). Other co-receptors have also been described that enhance triggering of NK cell cytotoxicity. These include CD2, LFA-1, CD40, CD69, 2B4 and NKp80. The precise biological role of these co-receptors in NK mediated killing is unclear (Farag et al. 2002).

NK cells, the killer immunoglobulin-like receptors (KIR) and the interaction of these receptors with class I MHC molecules and the role they play in NK alloreactivity in bone marrow transplantation (BMT) will be reviewed in more detail.

1.2 NATURAL KILLER CELLS

NK cells are bone marrow derived lymphocytes that are thought to originate from the same lineage as T cells (Spits et al. 1995). They are relatively large granular lymphocytes that comprise 10-15% of all circulating blood lymphocytes and can be found in the spleen, lymph nodes, liver, lung, intestine and placenta. NK cells are characterised by the expression of CD16 and CD56 and the absence of CD3 (Whiteside and Herberman 1994). NKp46 has more recently been proposed as a pan NK cell marker (Moretta et al. 2001).

The cell surface density of CD56 expression subdivides NK cells into 2 distinct subpopulations. The majority (90%) of NK cells are $CD56^{dim}$ and express high levels of CD16, while the remaining NK cells are $CD56^{bright}$ and express low levels or no CD16 (Cooper et al. 2001). The two subpopulations also differ in function and NK receptor repertoires. $CD56^{dim}$ NK cells, being CD16 positive, mediate antibody-dependent cell mediated cytotoxicty (ADCC). They express both killer immunoglobulin-like receptor (KIR) and C-type lectin NK receptors (reviewed in section 1.4). $CD56^{bright}$ NK cells are less cytotoxic than $CD56^{dim}$ NK cells but produce cytokines. They express high levels of C-type lectin receptors with only a small number expressing KIR (reviewed in Cooper et al. 2001, Farag et al. 2002).

NK cells recognise and lyse target cells either by ADCC or natural cytotoxicity (Borrego et al. 2002). In ADCC, the activating receptor CD16 (FcγRIII) recognises the Fc portion of IgG-antibodies specifically bound to the surface of targets resulting in NK cell degranulation and lysis of the target. Natural cytotoxicity does not require antibody and is governed by a balance of inhibitory and activating signals received via receptors on the NK cell interacting with ligands on target cells. The ligands for many inhibitory receptors are class I MHC molecules. The lack of expression of class I MHC molecules or the down-regulation of these molecules, as occurs in tumour cells or cells infected with virus, results in NK-mediated lysis of the cell (reviewed in Moretta et al. 2001).

1.3 "MISSING SELF" HYPOTHESIS & HYBRID RESISTANCE

The conventional laws of transplantation are based on T cell recognition of histocompatibility antigens. If two inbred parental mouse strains differ in their MHC genotypes, their F1 hybrids will express the MHC antigens of both parents. F1 hybrids should therefore accept grafts from either parent as the T cells in the F1 mouse are tolerant of the MHC antigens encoded by both parental MHC haplotypes whilst both parents should reject grafts from the F1 hybrid. These laws generally apply but exceptions were discovered for haematopoietic grafts. Rejection of parental bone marrow grafts by (A x B) F1 hosts was first described in mice by Cudkowicz et al in 1964 (Cudkowicz and Stimpfling 1964). This phenomenon was called hybrid resistance (reviewed in Bennett 1987). The cells responsible for hybrid resistance were discovered to be of marrow origin and a number of studies in the early 1970s confirmed these to be NK cells (reviewed in Yu et al. 1992).

The importance of class I MHC expression in preventing NK mediated lysis was demonstrated in a series of experiments using transplantable lymphomas. In experiments performed by Karre and Ljunggren (Karre et al. 1986), lymphoma cells with reduced expression of H-2 were selected and compared to wild type cells for the ability to grow as tumours in mice. The lymphoma cells with reduced H-2 expression were more frequently rejected than the H-2 positive wild types. The authors provided evidence that the reduced tumourigenicity of the cell lines with reduced class I expression was due to the rapid elimination by NK cells. In this model the H-2 deficient lymphoma cells failed to inhibit NK-mediated lysis. Karre and colleagues hypothesised that NK cells mediated hybrid resistance by recognising and eliminating target cells that failed to express self class I MHC molecules. This was referred to as the 'missing self' hypothesis (Ljunggren and Karre 1990).

1.4 NATURAL KILLER CELL RECEPTORS

As previously described, in humans, two families of NK receptors have been described: the C-type lectin-like family and the immunoglobulin superfamily (Ig-SF) which include the killer immunoglobulin-like receptors (KIR) and the natural cytotoxicity receptors (NCR) (Borrego et al. 2002). The C-type lectin family include the CD94/NKG2 heterodimers, NKG2D homodimers, and in the mouse, the Ly49 family. The C-type lectin receptor family include both inhibitory and activating receptors, and in humans these receptors interact with the non-classical HLA-E class I molecule on potential targets (Borrego et al. 1998). HLA-E is expressed at low levels on a broad range of cell types and tissues including peripheral blood mononuclear cells (the levels vary according to cell type), liver, skin, lung and placenta (Lee et al. 1998). The recognition of HLA-E

by CD94/NKG2A receptors is dependent on the expression of other class I molecules in the target cell. CD94/NKG2A receptors recognise peptides from the leader sequence of HLA-A, -B, -C and –G bound in the peptide binding groove of HLA-E (Brooks et al. 2000). Consequently, CD94/NKG2A receptors indirectly monitor a broad array of class I MHC molecules.

KIR belong to the immunoglobulin-super family (Ig-SF) and are encoded in the leukocyte receptor complex (LRC) on chromosome 19. As for the C-type lectin receptors, the KIR family of receptors includes both inhibitory and activating members that interact with class I MHC on potential targets. However, unlike the C-type lectin receptors, the KIR receptors are specific for products of a single class I locus and therefore enable NK cells to recognise decreased expression of a particular class I MHC locus (reviewed in Colonna and Samaridis 1995, Lanier 1998)). The KIR receptors will be reviewed in more detail in section 1.5.

The natural cytotoxicity receptors (NCR), NKp46, NKp44 and NKp30, also belong to the Ig-SF but are not encoded in the LRC. These receptors are important activating receptors for the lysis of virus infected tumour targets (Arnon et al. 2006). The ligands for the NCRs were unknown at the commencement of this thesis.

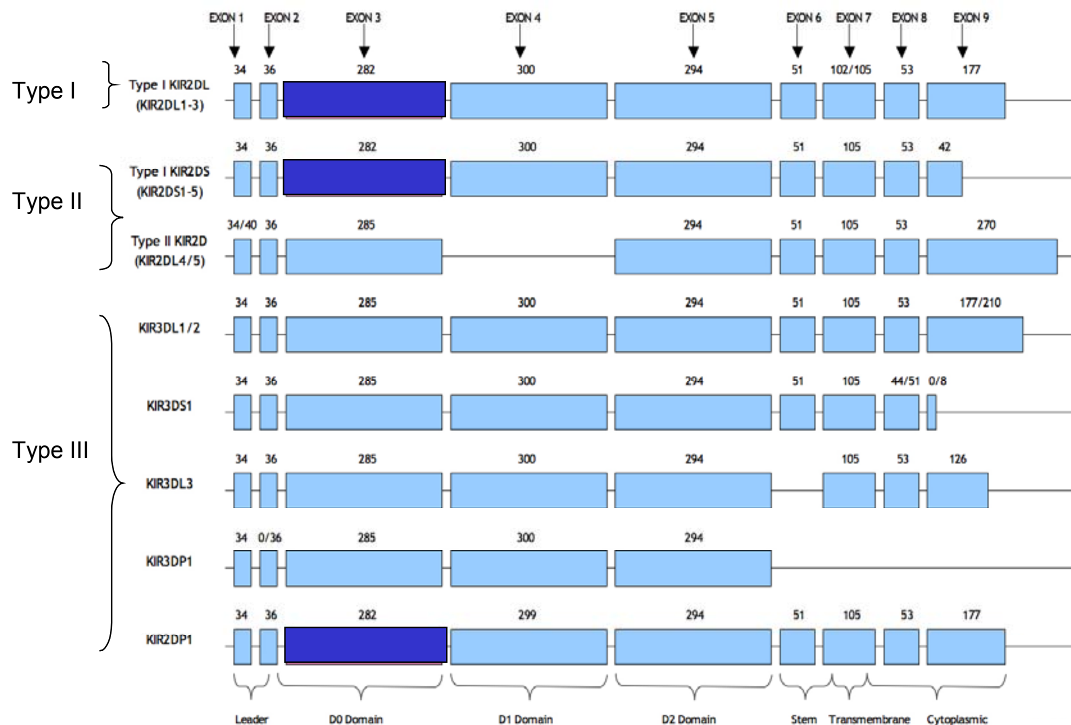
An individual NK cell clone does not express all NK receptors but NK clones may express receptors from more than one receptor family, resulting in NK clones with diverse patterns of receptor expression. The variegated pattern of

receptor expression allows different NK cells to discriminate among cells with different patterns of class I MHC expression (reviewed in Raulet et al. 2001).

1.5 KILLER IMMUNOGLOBULIN-LIKE RECEPTORS (KIR)

1.5.1 KIR receptor structure and nomenclature

KIR are type I transmembrane glycoproteins including a leader sequence, either two or three immunoglobulin-like (Ig-like) domains which form the extracellular region, a stem, a transmembrane region and a cytoplasmic region. The different members of the KIR family are named according to the number of Ig-like domains (2D or 3D), the length of the cytoplasmic tail (long (L) or short (S)) followed by a number to distinguish different members having the same structure but differing in amino acid sequence eg KIR3DL1, KIR3DL2 (Vales-Gomez et al. 2000). KIR receptors are further sub-classified into 3 groups (Type I, II and III) according to the configuration of their Ig-like domains. Type I receptors utilise a D1 and D2 domain and omit the D0 (membrane distal) domain. Type I receptors include all the KIR2DL and KIR2DS KIR, with the exception of KIR2DL4 and KIR2DL5 (Vilches et al. 2000a). Type II receptors utilise D0 and D2 domains and omit the D1 (middle) domain. Type II receptors include KIR2DL4 and KIR2DL5 (Vilches et al. 2000b). Type III receptors utilise all three Ig-like domains and include the KIR3DL and KIR3DS KIR (Fig 1.1)



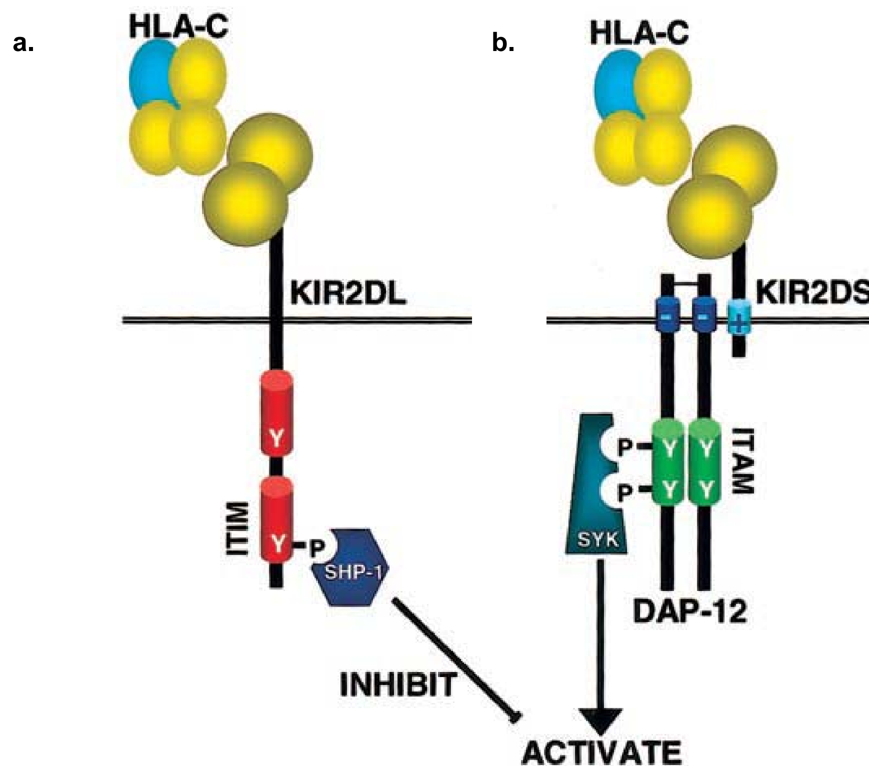
reproduced from www.ebi.ac.uk/ipd/kir

Fig1.1 KIR protein and gene organisation. KIR receptors are grouped together according to protein structure. The dark grey boxes represent pseudo-exons in type I KIR. The exons corresponding to each protein domain and region is indicated. The size in base pairs is shown above each box.

The Ig-like domains constitute the ligand binding site (Fan et al. 1997). KIR with long cytoplasmic tails generate inhibitory signals on ligand binding by virtue of two immune tyrosine-based inhibitory motifs (ITIM) in the cytoplasmic tail (Fig.1.2a). ITIMs consist of a tyrosine residue, an N-terminal isoleucine, valine, leucine or serine and C-terminal leucine or valine (I/V/L/SxYxxL/V). Interaction between the receptor and its HLA ligand results in phosphorylation of the tyrosines in the ITIM units and recruitment of the protein tyrosine phosphatase, SHP-1 or SHP-2. Through the activation of a further series of downstream events this results in inhibition of NK cell activation (Fig1.2a) (Bruhns et al.

1999, Burshtyn et al. 1997, Burshtyn et al. 1999). KIR with short cytoplasmic tails are activating KIR. Activating KIR lack ITIMs in the intracytoplasmic domain but contain a charged amino acid in the transmembrane domain (Fig.1.2b). The presence of a charged amino acid in the transmembrane domain of activating receptors enables them to bind non-covalently with the specialised signal transducing adapter molecule known as DAP-12 (Blery et al. 2000). DAP-12 is a disulfide-linked tyrosine phosphorylated homodimer containing an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. The activating receptor/adaptor molecule complexes in turn activate protein tyrosine kinases such as Syk or ZAP-70 which ultimately result in cellular activation (Lanier et al. 1998).

Despite differences in the transmembrane and intracytoplasmic domains that result in different signalling potential, some inhibitory and activating KIR receptors demonstrate high homology in the extracellular portion of the receptor suggesting similar ligand specificity (Biassoni et al. 1996).



reproduced from *Farag et al, 2002.*

Fig1.2. KIR receptors have either 2 or 3 immunoglobulin domains (2D or 3D) and a long cytoplasmic tail containing ITIM motifs or short cytoplasmic tails containing a charged amino acid residue in the transmembrane domain that interacts with DAP12 adaptor molecules.

1.5.2 KIR Genomic organisation

Sequencing of the LRC region containing the KIR gene family on human chromosome 19q13.42 identified 16 highly conserved, tightly clustered KIR genes and pseudogenes, arranged in head-to-tail orientation (Barten et al. 2001). All KIR genes share a similar exon-intron structure of nine exons and eight introns with each gene separated by approximately 2.4kb of intergenic sequence. The one exception is the intergenic region between KIR3DP1 and KIR2DL4 which is approximately 14kb.

KIR gene content varies from individual to individual. There are at least 36 unique genotypes based on the presence or absence of particular KIR genes (Hsu et al. 2002). KIR genes also exhibit variation at the sequence level such that there are several alleles at each KIR locus (a full list is available at www.ebi.ac.uk/ipd/kir). Three KIR genes are found in all individuals and are therefore termed “framework” genes; KIR3DL3 at the centromeric end of the KIR complex, KIR2DL4 in the middle and KIR3DL2 at the telomeric end. All three genes are located near unique sequences that may prevent loss of these genes by DNA looping and unequal crossing over during meiosis (Wilson et al. 2000). All KIR genes have the AG-GT exon-intron splice signal except for exon 3 of KIR2DL1 which has CG splice site. Exons 1 and 2 encode the signal peptide, exons 3, 4, and 5 encode the Ig-like domains (D0, D1 and D2 respectively), and exon 6 encodes the stem connecting the D2 domain to the transmembrane region which is encoded by exon 7. Exons 8 and 9 encode the cytoplasmic tail (Fig 1.1). In the Type I 2DL/S KIR genes exon 3 is a pseudoexon that is not expressed due to exon skipping. In the Type II 2DL KIR genes the region corresponding to exon 4 in all other KIR genes is deleted. The KIR3D genes have all nine exons. A striking difference between the KIR genes is their length which ranges from 3.9 to 16.3kb. This variation is primarily due to insertions/deletions of retroelements in the introns of these genes. The greatest number of non-synonymous substitutions is seen in exons 3, 4 and 5 which encode the D0, D1, D2 domains respectively and is most likely due to selective pressure operating on the interaction of these domains with different class I MHC molecules (Martin et al. 2000).

1.5.3 KIR haplotypes

Uhrberg et al (Uhrberg et al. 1997) first proposed two broad distinct KIR haplotype groups referred to as KIR A and B haplotypes. This classification was based on differences in the number of KIR genes present in 68 blood donors and the absence (KIR A haplotype) or presence (KIR B haplotype) of a 24kb HindIII fragment on Southern blotting. Subsequent analysis by Witt et al (Witt et al. 1999) of linkage disequilibrium between KIR sequences obtained in a panel of 147 individuals also showed that the KIR genes divided into two distinct haplotypes based on the presence or absence of KIR2DL1 and KIR2DL3 (KIR A haplotypes) or KIR2DL2 (KIR B haplotypes). It subsequently became clear that the approach of Witt et al was based on genes at the telomeric end of the KIR complex whereas the HindIII fragment resides in the centromeric end of the complex. The two approaches however resulted in a very similar classification of haplotypes due to the extensive linkage disequilibrium between the KIR genes. It is now agreed that the KIR group A haplotypes consists of the three framework genes KIR3DL2, KIR3DL3 and KIR2DL4, the inhibitory receptors KIR2DL1, KIR2DL3, KIR3DL1, the activating receptor KIR2DS4 and the pseudogene KIR2DP1. The KIR group A haplotype is the single most common haplotype group and accounts for ~50% of haplotypes in many populations. The KIR B haplotype group is more variable and defined as all haplotypes other than the common KIR A haplotype group (Barten et al. 2001, Uhrberg et al. 1997, Wilson et al. 2000, Witt et al. 1999). Hsu et al (Hsu et al. 2002) subsequently reported from family segregation studies, that the KIR A haplotype as defined by Uhrberg et al (Uhrberg et al. 1997) and Witt et al (Witt et al. 1999) was actually composed of two distinct subtypes. In one subtype the KIR2DS4 gene was intact and in the other the KIR2DS4 gene contained a 22 base pair deletion

likely to inactivate the gene. The latter subtype was demonstrated to be the more common, comprising 73% of KIR A haplotype and having a haplotype frequency of 39% in the Caucasian population. The haplotype with an intact KIR2DS4 gene had a haplotype frequency of 12%. The combined haplotype frequency of both KIR A haplotype subgroups in this study was 51%, consistent with previous reports (Uhrberg et al. 1997, Witt et al. 1999). This data suggested that some individuals would lack all functional activating KIR receptors, with the exception of KIR2DL4, a receptor which can have an activating function (Hsu et al. 2002).

Linkage disequilibrium (LD) analyses undertaken by Hsu et al (Hsu et al. 2002) supported the findings of Witt et al (Witt et al. 1999), ie KIR2DL1 and KIR2DL3 genes invariably occur together and were often absent when KIR2DL2 was present. KIR2DL2 was present in all samples negative for KIR2DL3 a finding suggesting that these inhibitory KIR segregate on different haplotypes and behave as alleles. Thus KIR2DL1 and KIR2DL3 were shown to be characteristic of the KIR A haplotype and KIR2DL2 characteristic of the KIR B haplotype. KIR2DL2 was shown to be in very high LD with KIR2DS2 suggesting that KIR2DS2 is also characteristic of KIR B haplotypes. KIR2DL1 and KIR2DL3 were shown to be in strong positive LD with KIR3DL1 and KIR3DL1 was strongly associated with KIR2DS4 suggesting that these two genes are also characteristic of the KIR A haplotype. It was also shown that KIR2DS4 and the variant of KIR2DS4 with the 22bp deletion were in strong negative LD suggesting that these two genes are possibly alleles of the same locus. Likewise KIR3DL1, which has high homology with KIR3DS1 in the ligand binding Ig-like domains, was shown to be in strong negative LD with KIR3DS1

suggesting they are related to each other as alleles and that KIR3DS1 is characteristic of KIR B haplotypes.

(More recently there has been agreement that the KIR haplotypes can be classified as KIR A or B group at either the centromeric or telomeric ends of the KIR gene complex (Martin et al. 2004).)

1.5.4 KIR ligands

Inhibitory KIR receptors interact with the classical class I molecules, HLA-A, -B and –C, to inhibit NK cell-mediated lysis. KIR recognition is not only locus-specific but also specific for certain groups of alleles sharing a common epitope. Type I KIR2D receptors interact with HLA-C alleles, while Type III KIR3D receptors interact with either HLA-B or -A alleles bearing the Bw4 epitope (KIR3DL1) or with HLA-A alleles (KIR3DL2). At the commencement of this thesis, the precise set of HLA-A alleles that bind to KIR3DL2 receptor was still unknown, however since then studies have shown that HLA-A3 and –A11 appear to be ligands for KIR3DL2 (Hansasuta et al. 2004).

Studies performed by Ciccone et al (Ciccone et al. 1992) provided the first direct evidence that different groups of NK clones recognised different class I HLA alleles thereby inhibiting NK cell function. Transfection of P815 murine cells with Cw3 but not other class I HLA alleles tested provided protection from NK clones designated as having “anti-NK2” specificity (Ciccone et al. 1992). The monoclonal antibody EB6 identified the relevant KIR receptors on such NK clones as having a molecular weight of 58 kDa. A second monoclonal antibody, GL183, recognised a similar receptor on other NK clones having the “anti-NK1” specificity. The receptors were named p58.1 and p58.2, respectively, based on

their molecular mass of 58 kDa (Moretta et al. 1993). p58.1 and p58.2 were later identified as receptors with two Ig-domains and a long cytoplasmic tail (Wagtmann et al. 1995). The receptors were later named KIR2DL1 and KIR2DL3 respectively (Uhrberg et al. 1997). It was shown that KIR2DL1 interacted with HLA-C alleles having a lysine (Lys) at residue 80 whereas KIR2DL2 and KIR2DL3 interacted with those having an asparagine (Asn) at residue 80 (Vales-Gomez et al. 1998). HLA-C alleles with a lysine at residue 80 were said to have the C2 epitope whereas those alleles with asparagine at residue 80 were said to have the C1 epitope (Colonna et al. 1992, Colonna et al. 1993a, Moretta et al. 1993). Those alleles having the C2 epitope include Cw2, Cw4, Cw5, Cw6, Cw17 and Cw18. Those alleles having the C1 epitope include Cw1, Cw3, Cw7, Cw8, Cw13 and Cw14.

HLA-B alleles can be divided into two groups based on the presence of either a Bw4 or Bw6 motif in the α1 domain at residues 77-83 of the molecule. Bw4 is present on approximately 35% of the known HLA-B alleles and HLA-B alleles that express the Bw4 epitope are present at a gene frequency of approximately 40%. The Bw4 epitope is also present on approximately 20% of HLA-A alleles (HLA-A23, A24, A25, A32). Cella and colleagues (Cella et al. 1994) identified NK cell clones that were selectively inhibited by Bw4+ alleles. Analysis of the cytotoxicity of two of these clones, termed NK3-specific, against class I HLA transfectants and PHA blasts, demonstrated that HLA-B alleles with the Bw4 epitope and a few HLA-A alleles with the Bw4 epitope inhibited NK cell lysis. The degree to which the Bw4 group alleles inhibit the NK3-specific cells correlated with amino acid residue at position 80. Isoleucine (Ile) at residue 80 was associated with strong NK cell inhibition, Threonine (Thr) with intermediate

inhibition and Asn 80 (Bw6) with complete susceptibility to lysis. Litwin et al, (Litwin et al. 1994) also identified an NK receptor, which they called NKB1, that recognised certain HLA-B alleles. NKB1^{+} NK clones were always inhibited by target cells expressing HLA-B*5801, HLA-B*5101 and B*2705 but not other HLA-B, or HLA-A or HLA-C alleles tested. Sequence analysis of the inhibiting alleles suggested that reactivity correlated with the Bw4 epitope. Gumperz et al (Gumperz et al. 1995) in experiments using 721.221 cell lines and 721.221 cell lines transfected with B*1513 (Bw4) and B*1502 (Bw6) showed that B*1513 but not B*1502 was able to inhibit lysis by NKB1+ NK clones. This study confirmed that the Bw4 epitope was critical for recognition by NKB1^{+} NK cells. The NKB1 receptor was later renamed KIR3DL1.

Colonna et al (Colonna and Samaridis 1995) first identified cDNA that encoded KIRs with three Ig domains (NKAT3 and NKAT4). Functional characterisation of NKAT4+ NK clones using a NKAT4-specific monoclonal antibody and the analysis of binding of soluble NKAT4-IgG1 fusion proteins to class I transfectants by flow cytometry showed that NKAT4 KIR was a receptor specific for HLA-A3 (Dohring et al. 1996). Pende et al (Pende 1996) later identified a receptor for HLA-A3 and –A11 expressed on a subset of NK cells as a disulphide-linked dimer of ~ 140kD named p140. In a cytotoxicity assays, polyclonally activated NK cells expressing the p140 receptor (as recognised by the Q66 monoclonal antibody) were tested against C1R-transfectants expressing HLA-A1, -A2, -A3 and -A24. This receptor could discriminate among HLA-A alleles, HLA-A1, -A2 and -A24 did not inhibit p140^{+} NK clones while HLA-A3 and –A11 protected target cells from lysis. NKAT4 and p140 were later named KIR3DL2. Although a number of studies have demonstrated specificity

of KIR3DL2 for HLA-A alleles, Valiante et al (Valiante et al. 1998) could not correlate the expression of KIR3DL2 and inhibition by HLA-A*0301. In cytotoxicity experiments, inhibition of NK cell cytotoxicity by a transfectant expressing HLA-A*0301 was not reversed by the addition of KIR3DL2-specific antibody. Further studies are required to clarify the interaction between HLA-A and KIR3DL2 as this remains a contentious issue.

1.5.4.1 Activating KIR receptors with class I HLA specificity

All NK cells with lytic capacity express at least one inhibitory receptor with specificity for a self HLA ligand thereby preventing lysis of autologous cells (Moretta et al. 1997). However, an NK cell may also express a number of activating KIR receptors. The activating receptors KIR2DS1 and KIR2DS2 exhibit high homology in the extracellular domains with their inhibitory counterparts KIR2DL1 and KIR2DL2/3, respectively, suggesting that the ligands for these receptors may be HLA-C alleles (Winter et al. 1998). There has however been little direct evidence that these activating receptors bind to HLA-C alleles. A soluble form of KIR2DS1 was shown to bind to HLA-Cw*0401 very weakly relative to the binding of KIR2DL1 with HLA-Cw*0401 (Biassoni et al. 1997, Moretta et al. 1995). Winter et al, (Winter et al. 1998) evaluated the binding of KIR2DS receptors to various class I HLA transfected cells using KIR2DS2-Ig and KIR2DS4-Ig fusion proteins. Neither KIR2DS receptor bound to any of the class I HLA alleles tested. KIR2DS2 differs from KIR2DL2 and KIR2DL3 by only four and three amino acids, respectively. Residue 45 in the extracellular domain of KIR2DS2 (tyrosine) is unique to this receptor. It has been shown that the replacement of tyrosine by phenylalanine at position 45 enabled the KIR2DS2-Ig protein to bind to the HLA-Cw*0304 transfectant. The

lack of binding of the activating KIR2D receptors may reflect a low affinity for class I HLA alleles. It may be possible that the affinity of activating receptors for HLA-C is lower than that of inhibitory receptors to ensure that inhibition overrides activation, yet it is sufficient to activate NK cells in the absence of an inhibitory signal (Moretta et al. 1995, Winter et al. 1998). Further investigation is required to determine the exact ligands for KIR2D activating receptors.

1.5.5 KIR Receptor expression

Human NK clones express different numbers and combinations of KIR receptors (Valiante et al. 1998). The only requirement for each NK clone with lytic activity is that it expresses at least one inhibitory receptor with specificity for a self class I MHC molecule (Valiante et al. 1998). The inhibitory receptor can belong to either the KIR or CD94/NKG2 family. The range of different KIR receptors expressed by the repertoire of NK clones in an individual is primarily determined by the KIR genotype but the individuals HLA genotype determines which KIR can be expressed as the only inhibitory receptor on individual NK cell clones (Uhrberg et al. 1997). Receptor selection occurs during NK cell development to ensure only NK cells with an inhibitory receptor for a self ligand are permitted to become armed for cytotoxicity. Thus lytic NK cells without an inhibitory receptor for self-HLA class I do not appear in peripheral circulation (Uhrberg et al. 1997, Valiante et al. 1998). (Recently however, it has been shown that such cells, though rare, can be identified. However they do not seem to be “armed” for cytotoxicity. It is thought that arming requires interaction between the inhibitory receptor and its ligand (Kim et al. 2005).)

1.5.6 NK Cell Allorecognition

Although KIR receptors determine the allospecificity of NK cells, the co-expression of CD94:NKG2A, which mediates inhibition when engaging the non-polymorphic HLA-E molecule, renders an NK cell non-alloreactive towards targets expressing HLA-E. To define the rules governing NK cell self-tolerance and alloreactivity, Valiante and colleagues (1997) studied the expression of KIR and CD94:NKG2 receptors on NK clones obtained from two blood donors PP and NV (Valiante et al. 1998). The two donors differed in their class I HLA and KIR genes. Both donors were heterozygous at HLA-A, -B and –C and shared one class I HLA haplotype. Donor PP's HLA type included only one HLA ligand for inhibitory KIR (C1), whereas donor NV's HLA type included all three ligands (C1, C2 and Bw4). NK clones from both donors were shown to lyse the class I HLA deficient 721.221 cell line but not their autologous B cell lines. 721.221 cells transfected with a single class I HLA allele were lysed to varying degrees but in a reproducible manner by the different NK clones. They showed that every cytotoxic NK cell expressed either an inhibitory KIR that recognised a self class I HLA epitope or CD94/NKG2A. Using antibody blocking experiments, the authors showed that the class I HLA-mediated inhibition of NK cell clones for donor PP, whose HLA type included only the C1 epitope, was accounted for by two receptors, KIR2DL3 and CD94:NKG2A. NK clones expressing KIR2DL3 were strongly inhibited by autologous HLA allotypes belonging to the C1 group (Cw*0304 and *0702). Donor NV's HLA type included all three HLA epitopes and the inhibitory KIR specific for each HLA-C epitope were individually or co-expressed on some 30-50% of the peripheral blood NK cells while KIR3DL1, the receptor specific for NV's Bw4 ligand (B*2708), was expressed on very few NK cells. KIR2DL1 was shown to have specificity for HLA-C alleles belonging to

the C2 group and KIR2DL2 specificity for the C1 group. All other inhibition not mediated through these receptors could be accounted for by CD94:NKG2A.

The extent to which NK cell clones from one donor are inhibited by the HLA ligands of another donor was also investigated. Some NK clones from donor NV (C1+, C2+, Bw4+) lysed targets from donor PP (C1+ only), whereas PP did not have any clones that could lyse targets from donor NV. The NK alloreactivity demonstrated could be explained by the repertoire of receptors and the HLA ligands expressed by the two donors. KIR2DL3 and CD94:NKG2A were the dominant inhibitory receptors in donor PP and since NV's cells had HLA ligands for both of these receptors there was no NK alloreactivity of donor PP's cells towards NV cells. In contrast, NV had a subset of NK cells that used KIR2DL1 as their only inhibitory receptor and since PP's cells did not express the ligand for this receptor (C2), these NK cells killed PP targets. Thus, the general rule emerged that NK cells from individual A will be alloreactive to cells from individual B if individual B lacks a KIR ligand expressed by individual A. Individual A's NK cells will be tolerant of cells from individual B if individual B has the same or additional KIR ligands as individual A (Valiante et al. 1998). In 1999, Ruggeri et al (Ruggeri et al. 1999) demonstrated the relevance of these findings for haematopoietic stem cell transplants (HSCT). If a recipient's class I HLA alleles do not inhibit all donor NK cells, the donor will have alloreactive NK clones that kill recipient target cells, including acute myeloid leukaemia (AML) cells. The implications of these findings for HSCT are discussed in detail in the next section of this chapter.

1.6 KIR AND HAEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

Allogeneic haematopoietic stem cell transplantation (HSCT) provides a possible cure for leukaemia. Before infusion of donor cells, transplant recipients undergo either myeloblative or non-myeloablative conditioning regimens and/or irradiation. This regimen first destroys the host bone marrow making way for the transplanted cells to engraft and secondly, destroys the leukaemic cells. Transplant recipients then receive infusions of haematopoietic stem cells from healthy donors to restore marrow function (reviewed in Little and Storb 2002).

1.6.1 Graft-versus-Leukaemia (GVL) effect

In the 1950s, it was first proposed that the successful elimination of leukaemia by allogeneic HSCT could be attributed not only to the pre-transplant chemoradiotherapy conditioning regimen, but also to an anti-tumour effect not explained by the conditioning regimen (Barnes et al. 1957). This effect was referred to as the graft-versus-leukaemia (GVL) effect. In human allogeneic transplantation, the introduction of T-cell depletion of the allograft to prevent graft-versus-host disease (GVHD) first implicated donor T cells in the GVL effect (Horowitz et al. 1990). Donor T cells exert their anti-tumour activity by the recognition of mismatched HLA antigens or minor histocompatibility antigens or leukaemia-specific antigens on the recipient's leukaemic cells. However, donor T cells not only attack leukaemic cells but also normal cells in the patient resulting in graft-versus-host disease (GVHD). More recently, alloreactive NK cells have been shown to be able to mediate a GVL effect without GVHD (vide infra).

1.6.2 Graft-versus-Host Disease (GVHD)

Graft-versus-Host disease occurs when donor T and NK cells in the allograft recognise HLA incompatibilities in the recipient and attack the tissues and organs of the recipient (including skin, gastrointestinal tract, liver and lungs) (Martin et al. 1990). GVHD has been classified into two types, acute GVHD, which usually occurs within 100 days of transplantation, and chronic GVHD, which develops after this time (Sullivan et al. 1992). The likelihood of GVHD is reduced by selecting a donor whose HLA type is identical to that of the recipient, however, even with a HLA identical donor, GVHD can still occur, usually mediated by T cells against minor histocompatibility antigens (Goulmy et al. 1996). Extensive T cell depletion of the allograft (Aversa et al. 1998) and the use of pharmaceutical agents (Storb et al. 1986) have reduced the occurrence of GVHD however with a higher risk of relapse (reviewed in Horowitz et al. 1990, Storb and Thomas 1985).

1.6.3 Donor NK Alloreactivity in HSCT

In HLA mismatched HSCT NK cells can also mediate alloreactivity. Donor-vs-recipient NK alloreactivity exists when donor NK cells recognise the absence of donor HLA class I epitopes on recipient cells. In HLA mismatched HSCT, three possible outcomes in terms of NK alloreactivity exist; (1) no NK alloreactivity, as the patient's HLA type includes only epitopes which are present in the donor and vice-versa, (2) NK alloreactivity in the GVH direction, as the patient's HLA type lacks an epitope present in the donor and for which some donor NK cells express the appropriate inhibitory KIR receptor and (3) NK alloreactivity in the HVG direction, where the donor's HLA type lacks an epitope present in the recipient and for which some recipient NK cells possess the appropriate

inhibitory KIR receptor. This model of NK alloreactivity has been called the ligand incompatibility model.

1.6.3.1 Graft-versus-Leukaemia (GVL) effects mediated by NK cells

In a series of studies published in 1999 (Ruggeri et al. 1999, Ruggeri et al. 2002), Ruggeri and colleagues showed that donor-vs-recipient NK cell alloreactivity resulted in less relapse, rejection, GVHD and increased survival in human haploidentical (one haplotype mismatched) transplants and murine transplant models. Most importantly in haploidentical transplants, NK cells were shown to mediate GVL effects without GVHD (Ruggeri et al. 1999).

Ruggeri et al (Ruggeri et al. 1999) tested the hypothesis that alloreactive NK cells could induce a GVL effect by comparing the susceptibility of leukaemia cells, PHA lymphoblasts and BLCLs from the same individuals to lysis by alloreactive NK cells. Leukaemic cells from 4 CML, 4 AML and 5 B-ALL ($CALLA^+$) patients were tested. Alloreactive NK clones efficiently killed all AML/CML targets if they lacked the relevant HLA epitopes but only 2 of the 5 B-ALL targets. Analysis of several adhesion molecules showed that the NK-resistant B-ALL cells had lower surface expression of lymphocyte function antigen-1 (LFA-1), compared with NK-susceptible B-ALL cells and CML and AML cells. These results showed that alloreactive NK cells could kill leukaemic cells providing the leukaemic cells lacked the relevant HLA epitope but also expressed the appropriate accessory molecules needed for NK cell mediated lysis.

In a study of 57 AML and 35 ALL patients who received haematopoietic transplants from HLA haploidentical family donors, Ruggeri et al (Ruggeri et al. 2002) showed that KIR ligand incompatibility in the GVH direction could confer a GVL effect. Donor-recipient pairs were divided into those with and without potential NK alloreactivity through KIR ligand incompatibility in the GVH direction and frequency of relapse assessed in each group. Transplantation from donors with potential NK alloreactivity protected patients from relapse in AML patients but not in ALL patients (Table 1.1). In AML patients, the probability of event-free survival at 5 years was 5% in those without KIR ligand incompatibility compared to 60% in the KIR ligand compatible group ($p < 0.0005$). Multivariate analysis of the data showed that KIR ligand incompatibility in the GVH direction was responsible for the lower relapse rate.

Table 1.1 Clinical and transplant outcome data in HLA haploidentical transplants with and without ligand mismatching in the GVH direction.

KIR ligand incompatibility in GVH direction Number of transplants	No 58	Yes 34
Donors displaying antirecipient NK clones	1/58	34/34*
Disease		
ALL	21	14
AML	37	20
Transplantation outcomes		
Rejection	15.5%	0%*
Acute GVHD, ≥ grade II	13.7%	0%*
Probability of relapse at 5 years		
ALL	90%	85%
AML	75%	0%**

$P \leq 0.01$; $**P < 0.0008$ (22).

reproduced from Ruggeri et al, 2002

The strong GVL effect seen in AML patients with KIR ligand incompatibility in the GVH direction was not present in the ALL patients. These data are consistent with their *in vitro* data suggesting ALL cells are resistant to NK cell mediated lysis due to the lack of LFA-1 expression (Ruggeri et al. 1999).

Since the human clinical study data showed an association between potential NK cell alloreactivity and GVL effect, the mechanism was then investigated in murine models (Ruggeri et al. 2002). Human AML-engrafted non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice infused with human AML cells developed advanced myeloid leukaemia in 5 to 6 weeks. The mice died if left untreated or given human non-alloreactive NK cells. When human NK cells shown to be alloreactive towards the AML cells were given, the leukaemia cells were eradicated and mice survived until the end of the experiment (day 120). The murine models together with the clinical transplant data provided evidence that alloreactive NK cells are able to lyse leukaemic cell targets susceptible to alloreactive NK cell killing (ie AML cells not ALL cells).

1.6.3.2 Alloreactive NK cells prevent Graft-versus-Host Disease

The reduced relapse rate demonstrated in AML patients receiving haploidentical HSCT from donors with potential NK alloreactivity was not accompanied by an increase in GVHD (Table 1.1) (Ruggeri et al. 1999). The authors hypothesised that the unexpected absence of GVHD in the presence of a GVL effect may be due to the fact that haematopoietic cells alone may be susceptible to NK cell killing mediated through HLA class I polymorphism. Non-haematopoietic tissues may lack the ligands which bind to and/or activate NK cells.

As they demonstrated in humans, Ruggeri et al (Ruggeri et al. 2000) in their murine models also showed that NK cell alloreactivity in the GVH direction did not result in GVHD (Ruggeri et al. 2002). Lethally irradiated H-2^b mice transplanted with fully allogeneic H-2^d bone marrow containing 10^6 T cells died

from GVHD in 2-4 weeks. However, when lethally irradiated H-2^b mice were given alloreactive human NK cells with increasing doses of H-2^d T cells, 100% of these mice survived until the end of the experiment (day 120) with no GVHD. The infusion of non-alloreactive NK cells, even in large numbers, provided no protection against GVHD. As recipient antigen-presenting lymphocytes (APCs) have been shown to initiate GVHD by interaction with donor T cells (Shlomchik et al. 1999), Ruggeri et al (Ruggeri et al. 2002) hypothesised that the protection against GVHD might also be mediated by the elimination of recipient APCs by alloreactive NK cells. To test this hypothesis, B6 X BALB/c → B6 bone marrow chimeras were made to replace the alloreactive NK cell-susceptible *H-2^b* mouse haematopoietic cells, including APCs, with *H-$2^{b/d}$* cells that were resistant to NK cell killing. Although the *H-2^d* allele would protect against alloreactive NK cells, the *H-2^b* cells could still present alloantigens to donor *H-2^d* T cells, therefore priming GVHD reactions. When the *H-$2^{b/d}$* chimeras were conditioned with TBI plus alloreactive NK cells and transplanted with *H-2^d* BMT containing 10^6 T cells, 100% of the mice died. Control chimeras (*H-2^b*) given 10^7 T cells survived with no signs of GVHD. This data indicated that alloreactive NK cells prevent GVHD by the elimination of recipient APCs.

1.6.3.3 NK Alloreactivity and Allograft Rejection

Using mouse models, Ruggeri et al, (Ruggeri et al. 2002) showed the successful engraftment of MHC haplotype-mismatched grafts following alloreactive NK cell conditioning. In F1 *H-$2^{b/d}$* to *H-2^b* parent mouse bone marrow transplants, mice conditioned with nonlethal irradiation ($\leq$ 7 grays) alone or with non-alloreactive NK cells, rejected donor marrow grafts. In contrast, all mice conditioned with nonlethal irradiation and alloreactive NK cells (2-4 x 10^5

alloreactive NK cells) engrafted with *H-2*$^{b/d}$ donor type haematopoietic chimerism. These data suggest that alloreactive NK cell infusions have the potential to control graft rejection.

1.6.3.4 Predicting Donor NK Alloreactivity Using HLA and KIR Genotype

Donor NK alloreactivity towards a recipient can largely be predicted by the HLA and KIR genotype of the NK donor and HLA genotype of the recipient. The absence in the recipient of one of the C1, C2 or Bw4 epitopes that is present in the donor results in the potential for donor NK alloreactivity, providing that the donor's KIR gene repertoire includes the appropriate inhibitory KIR gene (ie ligand incompatibility model of NK alloreactivity). However, this assumes firstly that all HLA-C and HLA-B alleles interact with the appropriate inhibitory KIR according to the presence of the relevant amino acids at residue 80 of the HLA molecule and secondly, that all alleles of each of the inhibitory KIR interact with the HLA epitopes in the same way. HLA-C alleles that appear to be exceptions to the definition of a C1 epitope have been reported. Soluble KIR2DL2-Ig and KIR2DL3-Ig fusion proteins have been reported not to interact with all C1 epitopes (Winter et al. 1998). Neither KIR2DL2-Ig or KIR2DL3-Ig fusion proteins bound to HLA-Cw*0801, an allele having the C1 epitope, even though binding of these fusion proteins was detected to other HLA-C alleles with the C1 epitope. Another HLA-C allele, HLA-Cw*1402, may not be a ligand for KIR2DL2/3 despite having the appropriate amino acid at position 80 for a C1-group allele (Colonna et al. 1993b). Colonna et al (Colonna et al. 1993b) generated NK cells expressing only NK-2 (inhibited by the C1 epitope in today's terminology) from homozygous C1 epitope NK donors and these were then

tested in a ^{51}Cr-release assay against homozygous C1 and C2, and heterozygous (C1/C2) target cells. Unexpectedly, of the nine heterozygous (C1/C2) targets tested, one was lysed and two showed borderline lysis. Sequencing of these heterozygous cells for HLA-C revealed that all these samples were HLA-Cw*14 positive and it was suggested that this allele may be incapable of NK inhibition. Therefore according to the genetic model, donors who express either HLA-Cw*0801 or HLA-Cw*1402 would be predicted to show NK alloreactivity towards target cells from recipients lacking the C1 epitope. However, as shown by Colonna et al (Colonna et al. 1993b) and Winter et al (6234} donors expressing HLA-Cw*0801 or HLA-Cw*1402 may not have a functional C1 epitope and therefore may not be able to generate NK clones dependent on KIR2DL2/3.

KIR alleles that are functionally unusual have also been described. KIR3DL1 is present in approximately 90% of individuals (Uhrberg et al. 1997). KIR3DL1 is an inhibitory receptor for the Bw4 epitope determined by amino acids 77-83 on some HLA-B alleles (Gumperz et al. 1995). KIR3DL1 was first identified and characterised using a KIR3DL1-specific monoclonal antibody DX9 (Litwin et al. 1994). This antibody was subsequently used to determine the cell surface expression of KIR3DL1 and individuals were characterised as either having high, low or no expression of DX9. The level of cell surface expression correlated with alleles of KIR3DL1. Four alleles, KIR3DL1*001, *002, *003 and *008, were associated with high levels of DX9 binding while KIR3DL1*005, *006 and *007 were associated with low binding and KIR3DL1*004 with no binding of DX9 (Gardiner et al. 2001). Lack of DX9 binding was subsequently shown to be due to lack of expression of the KIR3DL1*004 protein. The absence of the

KIR3DL1 gene or the lack of expression (KIR3DL1*004) could result in a subset of Bw4^{+} individuals who are unable to generate KIR3DL1 dependent NK clones.

The ligands of activating KIR and the role they play in mediating NK alloreactivity are still unclear. The amino acid sequence of activating KIR (KIR2DS1, KIR2DS2 and KIR3DS1) suggest that they might bind to similar ligands as their inhibitory counterparts (KIR2DL1, KIR2DL2 and KIR3DL1) but it has been difficult to demonstrate this interaction. Moretta and colleagues demonstrated that some NK clones that lacked KIR2DL2/3 and stained with the KIR2DL1/2DS1 antibody (anti-CD158a) lysed HLA-C C2 expressing targets. Activation of these clones by C2- homozygous targets was blocked by the addition of anti-CD158a suggesting that these NK clones mediated alloreactivity via the activating receptor KIR2DS1 (Moretta et al. 1995). The role of activating receptors in mediating NK alloreactivity requires further investigation.

1.7 HYPOTHESES AND AIMS

Hypothesis 1.

The benefits of NK cell mediated alloreactivity demonstrated in the haploidentical setting by Ruggeri et al, can be observed in other allo-transplant protocols.

This hypothesis will be evaluated by analysing clinical outcomes in haploidentical stem cell transplants and HLA-C mismatched unrelated donor transplants performed in the Hadassah Medical Centre, Jerusalem, Israel.

Aims in relation to hypothesis 1.

1.1 To develop an efficient and robust PCR-SSP KIR typing method.

1.2 To correlate clinical outcomes with donor-recipient KIR ligand incompatibility and donor KIR receptor repertoire in different transplant protocols.

<u>Hypothesis 2.</u>

There will exceptions to the simple rules for predicting NK alloreactivity based on the presence or absence of particular amino acids in the class I MHC molecules.

This hypothesis will be tested by systematically evaluating the ability of each of the common HLA-B alleles to inhibit NK cell cytotoxicity in *in vitro* functional assays, and by testing NK cells from a panel of NK cell donors with or without the appropriate ligand for alloreactivity using an *in vitro* functional assay.

Aims in relation to hypothesis 2.

2.1 To develop a functional assay of NK alloreactivity without the need for NK cloning.

2.2 To systematically evaluate the ability of the common HLA-B and $Bw4^+$ HLA-A alleles to interact with KIR3DL1.

<u>Hypothesis 3.</u>

Activating KIR can confer NK cell alloreactivity.

This hypothesis will be tested by examining the ability of NK clones expressing activating KIR to kill allogeneic targets.

Aims in relation to hypothesis 3

3.1 Identify NK clones expressing KIR2DS1.

3.2 Test the ability of KIR2DS1 expressing NK cells to kill allogeneic targets using both KIR2DS1 positive NK clones in a ^{51}Cr-release assay and by using the functional assay for NK alloreactivity developed in Aim 2.1.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS

2.1 DNA SAMPLES

2.1.1 PCR optimisation and standardisation of PCR-SSP reaction for KIR genes.

DNA used in the optimisation and standardisation of KIR PCR-SSP typing protocols was extracted from Ebstein-Barr Virus (EBV) transformed cells of the 10th International Histocompatibility Workshop (IHWS) (Wang et al, 1989). These cell lines are homozygous at the HLA loci but not at the KIR loci. They are predominantly of Caucasian origin. DNA from 20 IHWS cell lines that had been KIR genotyped by at least 2 other laboratories was selected for evaluation of KIR typing methods (Chapter 3). DNA was extracted from the cell lines by staff of the routine laboratory at the Department of Clinical Immunology & Immunogenetics, Royal Perth Hospital using either a commercial kit (Qiagen, Valencia USA) or a salting out method (Miller et al, 1988).

2.1.2 Haematopoietic Stem Cell Transplant samples

2.1.2.1 Haploidentical transplant donor-recipient pairs.

DNA samples from 62 consecutive patients transplanted with haematopoietic stem cells from haploidentical donors were obtained from the Hadassah Medical Centre, Jerusalem, Israel. The transplants were performed between January 1996 and June 2001 at the Department of Bone Marrow Transplantation & Cancer Immunotherapy, Hadassah Medical Centre, Jerusalem, Israel. The patient cohort included 39 males and 23 females with median age 24, ranging from 1-57 years. Twenty-four patients had acute lymphoblastic leukaemia (ALL), 15 had acute myeloid leukaemia (AML), 13 had chronic myeloid leukaemia (CML) and 10 had other diagnoses.

2.1.2.2 Matched unrelated donor (MUD) transplant donor-recipient pairs

DNA samples from 104 patients transplanted with peripheral blood stem cells (n=39) or bone marrow (n=65) were obtained from the Hadassah Medical Centre, Jerusalem, Israel. The transplants were performed between December 1992 and March 2003 at the Department of Bone Marrow Transplantation & Cancer Immunotherapy, Hadassah Medical Centre, Jerusalem, Israel. The patient cohort included 65 males and 42 females with median age 26, ranging from 1-74 years. Sixteen patients had ALL, 3 had AML, 23 had CML, 1 had chronic lymphoblastic leukaemia (CLL), 18 had acute non-lymphostic leukaemia (ANLL) and 46 had other diagnoses.

Each recipient and donor sample from both the haploidentical and unrelated transplant cohorts were typed for HLA-A and -B by serology and by SSO-PCR for HLA-C by the Tissue Typing laboratory at the Hadassah Medical Centre, Jerusalem, Israel. DNA from each pair was provided by the laboratory in Hadassah for KIR typing by SSP-PCR for the detection of KIR2DL1, KIR2DL3, KIR2DL2, KIR3DL1, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DL5, KIR2DS5 and KIR3DS1 using the typing method described below. The typing of the non-expressed variant of 2DS4 (2DS4*003) was performed only for the MUD transplant study.

2.2 SEQUENCE SPECIFIC PRIMING (PCR-SSP) ASSAY FOR KIR GENOTYPING

2.2.1 Oligonucleotide Primers (Geneworks, Adelaide)

Primers used for determining the presence or absence of the KIR genes KIR2DL1, KIR2DL3, KIR2DS1, KIR2DS2, KIR2DS3 and KIR3DS1 were those described by Uhrberg et al (Uhrberg et al. 1997). KIR2DS5 was amplified with primers described by Norman et al (Norman et al. 2001) as the KIRDS5 primers described by Uhrberg et al did not amplify KIR2DS5. KIR3DL1, KIR2DL2 and KIR2DL5 were amplified by primers described by Gomez-Lozano et al (Gomez-Lozano and Vilches 2002) because the primers described by Uhrberg did not amplify all alleles of KIR2DL2, did not distinguish between the transcribed and non-transcribed alleles of KIR2DL5 and the sense primer for KIR3DL1 described by Uhrberg did not amplify a novel polymorphism in KIR3DL1*006. Primer sequences are shown in Appendix II. The KIR2DL1 primers described by Uhrberg amplified all known alleles of KIR2DL1 excluding the KIR2DL1*004 variant. In order to amplify this allele, primers that amplify KIR2DL1*004 as described by Gomez-Lozano et al were used in addition to the Uhrberg KIR2DL1 primers. The presence of a PCR product amplified with the KIR2DL1*004 primers and the absence of a PCR product amplified with the Uhrberg KIR2DL1 primers defined a sample as homozygous for the 2DL1*004 variant. The KIR2DL5 primers described by Gomez-Lozano et al (Gomez-Lozano and Vilches 2002) were used as they detected only the expressed variants 2DL5A*001 and 2DL5B*003. KIR2DS4 and the allele 2DS4*003 that contains a 22bp deletion (it is expressed as a soluble product) were amplified using primers described by Hsu et al (Hsu et al. 2002). Primers that amplified

human growth hormone (HGH) were included in all KIR PCR-SSP reactions as an internal PCR control.

2.2.2 SSP-PCR Optimisation

KIR-SSP PCR typing protocols based on those described by Uhrberg et al (Uhrberg et al. 1997) were already available. However, these protocols needed checking and modification as new primer sets were to be used and internal PCR control primers designed to co-amplify the genetically invariant human growth hormone (HGH) gene were to be included. All SSP-PCR protocols were tested initially on 3 samples; DNA from a cell line known to be positive for a particular KIR gene, DNA from a cell line known to be negative of a particular KIR gene and sterile water (DNA contamination control). Having optimised each PCR reaction using these 3 controls, a cell line panel of 20 cell lines was used to further test the specificity of each reaction. Results for this panel are shown in chapter 3. Final reaction mixes and PCR thermocycling conditions used for each KIR gene are indicated below.

Reaction mixes:

20 μM sense primer	0.5μL per sample
20 μM antisense primer	0.5μL per sample
40 mM dNTP	1μL per sample
10 x PCR Buffer	2.5μL per sample
HGH - sense primer	0.5μL per sample
HGH - anti-sense primer	0.5μL per sample
5 U/μl AmpliTaq DNA Polymerase	0.3μL per sample
Deionised water	6.7μL per sample

12.5μL of reaction mix and 12.5μL of DNA at 20ng/μL were added to each PCR tube.

Reaction mixes for KIR2DL1, KIR2DL2, KIR3DL1 and KIR2DL5 were prepared with a final concentration of 2.0mM $MgCl_2$ while reaction mixes for KIR2DL3, KIR2DS1, KIR2DS2, KIR2DS3, KIR3DS1, KIR2DS5 and KIR2DS4 contained a final concentration of 2.5mM $MgCl_2$.

Amplification was performed on an Applied Biosystems GeneAmp PCR 9700 using the following cycling conditions:

1 cycle:	95ºC x 5min
5 cycles:	97ºC x 20sec
	64ºC x 45sec
	72ºC x 90sec
25 cycles:	95ºC x 20sec
	60ºC x 45sec
	72ºC x 90sec
1 cycle:	72ºC x 10min
HOLD:	4ºC

Annealing temperatures varied according to the KIR genes. KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1, KIR2DS1, KIR2DS2, KIR2DS3 and KIR3DS1 were amplified according to the conditions above. KIR2DS5 and KIR2DS4 were amplified using annealing temperatures at 66ºC (5 cycles) and 62ºC (25 cycles), while KIR2DL5 was amplified at higher annealing temperatures, 67ºC (5 cycles) and 63ºC (25 cycles).

2.3 STATISTICAL ANALYSIS

The relationship between KIR genotype and transplant outcomes was tested using the Fisher's exact or chi-square test. The relationship between KIR genotype and survival was analysed by Kaplan-Meier analysis and the significance tested by Cox proportional hazard using the SPSS statistical package (SPSS, Chicago, Illinois). A p-value threshold for significance was set at $p < 0.05$.

2.4 TARGET CELL LINES FOR NK CELL MEDIATED CYTOTOXICITY

2.4.1 Cell Lines

EBV-transformed B-Lymphoblastoid Cell Lines

EBV-transformed BLCL to be used as targets in the CD107a assay of NK cell cytotoxicity were selected from a panel at the Department of Clinical Immunology and Immunogenetics at Royal Perth Hospital (RPH) according to the HLA types encoding the presence or absence of the C1, C2 and Bw4 epitopes (Table 2.1).

Table 2.1 HLA types and KIR epitopes of BLCL used as target cells used in the CD107a assay.

IDENTIFIER	KIR EPITOPES			HLA-A	HLA-B	HLA-C
	C1	C2	BW4			
“All”	+	+	+	01, 03	27, 44	01, 04
“C1^{-}“	-	+	+	02	57	06
“C2^{-}“	+	-	+	33	58	03
“Bw4^{-}“	+	+	-	03, 11	07, 35	04, 07

Cell lines were maintained in RPMI 1640 supplemented with Penicillin-Streptomycin (100IU/mL), L-glutamine (2mM) and 10% sterile heat inactivated FCS (RPMI/10%HIFCS) and subcultured every 2-3 days to 1 x 10^5 cells/mL. BLCL to be used as targets in the CD107a cytotoxicity assay were subcultured and fed the day before use. On the day of the assay the cells were counted and adjusted to 1 x 10^6/mL ready for use.

721.221 Cell Line

The 721.221 cell line is a class I negative human BLCL (kindly provided by Jim McCluskey, University of Melbourne). 721.221 cells were maintained in RPMI/10%HIFCS and subcultured every 2-3 days at a concentration of 1 x 10^5 cells/mL.

2.4.2 Feeder Cells for In Vitro Expansion of NK cells

2.4.2.1 Preparation of Peripheral Blood Lymphocyte (PBMC) feeder cells

Peripheral blood lymphocytes (PBMC) were isolated from 10 random buffy coat bags (50mLs/bag) or from unprocessed blood from blood donors (with informed consent) obtained from the Australian Red Cross Blood Service. Blood was diluted 1:3 in PBS containing 2% HIFCS. 30mLs of the diluted blood was overlayed onto 15mLs Ficoll-paque™ PLUS (Pharmacia, Uppsala Sweden) and centrifuged at 1200g for 20 minutes with no brake. The cells from the interface were then harvested and washed 3 times with PBS/2%HIFCS at 300g for 7 minutes. The cell pellet was resuspended in 1mL RPMI/10%HIFCS and counted. The cell suspension was stored at concentrations varying from 20-50 x 10^6cells/mL and cryopreserved in 1 mL of freezing medium containing

RPMI/10%HIFCS and 10% DMSO in CryoTubeTM Vials (Nalgene Nunc International, Albertslund Denmark). The vials were stored in liquid nitrogen until use.

2.4.2.2 Preparation of RPMI-8866 feeder cells

The RPMI-8866 lymphoblastoid cell line (American Type Cell Collection, Manassas, USA) was maintained in RPMI/10%HIFCS and split every 2-3 days to 1 x 10^5 cells/mL.

2.4.3 Irradiation of allogeneic PBMC and RPMI-8866 cells

Cells were removed from liquid nitrogen and thawed quickly in a waterbath at 37°C and immediately transferred to RPMI/10%HIFCS. The cells were pelleted by centrifugation at 300g for 7 minutes and resuspended in 1mL RPMI/10%HIFCS. Cells were diluted either 1/2 or 1/5 in trypan blue and counted using a Neubauer haemocytometer and adjusted to 10^6/ml. Both pooled allogeneic PBMC and RPMI-8866 cells were irradiated at a concentration of 10^6/mL. Pooled allogeneic PBMC were irradiated at 30Gy and RPMI-8866 cells at 60Gy using the GammaCell 3000 ELAN (MDS Nordion, Ottawa Canada). After irradiation, the cells were washed twice with RPMI/10%HIFCS at 300g for 7 minutes to remove any free radicals in cell suspension generated by the irradiation process.

2.5 NATURAL KILLER CELL CLONING

2.5.1 Enrichment of Natural Killer Cells

Natural killer cells were purified using Rosette-Sep Human NK cell enrichment cocktail (StemCell Technologies, Vancouver Canada) designed to enrich NK

cells by negative selection. 50ul of the enrichment cocktail was added to each mL of donor blood followed by incubation for 20 minutes with gentle mixing. The enrichment cocktail contains a combination of mouse and rat monoclonal antibodies bound in bi-specific tetrameric antibody complexes in which one arm of the antibody is specific for cell surface antigens on human haematopoietic cells (CD3, CD4, CD19, CD36, CD66b) and the other arm specific for glycophorin A on red blood cells. The antibody cocktail cross-links unwanted cells in human blood to multiple red blood cells, forming immune-rosettes. The unwanted rosetted cells (T and B lymphocytes, monocytes, granulocytes and dendritic cells) along with free red blood cells pass through the ficoll layer during centrifugation. After incubation and centrifugation the NK cells were harvested from the interface of a ficoll gradient as described in section 2.4.2. After the final wash the cells were resuspended in 1mL NK culture medium containing RPMI 1640 medium supplemented with Penicillin (100IU/mL), Streptomycin (100μg/mL), L-glutamine (2mM), 10% HIFCS, 1% non-essential amino acids, 1% pyruvate and 2-mercaptoethanol. Cells were then counted and adjusted to 10^6/mL.

2.5.2 Natural Killer Cell Cloning

Following NK cell enrichment, NK cell preparations containing 40 cells/100µL, 20 cells/100µL, 10 cells/100µL, 5 cells/100µL and 2 cells/100µL were prepared and dispensed into 96 well round bottom plates (BD, Franklin Lakes USA). Irradiated pooled allogeneic PBMC feeder cells or RPMI-8866 cells at a concentration of 1 x 10^5 cells/100µL were then added to each well to a final volume of 200µL/well. On day 2 (24 hours after plating cells), 100μL of supernatant from each well was removed and replaced with 100μL of fresh NK

medium supplemented with 400IU/mL IL-2 (Chiron, Emeryville USA). On day 5, 100μL of supernatant from each well was removed and replaced with 100μL of irradiated feeder cells (8 x 10^5 cells) supplemented with 400IU/mL IL-2. On day 11, 100μL of supernatant from each well was removed and replaced with 100μL of fresh NK medium supplemented with 200IU/mL IL-2. On days 14 to 20, NK clone growth was evaluated either by light microscopy or by eye. Plates with less than 30 positive wells per 96 well plate were likely to contain the progeny of a single cell in positive wells based on the Poisson distribution. Based on the Poisson distribution, when 67% of wells are negative, 27% of positive wells will contain the progeny of a single cell (clonal) and 6% will contain the progeny of two cells (polyclonal) (Remington et al. 1985). Therefore the Poisson estimate of clonality was 26/(27+6) = 81%. Cells in positive wells were subcultured and further expanded in a new 96 well plate. To subculture NK clones, clones were resuspended thoroughly and 100μL of each clone was added to 100μL of fresh NK medium supplemented with 200IU/mL IL-2 and 2 x 10^4 irradiated RPMI-8866 cells in two new wells. NK clones used in functional assays were grown to numbers sufficient (at least 10^6 cells) for evaluating KIR and NKG2A expression by flow cytometry. NK clones were checked for clonality by demonstrating a uniform KIR/NKG2A receptor profile by flow cytometry.

2.5.3 Natural Killer Cell Polyclonal Cultures

Following NK cell enrichment using the RosetteSep method, polyclonal NK cells were diluted to a final concentration of 3 x 10^4cells/mL in fresh NK medium supplemented with 200IU/mL IL-2. 100μL of NK cells were added to each well of a 96 well plate. Irradiated PBMC feeder cells or RPMI-8866 cells at a concentration of 3x10^5/mL were then added to each well to a final volume of

200μL/well. On day 3 and every third day, 100μL of supernatant from each well was removed and replaced with 100μL of fresh NK culture medium containing 200IU/mL IL-2. After 12 days, polyclonal NK cells were counted and frozen at a concentration of 2-3 x 10^6 cells/mL for use in cytotoxicity assays.

2.6 NK CYTOTOXICITY ASSAYS

2.6.1 ^{51}Cr-release cytotoxicity assay

2.6.1.1 Preparation of EBV Target Cells

EBV-transformed BLCL were used as target cells in the ^{51}Cr-release cytotoxicity assay. Target cells were diluted 1 in 3 two days before labelling with ^{51}Cr to ensure cells were growing rapidly at the time of labelling. To label cells with ^{51}Cr, cells were washed in RPMI/5%HIFCS, resuspended in RPMI/10%HIFCS at 5 x 10^5cells/mL and transferred to a 24-well tray (1mL/well). 50μCi per 1 x 10^6 cells/mL of ^{51}Chromium sodium chromate in a sodium chloride solution (2mCi, Amersham, Buckinghamshire UK) was added to each well (50-100μL/mL of cells). The amount of ^{51}Cr added to target cells was increased as the specific activity of the radioisotope decreased in order to maintain a constant level of specific activity of the labelled cells. The cells were then incubated overnight at 37°C 5% CO_2. Approximately 16 hours following the addition of ^{51}Cr cells were harvested into a centrifuge tube and washed with RPMI/5%HIFCS for 7 minutes at 300g at room temperature. The supernatant was then removed and cells were washed twice in 5ml RPMI/5%HIFCS. Following the final wash, cells were resuspended in 1ml of RPMI/10%HIFCS and counted. The target cells were then adjusted to a final concentration of 5 x 10^4 cells/mL.

2.6.1.2 Preparation of Effector NK Cells

NK clones were used as effector cells in the ^{51}Cr-release cytotoxicity assay. Cells were counted and the concentration adjusted to obtain the desired effector:target ratio. A 1:1 ratio was used in most experiments with NK clones.

2.6.1.3 ^{51}Cr-Release Assay

NK clones (effector cells) and BLCL (target cells) were added to a 96 well round bottom plate to a final volume of 200µL. Wells for estimating background chromium release (BLCL targets with medium alone and no effector cells) and maximum release (BLCL targets with 0.5% tritonX-100) controls were also set up. After adding target cells to the effector cells the tray was incubated at 37°C 5% CO_2 for 4 hours. Following incubation, the plates were centrifuged for 3 minutes at 300g with low brake to pellet cells. 100ul of supernatant was then harvested without disturbing the pellet and placed into tubes for counting by the Medical Physics department of Royal Perth Hospital. ^{51}Cr-release was measured in a 1282 Compugamma Universal Gamma Counter (LKB Wallace, Turku Finland) and expressed in counts per minute. The relative specific lysis of targets was calculated as:

$$\text{Relative specific lysis} = \left\{ \frac{\text{(specific release (cpm) - background release (cpm)}}{\text{(maximum release (cpm) - background release)}} \right\} \times 100$$

An NK clone was defined as cytotoxic if it gave > 80% relative specific lysis (RSL) against the 721.221 HLA negative target. The specificity of the NK clone was determined by the following cytotoxic reaction patterns against the panel of

BLCL targets (Table 2.2); positivity was defined as > 20% RSL and negativity was defined as < 5% RSL.

Table 2.2 The specificity of NK clones was determined by lyse of specific target cells.

	Cytotoxicity pattern			
Specificity	**721.221**	**C1+**	**C2+**	**C1+Bw4+**
Anti-C1	+	-	+	-
Anti-C2	+	+	-	+
Anti-Bw4	+	+	+	-

2.6.2 CD107a Flow Cytometric Cytotoxicity Assay

NK cells consist of a large number of preformed cytolytic granules in their cytoplasm. Following activation from signals from both activating and inhibitory receptors on the surface of these cells, NK cells release these granules to the site of cell-cell contact (immunological synapse). These granules contain cytolytic proteins such as perforin and granzyme that are involved in inducing target cell death (Djeu et al. 2002). Lining the membrane of the granules is the lysosomal-associated membrane protein, LAMP-1 or CD107a (Fukuda 1991). CD107a is a glycoprotein representing approximately 50% of the proteins in the lysosomal membrane. During degranulation, CD107a on the luminal membrane is exposed in the immunological synapse and therefore accessible for antibody binding. Betts et al (Betts et al. 2003) showed CD107a expression on the cell surface was a marker of cytotoxic CD8+ T cell degranulation. Alter et al (Alter et al. 2004) showed that CD107a is significantly upregulated on the surface of NK cells following stimulation with target cells lacking HLA ligands. CD107a expression was detected by incubating freshly isolated PBMCs with HLA bare target cells or PMA/ionomycin in the presence of monensin and CD107

antibody. NK cells were stained for CD3 and CD56. Surface expression was low in unstimulated NK cells (0.33%). In the presence of the HLA bare target, CD107a expression on the surface of NK cells was increased, resulting in 17.4% of NK cells expressing CD107a. Following maximal stimulation of NK cells with PMA/ionomycin, CD107a expression on NK cells was 29.17%. The expression of CD107a correlated with both cytokine secretion and NK cell mediated lysis of target cells.

By modifying the method described by Alter et al (Alter et al. 2004) I developed a rapid flow cytometric assay to detect KIR dependent-alloreactive NK cells. Initially the ability to detect alloreactive NK cells was determined by testing NK cells isolated from individuals who expressed the C1, C2 and Bw4 epitopes against targets lacking a single epitope. Day-12 polyclonally expanded NK cells isolated from these NK donors were incubated alone (background control), with the HLA class I negative 721.221 target (positive control), a target expressing all epitopes (negative control) and target cells lacking only C1 ($C1^-$), only C2 ($C2^-$), or only Bw4 ($Bw4^-$). KIR2DL1-dependent alloreactive NK cells were identified as $CD107a^+/CD56^+/CD158b^-/CD158a^+$ following incubation with the 721.221 and $C2^-$ BLCL target. KIR2DL2/3-dependent alloreactive NK cells were identified as $CD107a^+/CD56^+/CD158a^-/CD158b^+$ following incubation with the 721.221 and $C1^-$ BLCL target. KIR3DL1-dependent alloreactive NK cells were identified as $CD107a^+/CD56^+/ CD158a^-/CD158b^-/DX9^+$ following incubation with the 721.221 and $Bw4^-$ BLCL target. The CD107a method I developed for the detection of alloreactive NK cells is described in detail below. The method was then validated by testing a panel of 42 donors representing NK donors who

expressed all epitopes, or only C1 and C2, or only C1 and Bw4, or only C2 and Bw4. The results of this validation are presented in chapter 6 of this thesis.

Cultured polyclonal NK cells were used in the CD107a flow cytometric cytotoxicity assay. Before use in the CD107a assay, 12-day cultured polyclonal NK cells were cultured at 1 x 10^6 cells/mL for a further 48 hours with 400IU/mL IL-2. To each well of a 96 well plate, 10^5 cultured polyclonal NK cells and 10^5 BLCL target cells were added to a total volume of 200µL. To the effector/target mix, 5µL of anti-CD107a-FITC (BD Biosciences, Franklin Lakes USA) antibody was added and incubated at 37ºC 5% CO_2 for 1 hour. After the hour incubation, 20µL of monensin (6ug/mL, BD GolgiStop™ BD Biosciences) was added to the cells at 1/15 dilution and cells were further incubated for 5 hours at 37°C 5% CO_2. Following incubation, cells were washed with flow buffer (PBS/2%HIFCS/0.1% sodium azide) by centrifugation at 200g for 5 minutes and incubated with 1/40 dilution of anti-CD56-PECy7 (BD Biosciences) and 1/20 dilution of the appropriate KIR antibody (Appendix 4) in the dark for 15 minutes at 4°C. Cells were then washed twice in flow buffer by centrifugation at 200g for 5 minutes. Cells were resuspended in 300µL flow buffer and analysed.

Duplicate tubes were analysed on a BD FACSCanto™ flow cytometer using BD FACSDiva software. Events were initially gated on forward and side scatter (SSC) to identify lymphocytes. A bivariate plot of CD56 versus SSC was used to acquire at least 10,000 $CD56^+$ cells. $CD56^+$ cells were further gated to identify cells expressing the KIR phenotype of interest eg. $CD158a^+$ or $CD158a^+/158b^-$. The cells of interest were then analysed in a bivariate plot showing the receptor of interest (eg CD158a) on one axis and CD107a on the

other axis to determine the percentage of cells with the KIR phenotype of interest that were also $CD107a^+$. I found the analysis method to be critical in the detection of NK alloreactivity. To minimise the effect of irrelevant receptors we restricted the analysis to only NK cells expressing the inhibitory KIR receptor relevant to the missing epitope. The details related to the analysis method are described in detail in chapter 6 of this thesis.

2.7 IMMUNOPHENOTYPING OF NK CELLS

NK clones or cells were adjusted to a concentration of at least 1 x 10^4 cells/mL. Cells were harvested from 96 well plates into 5ml round bottom FACS tubes (BD Biosciences) and the volume adjusted to 150μL per antibody using Flow buffer. The cells were then centrifuged for 5 minutes at 200g and supernatant removed. To each flow tube 50μL of the appropriate fluorescently labelled antibody diluted 1/20 in flow buffer was added and incubated at 4°C for 15 minutes. Following incubation, 100μL of flow buffer was added per tube and centrifuged for 5 minutes at 200g. The buffer was then removed and the wash step repeated using 150μL of flow buffer. After final wash, the buffer was removed and 100μL of flow buffer and 100μL of 2% paraformaldehyde were added. Tubes were wrapped in foil to protect cells from the light and stored at 4°C or analysed immediately. If cells were to be analysed immediately a further 100μL of flow buffer was added and the cells analysed using Beckman XL flow cytometer or BD FACSCanto™ flow cytometer using BD FACSDiva software. At least 2000 cells were collected.

CHAPTER 3: A MULTI-LABORATORY EVALUATION OF REFERENCE CELLS FOR KIR TYPING

De Santis D, Witt CS, Gomez-Lozano N, Vilches C, Garcia CA, Marsh SGE, Williams F, Middleton D, Hsu K, Dupont B and Christiansen FT

In 1997, Uhrberg et al published an SSP system for the detection of each KIR gene. The re-optimisation of the existing Uhrberg thermocycling conditions was required to include an internal PCR control reaction (amplification of the growth hormone gene). The optimisation of the new primers to amplify KIR2DS5 (Norman et al), KIR3DL1, KIR2DL2 and KIR2DL5 (Gomez-Lozano et al) was also performed as the primers described by Uhrberg et al did not amplify new alleles or distinguish between expressed and non-expressed alleles. Once the primers were optimised it was important to validate our PCR-SSP typing method against a DNA reference panel. At the commencement of this study a DNA reference panel was not available. However, in May 2002 I attended the 13th International Histocompatibility Workshop - KIR workshop. At this workshop, it was clear that a number of KIR genotyping methods were being used by each of the research groups but no reference material was available to validate the typing methods. It was agreed at this workshop that a DNA reference panel be established. The DNA reference panel would need to be DNA that was accessible to laboratories all over the world, be molecularly typed by as many of the existing KIR genotyping methods and results collated and published for future reference. It was agreed by the international working group that I co-ordinate this study. Each laboratory was asked to type the same 20 BLCL (a subset of the 10th IHWS cell panel) for the presence of 16 KIR genes including the KIR alleles, KIR2DL1*004 and KIR2DS4*003. Five international laboratories participated, four of which used PCR-SSP methods (of which we

were one) and one a PCR-SSOP method. Our participation in this multi-laboratory evaluation was used to validate our SSP-PCR KIR genotyping method. The results of which are presented in Chapter 3 and published in;

Immunobiology of the Human MHC-Proceedings of the 13th International Histocompatibility Workshop and Conference, (Ed: Hansen JA.), vol 1 p1233-1236, International Histocompatibilitiy Working Group Press.

Contributions:

DDS performed the optimisation of the KIR PCR-SSP genotyping method used in the Perth laboratory, genotyped the 20 cell line DNA panel for the multi-laboratory evaluation, coordinated this international collaboration study including selection of the appropriate cell lines, collated all the KIR genotype results from the five laboratories and prepared the manuscript.

CW assisted in the study design and in the preparation of the manuscript.

CV, NGL, CAG, SGEM, FW, FW, KH, BD: Performed KIR genotyping on the 20 cell line DNA panel and submitted results to DDS.

FTC assisted in the study design and in the preparation of the manuscript.

A MULTI-LABORATORY EVALUATION OF REFERENCE CELLS FOR KIR TYPING

De Santis D [1,2], Witt C [1], Gomez-Lozano N [3], Vilches C[3], Garcia CA[4], Marsh SGE[4], Williams F[5], Middleton D[5], Hsu K[6], Dupont B[6] and Christiansen F [1,2]

1. Department of Clinical Immunology and Biochemical Genetics (DCIBG), Royal Perth Hospital (RPH), Perth, Western Australia, Australia
2. Department of Pathology, University of Western Australia, Perth, Western Australia, Australia
3. Servicio de Inmunologia, H.U. Clinica Puerta de Hierro, Madrid, Spain
4. Anthony Nolan Research Institute, Royal Free Hospital, London, United Kingdom
5. Northern Ireland Histocompatibility & Immunogenetics Laboratory, Belfast, Northern Ireland.
6. Memorial Sloan Kettering Cancer Center New York, United States of America.

Correspondence and requests for materials to: Dianne De Santis, DCIBG, RPH, GPO Box X2213, Perth, Western Australia, Australia;

email: dianne.desantis@health.wa.gov.au

frank.christiansen@health.wa.gov.au

INTRODUCTION

Studies on the role of KIR in transplantation (1,2,3) and viral infections such as HIV (4) highlight the need for KIR typing techniques that provide robust and accurate results. Currently there are a number of different typing methods available but little in the way of reference material on which these methods can be assessed. In an effort to provide reference material, a multi-laboratory evaluation of KIR in a subset of the 10th IHWS cell panel was undertaken. The five participating laboratories were those of Dr F Christiansen (CHR), Dr C Vilches (VIL), Dr S Marsh (MAR), Dr D Middleton (MID) and Dr B Dupont (DUP). Each laboratory was asked to type the same 20 B cell lines for the presence of 16 KIR genes including the KIR variants, 2DL1 and 2DS4. Only results for a series of other cell lines were also submitted however only cell lines with data from 3 or more laboratories were included in this analysis (Figure 1). Four of five laboratories used PCR-SSP typing technique (5-8) while the fifth (MID) used PCR-SSOP (9).

RESULTS AND DISCUSSION

The consensus results for the KIR genotypes of the 20 cell lines are given in Figure 2. There was complete concordance between the five laboratories for 16 out of 20 cell lines tested. There were also complete concordance for detection of 2DL1, 2DL2, 2DL3, 2DL4, 3DL2, 2DL5, 2DS4 and 3DS1. However, there were a number of discrepancies for 3DL1, 2DS1, 2DS2, 2DS3, 2DS4-del-22 (the 2DS4 gene containing a 22 bp deletion) and 2DS5. Typing for 2DS2 provided the most inconsistent results between the five laboratories.

The aim of the study was to provide a well-characterised panel for future use by laboratories wanting to develop KIR typing. This aim was achieved for the cells and genes for which all laboratories were in agreement. Some analysis of the discrepant results is given below.

2DS1: The MID laboratory reported the absence of 2DS1 in cell WT100BIS while CHR, MAR and DUP reported the presence of 2DS1. The MID laboratory uses PCR-SSOP in which 3 probes are used to detect 2DS1 and the gene is assigned if all 3 probes are positive as 2 of these also detect a variant of 2DL1. In this instance only the 2 probes were positive, suggesting the presence of 2DL1 variant. However, the other 3 labs did not detect 2DL1v. It seems unlikely that either the PCR-SSP or PCR-SSOP assays consistently give false positive reactions with 2DL1v as many other cells in the panel were positive for 2DL1v and negative for 2DS1 or negative for 2DL1v and positive for 2DS1 (Figure 2) and all labs were concordant for these cells.

2DS2: KIR2DS2 typing provided the most discrepancies within the 20-cell line panel, with discrepant results in 2 out of the 20 cell lines. The results for each of the laboratories that typed these cell-lines for 2DS2 are shown in Table 1. All laboratories with the exception of the PCR-SSOP laboratory use the Uhrberg (5) primers. These results suggest that the assay for 2DS2 is not robust. In our laboratory (CHR), further optimisation of the 2DS2 PCR using the same primers (5) was performed subsequent to this inter-laboratory comparison. The concentration of $MgCl_2$ was increased and samples were retested. The subsequent testing of the above 2 cell lines revealed the presence of 2DS2 in both.

2DS3: The DUP and MID labs reported the presence of 2DS3 for cell line WT100BIS, while the CHR and MAR laboratories typed 2DS3 as absent. In this instance the primers used by each of the laboratories may explain the discrepancy. The CHR and MAR laboratories both use Uhrberg (5) primers to amplify 2DS3 whereas the DUP lab use the same 3' primer (5) but a different 5' primer described by Norman et al (7).

The Uhrberg (5) primers may not amplify a variant of 2DS3 detected by the Norman primers. This possibility could be confirmed that by sequencing the PCR product generated using the 5' Norman primer and the 3' Uhrberg primer.

2DS4-del-22: Four of five labs typed for the 2DS4 variant containing a 22-bp deletion in exon 5, referred here as 2DS4-del-22. The DUP lab reported the absence of this variant but the presence of the intact 2DS4 in the cell line JBUSH whereas CHR and VIL reported the presence of both 2DS4 and 2DS4-del-22. The MID lab can not define 2DS4 in the presence of 2DS4-del-22. Interestingly, both the CHR and DUP laboratories use the same primers to detect 2DS4-del-22 however CHR detected the variant while DUP did not. This suggests that primer sequence is a less likely explanation than a technical problem.

2DS5: The MID lab reported the absence of 2DS5 for cell line PITOUT. The VIL lab reported the presence of a weak amplification band whereas the CHR and MAR laboratories both reported the presence of 2DS5. As the VIL and MAR laboratories use the same primers but obtained discrepant results for 2DS5, the discrepancy cannot be ascribed to differences in primer sequence but rather, must be technical.

3DL1: Laboratories VIL, MAR, CHR and MID reported the absence of 3DL1 in the cell line CB6B. This cell line was initially typed as weakly positive for 3DL1 by the CHR laboratory using the Uhrberg et al primers (5) but CHR found this cell line to be negative in subsequent testing with the Vilches 5' primer (8) and Uhrberg (5) 3' primer. Likewise, VIL and MAR tested HOR negative for KIR3DL1 while the CHR lab obtained a weak positive reaction using the Uhrberg (5) primers. Subsequently the CHR lab found HOR to be negative using the Vilches 5' and Uhrberg 3' primers. Labs CHR, VIL and MID reported the presence of 3DL1 in cell line CF996 while MAR lab reported the absence of 3DL1. To type for 3DL1 the MAR lab used allele specific primers (6) suggesting that the generic primers used by the other labs may amplify a variant not detected by the allelic primers.

Allelic typing for the KIR genes on the 20 cell lines was performed by MAR laboratory and is reported elsewhere in this issue (10). Allelic typing results for 2DL1 and 2DL2 were analysed in this report. However typing results for 2DL2 were submitted by less than three laboratories and a multi-lab evaluation similar to that performed for the generic primers needs to be conducted. 2DL1v was identified in the two cell lines CB6B and WT47 by three laboratories, CHR, MAR and VIL laboratories. The SSOP-PCR typing protocol for 2DL2 used by the MID laboratory included the identification of the 2DL2v. The 2DL2v was only identified in 3 cell lines, LBUF, RML and T7527.

Ninety eight percent of the typings in this inter-laboratory comparison were concordant. However, discrepancies were seen in 5 of 16 genes suggesting that the current KIR typing methods are insufficiently standardised and robust. The discrepancies between different laboratories using the same primers suggest that these methods are not completely robust. As with all SSP assays the inclusion of positive control primers in each PCR tube lessens the chance of misinterpretation of false negative results. Only 2 out of 5 laboratories (CHR and VIL) used internal control primers in their assays. In our experience the use of high quality DNA is essential to reduce false negative reactions. Even with the inclusion of positive control primers, we have observed that positive reactions were always weak for some samples and a negative reaction with KIR primers in the presence of a weak positive reaction with the positive control primers is difficult to interpret. The discrepancies seen here in 20 cell lines also raises the possibility that the high number of different KIR genotypes observed may result partly from inaccurate typings.

Figure 1. Shaded boxes indicate KIR typing data received by each laboratory. Data was included in the analysis if received by 3 or more laboratories. A total of 20 cell lines were analysed (1-20).

SAMPLE NO.	CELL NAME	CHR (SSP-PCR)	VIL (SSP-PCR)	MAR (SSP-PCR)	DUP (SSP-PCR)	MID (SSOP-PCR)
1	JBUSH					
2	TAB089					
3	BTB					
4	KAS116					
5	SAVC					
6	PLH					
7	E4181324					
8	PE117					
9	BOLETH					
10	OLGA					
11	EJ32B					
12	HOR					
13	PITOUT					
14	LBUF					
15	WT100BIS					
16	RML					
17	CF996					
18	T7527					
19	CB6B					
20	WT47					

Cell Name	10IHWS No.	2DL1	[a]2DL1v	2DL2	[b]2DL2v	2DL3	2DL4	3DL1	3DL2	2DS1	[c]2DL5	2DS2	2DS3	2DS4	2DS4-del-22	2DS5	3DS1
JBUSH	9035														k		
TAB089	9066																
BTB	9067																
KAS116	9003																
SAVC	9034																
PLH	9047																
E4181324	9011																
PE117	9028																
BOLETH	9031																
OLGA	9071																
EJ32B	9085																
HOR	9053																
PITOUT	9051											f				j	
LBUF	9048																
WT100BIS	9006									e		g	h				
RML	9016																
CF996	9104							d									
T7527	9077																
CB6B	9059																
WT47	9063																

Figure 2. Consensus KIR results for 20 cell lines with results from 3 or more laboratories. Dark shaded boxes represent the presence of KIR gene, white boxes represent the absence of KIR gene and the lighter shaded boxes represent discrepancies between the five laboratories.

[a] The 2DL1v was typed by less than 3 laboratories. [b] The 2DL2v was typed by only 1 laboratory (MID). [c] 2DL5 typing does not discriminate between the variants. [d] CHR, VIL and MID reported presence of 3DL1, MAR reported the absence of 3DL1. [e] MID lab reported the absence of 2DS1 all others reported the presence of 2DS1. [f, g] Refer to figure 3. [h] The DUP and MID labs reported the presence of 2DS3 while the CHR and MAR reported the absence of 2DS3. [k] DUP lab reported the absence of 2DS4-del-22 while CHR, VIL and MID reported the presence of 2DS4-del-22. [j] MID reported the absence of 2DS5, VIL reported weak amplification while CHR and MAR reported the presence of 2DS5.

	CHR	DUP	MAR	MID	VIL
PITOUT	-	NT	-	+	-
WT100BIS	-	+	-	+	NT

Table 1. KIR2DS2 results provided by each of the laboratories for 2 cell lines. + indicates the presence of 2DS2, - indicates the absence of 2DS2 and NT indicates this cell line was not tested.

REFERENCES

1. De Santis D, Witt C, Nagler A, Brautbar C, Christiansen F, Bishara A. NK epitope matching and KIR genotyping in one haplotype matched haematopoietic transplants. In HLA 2002
2. Ruggeri L, Capanni M, Urbani E et al. Effectiveness of donor natural killer cell alloreactivity in mismatched haematopoietic transplants. Science 2002 Mar 15;295(5562):2097-2100.
3. Velardi A, Ruggeri L, Moretta A et al. NK cells: a lesson from mismatched haematopoietic transplantation. Trends Immunol 2002 Sep; 23(9): 438.
4. Martin M, Xiaojiang Gao, Jeong-Hee Lee et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet. 2002 Aug;31(4):429-34.
5. Uhrberg M, Valiante NM, Shum BP et al. Human diversity in killer cell inhibitory receptor genes. Immunity 1997 Dec;7(6):753-63
6. Gardiner CM, Lisbeth AG, Shilling HG, et al. Different NK cell surface phenotypes defined by the DX9 antibody are due to KIR3DL1 gene polymorphism. J Immunol. 2001 Mar 1;166(5):2992-3001.
7. Norman PJ, Stephens HA, Verity DH, Chandanayingyong D, Vaughan RW. Distribution of natural killer cell immunoglobulin-like receptor sequences in three ethnic groups. Immunogenetics 2001;52(3-4):195-205
8. Gomez-Lozano N, Vilches C. Genotyping of human killer-cell immunoglobulin-like receptor genes by polymerase chain reaction with sequence-specific primers: An update. Tissue Antigens 2002: 56: 184-193.
9. Crum KA, Logue SE, Curran MD, Middleton D. Development of a PCR-SSOP approach capable of defining the natural killer cell inhibitory (KIR) gene sequence repertoires. Tissue Antigens 2000: 56:313-326.

10. Garcia CA, Robinson J, Schilling HG, Guethlein LA, Parham P, Madrigal JA, Marsh SGE. KIR gene characterisation of HLA homozygous cell line. In HLA 2002.

ACKNOWLEDGMENTS

We would like to thank all the laboratories that participated in this evaluation. The work performed in the Carlos Vilches laboratory was supported by grant FIS 01/0381.

CHAPTER 4: THE BENEFICIAL ROLE OF INHIBITORY KIR GENES OF HLA CLASS I NK EPITOPES IN HAPLOIDENTICALLY MISMATCHED STEM CELL ALLOGRAFTS MAY BE MASKED BY RESIDUAL DONOR-ALLOREACTIVE T CELLS CAUSING GVHD.

Bishara A, De Santis D, Witt CS, Brautbar C, Christiansen FT, Or R, Nagler A, Slavin S

Once the KIR genotyping PCR-SSP method was optimised and validated against the 20 cell line panel in the multi-laboratory evaluation (Chapter 3), we used this PCR system in transplant and disease studies. My supervisors established a collaboration with the transplant group at the Tissue Typing laboratory at the Hadassah University, Jerusalem, Israel to KIR genotype donor and recipients from haploidentical transplants performed in their transplant centre. DNA samples, HLA typing and clinical data was provided by the Jerusalem transplant group on a cohort of 62 one haplotype-matched stem cell transplants in order to evaluate the impact of NK cell alloreactivity on transplant outcome. Recent reports at the time by Ruggeri et al (Ruggeri et al. 1999,Ruggeri et al. 2002) from Perugia Italy demonstrated the beneficial effects of NK alloreactivity in the GVH direction. The human transplant data published by this group and confirmed in murine models demonstrated the potential for NK alloreactive cells to reduce relapse and rejection in the haploidentical transplant setting. These results provided an exciting new approach to haploidentical transplants. However these findings appeared to be in contrast to previously published data in matched unrelated donor settings where HLA-C mismatching was demonstrated to be associated with increased GVHD, and poorer survival (Bishara et al, 1995 and Nagler et al 2001). Davies et al had also demonstrated that mismatching for HLA class I NK epitopes in the GVH

direction increased the likelihood of GVHD (Davies et al. 2002). Data suggesting that the number of KIR genes in the donor and patient in the unrelated setting also influenced the probability of GVHD had also been published by Gagne et al (Gagne et al. 2002). The data generated from the collaborative study with the Israeli haploidentical transplants examined whether the published findings by the Perugia group were generally applicable.

The findings were published in;

Tissue Antigens. 2004 Mar;63(3):204-11

Contributions:

AB performed the HLA typing of donor-recipient pairs and prepared the manuscript.

DDS performed the KIR PCR-SSP genotyping of donor-recipient pairs, collated HLA and KIR genotype data, performed statistical analysis and assisted with the preparation of manuscript.

CSW assisted in the study design, performed statistical analysis and assisted in the preparation of the manuscript.

FTC, CB, RO, AN and SS assisted in the study design and in the preparation of the manuscript.

A. Bishara
D. De Santis
C.C. Witt
C. Brautbar
F.T. Christiansen
R. Or
A. Nagler
S. Slavin

The beneficial role of inhibitory *KIR* genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD

Key words:
activating receptors; haploidentical; hematopoietic stem cell transplantation; inhibitory receptors; natural killer cells

Abstract: HLA allele mismatches will provoke T-cell alloreactivity after allogeneic stem cell transplantation. As donors and recipients are usually HLA matched, the public HLA epitopes that are recognized by natural killer (NK) cells (NK epitopes) are rarely mismatched, and therefore there is rarely potential for NK alloreactivity arising from the absence of ligands for inhibitory killer immunoglobulin-like receptors (*KIR*). Transplants using related donors sharing only one haplotype (haploidentical donors) represent a setting in which NK epitopes are often mismatched, thus resulting in the potential for NK alloreactivity. We have analyzed engraftment, acute graft *vs* host disease (GVHD), leukemia relapse, and survival in 62 haploidentical transplants in relationship with potential NK alloreactivity, inhibitory, and activating *KIR* genes of class I HLA NK epitopes. Potential NK alloreactivity in the rejection direction was not associated with any outcome variable. Potential NK alloreactivity in the GVHD direction was associated with an increased incidence of severe GVHD and poorer patient survival but not with non-engraftment nor leukemia relapse. A higher number of activating *KIR* receptors in the genome of the donor was associated with a higher prevalence of GVHD. These results suggest that lack of extensive T-cell depletion in haploidentical transplantation is associated with high GVHD rates and diminishes the benefits of NK-cell alloreactivity.

Authors' affiliation:

A. Bishara,[1]
D. De Santis,[2]
C.C. Witt,[3]
C. Brautbar,[1]
F.T. Christiansen,[2,3]
R. Or,[4]
A. Nagler,[4]
S. Slavin[4]

[1]Tissue Typing Unit, Hadassah University, Jerusalem, Israel

[2]School of Surgery and Pathology, University of West Australia, Perth, Western Australia, Australia

[3]Department Clinical Immunology and Biochemical Genetics, Royal Perth Hospital, Perth, Western Australia, Australia

[4]Department of Bone Marrow Transplantation & Cancer Immunotherapy, Hadassah University, Jerusalem, Israel

Correspondence to:
Amal Bishara, PhD
Tissue Typing Unit
Hadassah Medical Center
Jerusalem, Israel
Tel.: 972-2-6777513
Fax: 972-2-6433165
e-mail: amal@hadassah.org.il

Hematopoietic stem cell transplantation (HSCT) is the treatment of choice for a variety of hematological malignancies (1). Allogeneic transplants are more efficacious than autotransplants due to the graft *vs* leukemia effect (GVL), which results in a lower relapse rate (2). HLA-matched sibling donors are still regarded as ideal allogeneic donors because they offer an acceptable compromise between an effective GVL effect and modest graft *vs* host disease (GVHD), which often accompanies the GVL effect (3). In the absence of an HLA-matched sibling, HLA-matched unrelated donors (MUD) are often used as the second best choice (4). However, as European Caucasians represent the majority of donors on bone marrow registries, it can be difficult to find a well-matched donor in countries where the HLA antigen and haplotype frequencies differ from those

Received 27 June 2003, revised 29 September 2003, accepted for publication 6 October 2003

Copyright © Blackwell Munksgaard 2004
Tissue Antigens.

Tissue Antigens 2004: **63:** 204–211
Printed in Denmark. All rights reserved

on the registries (5). In order to enable HSCT across the HLA barrier, protocols have been developed for the use of HSCT from one haplotype-matched (haploidentical) family members. Generally, such transplants require large doses of $CD34^+$ stem cells for successful engraftment and effective T-cell depletion or positive selection of stem cells in order to eliminate or minimize severe GVHD (6). There are no histocompatibility criteria for selecting the optimal haploidentical donor, as it is difficult to predict which mismatches will elicit the strongest resistance to engraftment by residual immunocompetent lymphocytes of host origin or GVHD by immunocompetent lymphocytes inoculated with donor stem cells.

Recently, Ruggeri et al. (7, 8) demonstrated the potential for alloreactive natural killer (NK) cells to reduce relapse and allograft rejection. This has renewed interest in reinvestigating their potential roles in GVL and GVHD. In a murine model, it has been known for many years that recipient NK cells mediate hybrid resistance and rejection of SCT if the donor stem cells do not include the recipients' class I major histocompatibility complex (MHC) alleles (missing self hypothesis) (9). However, direct evidence that this occurs in human transplants is still lacking. Furthermore, it is still controversial whether NK cells can provide GVL effects or participate in GVHD (10). NK activity is controlled by several NK-cell receptors including killer immunoglobulin-like receptors (KIR) which include both inhibitory and activating receptors that interact with HLA class I antigens (11, 12). The class I HLA epitopes involved in NK-cell allorecognition are now known to include the Bw4 epitope, which is present on approximately 40% of HLA-B alleles (13) and the allelic C1 and C2 epitopes which have approximately equal frequencies and one or other of which is present on all HLA-C alleles (14). There is evidence for NK-recognized epitopes on some HLA-A alleles, but there is no universal agreement as to which alleles carry the epitope (15).

There would seem to be little potential role for NK alloreactivity in HLA-matched sibling transplants where the donor's HLA type always includes the recipient's class I HLA alleles and vice versa. Historically, transplants from MUD have generally been sufficiently well matched at HLA-B and HLA-A to avoid NK alloreactions, but many HLA-C-mismatched transplants have been performed due to an inability to type accurately at the HLA-C locus. We have shown that mismatching for alleles of HLA-C is associated with *in vitro* NK-mediated lysis, increased GVHD, and poorer survival in the MUD (16) and haploidentical (17) settings. Recent data from Minnesota also suggests that mismatching for class I HLA NK epitopes in the GVHD direction increases the likelihood of GVHD (18). Gagne et al. (19) reported increased GVHD incidence in unrelated settings when recipient *KIR* genes are included in those of the donor. One haplotype-matched transplants would appear to provide the greatest potential for NK epitope mismatching and might therefore be expected to reveal the role of alloreactive NK cells, particularly as the donor stem cells are usually depleted of T cells that are the main effector cells of GVHD (20).

In the present study, we have analyzed the *KIR* genotypes and their ligands (HLA-C and Bw epitopes) in our cohort of 62 recipients of one haplotype-matched stem cell transplants in an attempt to evaluate their impact on the outcome.

Patients and methods

Patients

A total of 62 consecutive leukemia patients (39 males, 23 females, median age 24, range from 1 to 57 years) were included in this study: 24 with acute lymphoblastic leukemia (ALL), of whom six were in first, five in second, two in third and one in fourth relapse, three and two in complete remission 2 and 3, respectively, and three in resistant and two in refractory disease; 15 with acute myeloid leukemia (AML), six of whom were in relapse, six in resistance stage, and three in refractory stage; 13 with chronic myeloid leukemia (CML), of whom seven were in blast crises, three in chronic, and three in accelerated phase; and 10 with other diagnoses. All of these patients underwent HSCT from haploidentical related donors between January 1996 and June 2001 at the Department of Bone Marrow Transplantation & Cancer Immunotherapy, Hadassah Medical Center, Jerusalem, Israel. Thirty-one recipients were transplanted from sex-matched and 31 from sex-mismatched donors. Fourteen female recipients were transplanted from male donors and 17 male recipients from female donors.

HLA typing

Patients and family members were typed for HLA class I by serology (21) and for HLA-DRB1 and HLA-DQB1 by molecular techniques (sequence-specific oligonucleotide primers) (22). All donor/recipient pairs were typed for HLA-C using sequence-specific primer (SSP) kits (Pel-freeze, Deerbrook Trail, WI). All donor/recipient pairs were mismatched for a full haplotype (haploidentical) in the HLA system.

Conditioning and transplantation protocols

All recipients were conditioned with intravenous fludarabine 30 mg/m^2 for 6 days and oral busulfan 4 mg/kg for 2 days, intravenous Fresenius anti-thymocyte globulin (ATG) 10 mg/kg for 4 days, and total body irradiation 750 cGY on day −1 with 50% dose reduction over the lung fields. All transplants were performed using granulocyte colony-stimulating factor-mobilized peripheral blood stem cells

(23). T-cell depletion for the prevention of GVHD was achieved by positive selection of $CD34^+$ cells using Dynal immunomagnetic beads (24) (Isolex, Baxter healthcare, Deerfield, IL) or Milenyi's CliniMACS (Miltenyi Biotec GmbH, Bergisch Gladback, Germany) (25). Median mononuclear cell count, $CD34^+$, and $CD3^+$ cell doses were 11.5 (range 1.5–60.2) $\times 10^8$/kg, 8.6 (range 1.9–31.5) $\times 10^6$/kg, and 10.8 (range 0.9–50) $\times 10^4$/kg, respectively. No cyclosporine A or hematopoietic growth factors were administrated prior to or following SCT.

Assessment of engraftment, rejection, and GVHD

Engraftment was defined as an absolute neutrophil count $>0.5 \times 10^9$/l, a white blood cell count $>1 \times 10^9$/l for three consecutive days, and unsupported platelet count $>20 \times 10^9$/l for three consecutive days, combined with molecular methods VNTR-PCR (26). Rejection was defined as loss of the allograft by polymerase chain reaction (PCR), using the ameloginin gene in male to female transplants, or by cytogenetic analysis, or by VNTR-PCR in sex-matched transplants (26). Acute GVHD was defined according to the Seattle criteria (27). Acute GVHD status could be determined in 40 patients.

KIR PCR-SSP typing

Polymerase chain reaction (PCR)-specific priming (SSP) assays were used to detect the presence or absence of 12 *KIR* genes. PCR-SSP for *2DL1*, *2DL1v*, *2DL2*, *2DL3*, *2DL5*, *3DL1*, *2DS1*, *2DS2*, *2DS3*, *2DS4*, *2DS5*, and *3DS1* genes were amplified separately in a single reaction using primers and conditions published by Uhrberg et al. (28), with some modifications to *2DS5*. Primers for the amplification of the transcribed 2DL5 variants and the 2DL1v were based on those described by Gomez-Lozano et al. (29). Each reaction contained 0.4 μM specific primer, 0.4 mM dNTPs (Invitrogen, Carlsbad, CA), 2.5 μl PCR buffer (2 mM $MgCl_2$ or 2.5 mM $MgCl_2$), and 0.5 U Ampli-Taq polymerase (Perkin-Elmer, Boston, MA). All reactions contain internal positive control primers specific for human growth hormone (forward and reverse primers at 0.15 μM). PCR amplification was performed in a 9700 thermal cycler (Perkin-Elmer) under the same conditions as described by Uhrberg et al. (28) except that annealing temperatures were increased to improve specificity. PCR products were analyzed on an ethidium-prestained 1% agarose gel.

Data analysis

The relationship between epitope matching and GVHD, engraftment, rejection, and relapse were tested using Fisher's exact or Chi-square tests (30, 31). The relationship between mismatching and survival was determined by Kaplan–Meier analysis and the significance tested by Cox proportional hazard (32). Analysis of engraftment was performed on 56 recipients that survived more than 28 days. Analysis of GVHD was performed on all engrafted recipients who survived more than 28 days ($n = 37$) and on those who engrafted and developed GVHD even if they survived less than 28 days ($n = 3$). Analysis of relapse was performed on all recipients who survived more than 28 days ($n = 54$). Analysis of survival was performed on all 62 recipients.

Results

Classification of donor–recipient pairs in terms of potential NK alloreactivity

Based on HLA-Cw typing, the recipients and donors were classified as having HLA-C alleles belonging to group 1 (alleles with Asn^{77}-Lys^{80}) and/or group 2 (alleles with Ser^{77}-Asn^{80}). HLA-B alleles were classified as having the Bw4 epitope or not. As there is no universal agreement as to which HLA-A alleles interact with the *KIR*3DL2 receptor, HLA-A alleles were not considered. In examining the role of NK alloreactivity, three kinds of compatibility were defined: (i) recipient/donor pairs having potential for NK alloreactivity in the GVHD direction as the recipient's HLA type did not include one of the donor's C1, C2, or Bw4 epitopes ($n = 14$) and recipient/donor pairs having no potential for NK alloreactivity in GVHD direction ($n = 42$); (ii) recipient/donor pairs having potential for NK alloreactivity in the rejection or host *vs* graft (HVG) direction as the donor's HLA type did not include a C1, C2, or Bw4 epitope which was present in the recipient ($n = 18$) and recipient/donor pairs having no potential for NK alloreactivity in the rejection direction ($n = 38$); (iii) recipient/donor pairs having potential for alloreactivity in either direction as donor and recipient did not share the same C1, C2, or Bw4 epitopes ($n = 36$) and recipient/donor pairs having no potential for alloreactivity in either direction as donor and recipient shared the same C1, C2 or Bw4 epitopes ($n = 26$). Two recipient/donor pairs had potential for NK alloreactivity in both the directions. These two pairs were included as having potential alloreactivity in the GVH direction, when correlation between GVH direction and NK alloreactivity was analyzed, and included as having potential alloreactivity in the HVG direction, when correlation between NK alloreactivity and rejection was analyzed.

Engraftment and rejection

As it was difficult to distinguish between primary non-engraftment and early graft rejection, it was decided to pool the data obtained

from patients with no durable engraftment together. Analysis of potential NK alloreactivity in either direction, in a GVH direction alone and in the HVG direction alone, did not show any correlation between engraftment rates and NK alloreactivity (Table 1). There were no significant differences in the distribution of *KIR*-inhibitory and -activating receptor genes between those who engrafted and those who did not (data not shown).

Relapse

Relapse rates were not correlated with potential NK alloreactivity in either direction, in the GVHD direction alone and in the HVG direction alone, although as summarized in Table 1, there was a trend toward less relapse in pairs with an NK epitope mismatch in either direction ($P = 0.09$). In view of the previous report by Ruggeri et al. (7) indicating NK sensitivity of AML and CML cells but not ALL cells, relapse rates were analyzed separately in the 26 recipients with AML and CML. Ten of 19 (53%) AML/CML recipients with no potential NK alloreactivity in the GVHD direction relapsed as compared with four of seven (57%) recipients with potential NK alloreactivity in the direction of GVHD. Interestingly, all the three AML patients who had potential alloreactivity in the GVHD direction relapsed. There was no significant correlation between the number of inhibitory and activating receptors and relapse.

Graft *vs* host disease

There was no significant relationship between the incidence of GVHD and potential NK alloreactivity in either GVH or HVG direction (data not shown). However, potential for NK alloreactivity in the GVH direction was associated with an increased prevalence of moderate-to-severe GVHD (Fig. 1). Of the seven pairs with potential NK alloreactivity in the GVHD direction, five (71%) developed grades III or IV GVHD compared to only seven of 33 (21%) in the group with no potential NK alloreactivity ($P < 0.02$). Four of the seven (57%) patients with potential NK alloreactivity developed grade IV GVHD as compared with four of 33 (12%) among patients with no potential NK alloreactivity ($P < 0.005$). As the ability of NK cells to detect class I HLA epitopes might be related to the number of *KIR* receptors present in the donor, we examined the relationship between the number of inhibitory or activating *KIR* in the donor and the prevalence of GVHD. There was very little variation in the number of inhibitory receptors present in the donors, and there were no significant differences in the number of inhibitory *KIR* genes between recipients who developed and those who did not develop GVHD (data not shown). However, as shown in Fig. 2, GVHD was more prevalent when donors had more than four activating receptors. Nine of 10 (90%) patients whose donor had more than four activating receptors developed grade II–IV GVHD compared to only seven of 21 (33%) of recipients whose donors had less than four activating receptors ($P < 0.005$) (Fig. 2). The analyses of the number of activating or inhibitory receptors were repeated taking into account the ligands present in the recipients, but the number of subjects in each category was too small for a meaningful analysis.

Survival

Ten recipients survived from 24 to 64 months. There was a trend toward inferior survival of patients with potential NK alloreactivity in either GVH or HVG direction ($P = 0.05$) (Fig. 3A). Potential for NK alloreactivity in the GVH direction appeared to be more important ($P < 0.02$) than the HVG direction (Fig. 3B,C). The age of the survivors ranged from 1 to 43 years (mean of 20 years), which is not different from the mean age of the cohort of the recipients and of the non-survivors. The diagnoses of the survivors were also similar to those of the study group as three had CML (one in blast crisis and one in chronic phase), two AML (two in relapse), two ALL (one in first and one in second relapse), and three with other diagnoses. There was no significant correlation between the number of inhibitory and activating receptors and survival.

Analysis of engraftment and relapse in relation to potential natural killer (NK) alloreactivity

	Potential for NK alloreactivity					
	GVH direction		HVG direction		Any direction	
	Yes	No	Yes	No	Yes	No
Engraftment*	9/14 (64%)	33/42 (79%)	14/18 (78%)	28/38 (74%)	21/30 (70%)	21/26 (81%)
Relapse†	5/12† (42%)	21/42 (50%)	9/18 (50%)	17/38 (45%)	10/28* (36%)‡	13/26 (50%)‡

*Engraftment was analyzed in 56 recipients who survived more than 28 days.
†Two recipients engrafted and died before 28 days; therefore, they were not included in the relapse analysis.
‡$P = 0.09$.
HVG, host *vs* graft; GVH, graft *vs* host.

Table 1

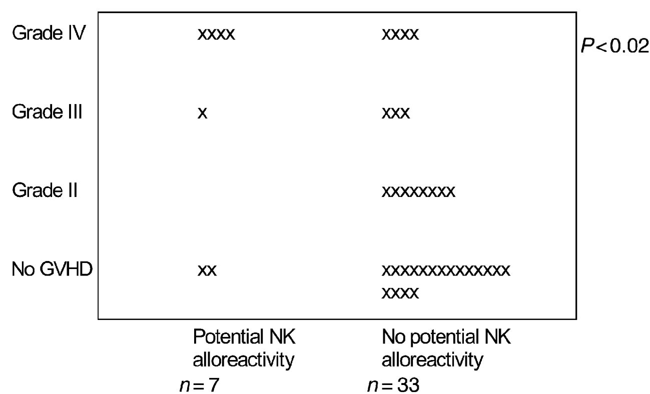


Fig. 1. Potential natural killer (NK) alloreactivity in the graft *vs* host (GVH) direction was associated with grades III–IV GVHD. Each X represents a patient.

Discussion

This study focused on the role of compatibility for class I HLA NK epitopes and donor/recipient *KIR* genotypes following single haplotype-mismatched SCT. Our data suggest that compatibility for class I HLA NK epitopes may have an important effect on outcome following such transplants. Mismatching of class I HLA alleles giving rise to potential NK alloreactivity in the GVHD direction resulted in significantly poorer survival that appears to be due, at least in part, to a higher incidence of GVHD. It was not possible to analyze the individual influence of HLA-C epitope and Bw4 mismatches, as there were only three pairs with only a Bw4 mismatch in the GVHD direction and four pairs with only Bw4 mismatch in the rejection direction. It should also be mentioned that many other analyses that did not show significant correlations in this manuscript involved relatively small numbers of transplants and they may not have been enough power to detect a correlation.

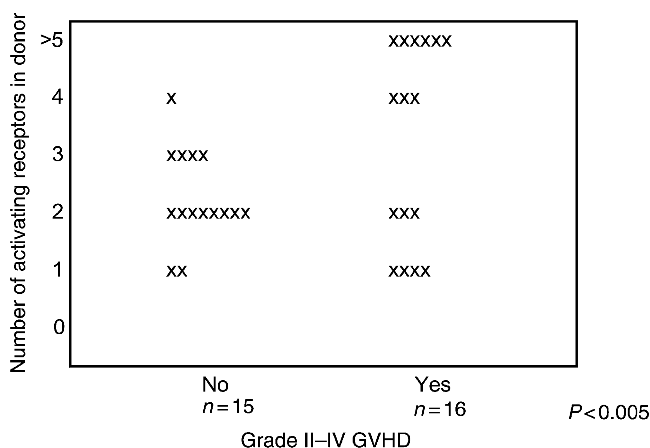


Fig. 2. A higher number of activating killer immunoglobulin-like receptors (*KIR*) in donors is associated with grade II–IV graft *vs* host disease (GVHD). Each X represents a patient.

The findings in the current study are at variance with the recent report from Ruggeri et al. (7, 8). We found that potential NK alloreactivity in the GVHD direction was associated with a higher prevalence of GVHD, whereas Ruggeri et al. reported a 0% prevalence of more severe GVHD in such SCT recipients. In a recent study of matched unrelated transplants, Davies et al. (18) reported findings similar to the current study. It is possible that these contradictory findings may be explained by differences in the transplant protocols. The haploidentical transplants described by Ruggeri et al. were specifically and extensively T-cell depleted, whereas a minority of the MUD transplants analyzed by Davies et al. were T depleted. The much higher frequency of GVHD in our study compared to the Italian series suggests that significant differences must exist between the protocols used for GVHD prevention. Although the conditioning was somewhat different between the two transplant centers, the dose of CD34 cells was similar in the two series. However, there were differences in the proportion of contaminating T lymphocytes inoculated. Indeed, composition of the grafts in the Italian study and our own, both positively selected for $CD34^+$ cells were not identical. In the Italian study, T-cell depletion was much more vigorous, with the stem cell inoculum containing an average of 3×10^4 T cells/kg, as compared with an average of 10.8×10^4 residual T cells/kg in Jerusalem. This difference can certainly explain a higher incidence of GVHD in Jerusalem, and therefore, the role of NK alloreactivity may be less clear in the presence of GVHD caused by residual alloreactive T cells. The number of NK cells present in the two studies is not known, but it is likely that in addition to the lower total T-cell number in the Italian study, different stem cell-enrichment protocols which included specific T-cell depletion in Italy could produce a higher NK : T-cell ratio compared to that obtained by positive selection of $CD34^+$ cells alone in Jerusalem.

Differences in NK : T-cell ratios may be crucial in determining the importance of NK allorecognition in HSCT. Investigations of the roles of T cells and NK cells in murine models of GVHD have revealed that donor NK cells can ameliorate GVHD if introduced early in the transplant or exacerbate GVHD if introduced after the transplantation (33). On the other hand, enhancing the number and function of NK cells by administration of linomide to recipients of donors of mismatched bone marrow allografts across MHC did not affect the incidence of GVHD or the outcome of the recipients following transplantation (34). Recent murine data of Ruggeri et al. (8) indicate that T cells initiate GVHD by interaction with recipient antigen-presenting cells (APCs), but alloreactive NK cells introduced prior to the transplant can prevent GVHD induction most likely by eliminating recipient APCs. In human transplants, T cells and NK cells are introduced simultaneously with the stem cells, and hence timing is not a variable. Hence, it can be envisaged that a high NK : T-cell ratio

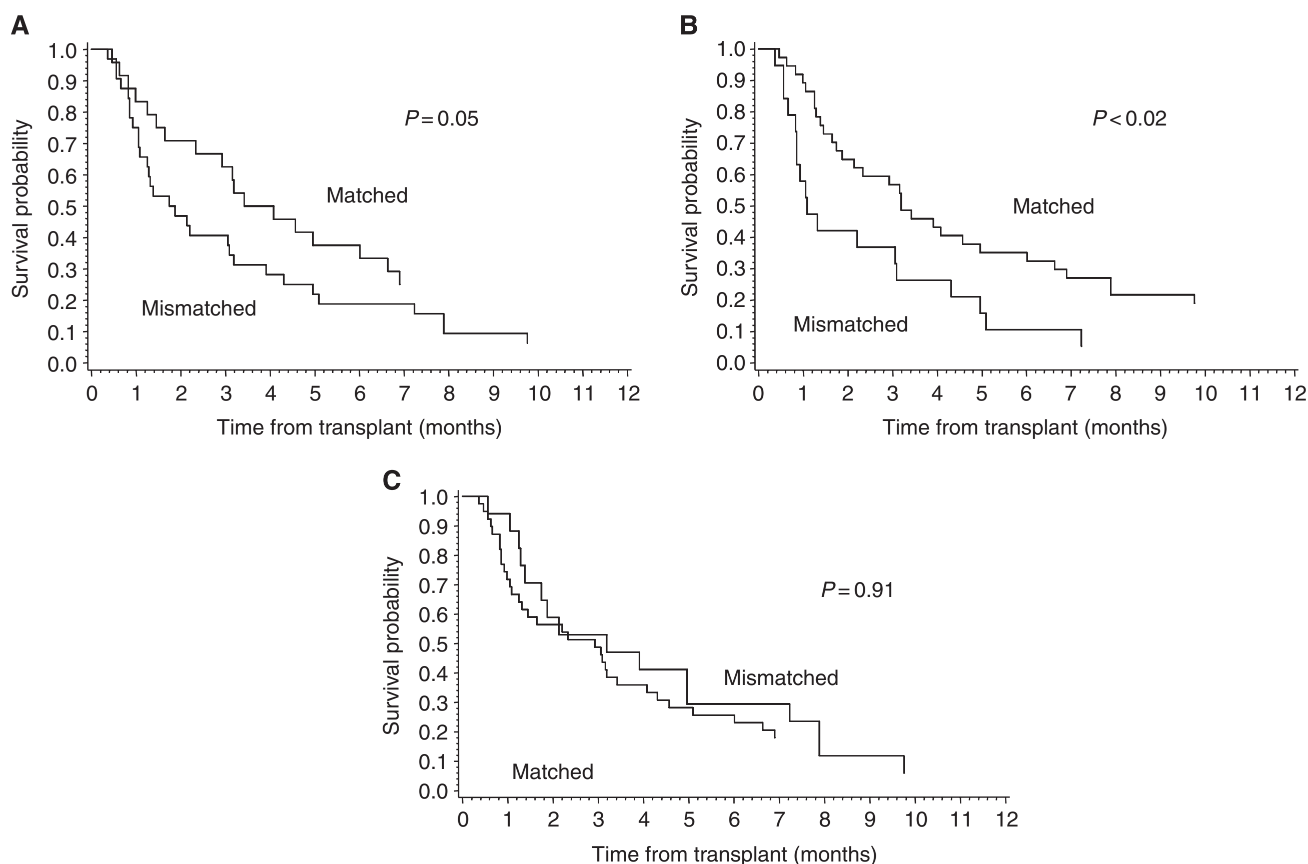


Fig. 3. Patients matched with their donors in both graft *vs* host (GVH) and host *vs* graft (HVG) directions had significantly better survival (A). Matching in the GVH direction (B) is more important for survival than matching in the HVG direction (C).

would favor the elimination of recipient APCs before donor T cells have a chance to induce GVHD, whereas at a low NK : T-cell ratio, induction of GVHD by resident T cells in the graft, especially in recipients not treated with anti-GVHD prophylaxis, may be unavoidable. It is not clear whether the difference in T-cell numbers and NK : T-cell ratios is sufficient to explain the difference in the incidence of GVHD, but in view of the recent experimental data, it must be considered as a likely possibility.

Based on their murine model, Ruggeri et al. suggested that the beneficial effect of NK alloreactivity in the GVHD direction on engraftment rates observed in humans may be due to donor NK-cell-mediated elimination of recipient T cells. Indeed, Slavin et al. (34, 35) have documented that engraftment across MHC may be facilitated following reduced intensity conditioning by activation of NK and T cells with recombinant interleukin-2. NK-like cells also resist normal as well as malignant hematopoietic cells of host origin, as can be best documented in parent into F1 murine models, known as hybrid resistance (9), thus suggesting that even NK or NK-like cells and not alloreactive T cells may be capable of eliminating residual hematopoietic cells of host origin in the setting of haploidentical SCT. Unfortunately, such effect could not be reproduced in the current study. Likewise, we were unable to show in a mixed group of leukemia patients a beneficial effect of NK alloreactivity on leukemia-relapse rates. As Ruggeri et al. reported this effect in AML patients but not ALL patients, we have analyzed our data according to the type of leukemia. Although there were only three AML patients with potential NK alloreactivity in the GVHD direction in this study, it is noteworthy that all three relapsed.

The differences observed can be explained by either the intensity of the conditioning that may have eliminated a larger number of tumor cells in the Italian series or by the fact that patients in Jerusalem had higher number of tumor cells and more aggressive leukemia to start with, as only high-risk patients were referred for haploidentical bone marrow transplantation program in Jerusalem. Such differences could explain better eradication of smaller residual host hematopoietic cells, with less rejection on the one hand and lower relapse rates on the other, found in the Italian study. On the other hand, one would anticipate that due to the heavier T-cell contamination in our study group, GVL effects would be more evident; however, the onset of GVHD in a mismatched setting necessitates immediate onset

of immunosuppressive treatment, which negates GVL effects induced by the allograft (36). Taken together, the data suggest that in order to observe the beneficial effects of alloreactive NK cells, patients should be treated with a lower tumor burden and with more vigorous T-cell depletion to allow alloreactive NK cells to mediate GVL effects independent of GVHD, as was recently demonstrated by Giebel et al. (37) who used ATG as a part of GVHD prophylaxis.

Although the number of inhibitory *KIR* in the patient or donor was not related to transplantation outcome, this might be explained by the fact that there was minimal variation in the number of inhibitory *KIR* present in different donors. Most donors had inhibitory receptors for the C1, C2, and Bw4 epitopes present in the recipient, thus ensuring that adequate NK inhibition is likely. However, patients whose donors had four or more non-inhibitory *KIR* had a higher incidence and more severe GVHD. We were unable to perform subtyping for *KIR*2DS4 to detect the non-coding alleles, as there was insufficient DNA available. We believe that if this finding can be confirmed by other groups, it will provide another criterion that can be used to select among potential haploidentical donors. The ligands for activatory *KIR* are currently not yet well established. Although DNA sequence analysis suggests that it is likely that their ligands will be similar to those of the inhibitory receptors, the activatory receptors appear to have a much lower affinity for these ligands.

In summary, the findings of Ruggeri et al. (8) and Giebel et al. (37) provide an exciting potential for more effective clinical application of alloreactive NK cells. However, our current study and that of Davies et al. (18) in matched unrelated transplants indicate that the type of disease status, conditioning regimen, composition of the allograft, and genetic background of the recipient and the donor may all have a major influence on the result of mismatching for class I HLA NK epitopes and the outcome of SCT. Therefore, the findings of Ruggeri et al. cannot be generalized and must be validated in each transplant center, in view of all of the variables that may play a role in engraftment, GVHD, late rejection, relapse, and overall disease-free survival. Better understanding of all the factors involved and their exact effect on the mechanisms of bilateral transplantation tolerance of HVG and GVHD on the one hand and GVL effects on the other will provide a better insight into the role of NK alloreactivity and development of protocols designed to exploit NK alloreactivity to improve the outcome of SCT. Meanwhile, our data suggest that lack of extensive T-cell depletion in haploidentical transplantation is associated with high GVHD rates and diminishes the benefits of NK-cell alloreactivity.

References

1. Powles R, Mehta J, Kulkarni S et al. Allogeneic blood and bone marrow stem cell transplantation in hematological malignant diseases. *Lancet* 2000: **355**: 1231–7.
2. Weiden PL, Sullivan KM, Flournoy N, Storb R, Thomas ED. Antileukemic effect of chronic graft-versus-host-disease: contribution to improved survival after allogeneic marrow transplantation. *N Engl J Med* 1981: **304**: 1529–33.
3. Anasetti C, Amos D, Beatty PG et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med* 1989: **320**: 197–205.
4. Hansen JA, Petersdorf E, Martin PJ, Anasetti C. Hematopoietic stem cell transplants from unrelated donors. *Immunol Rev* 1997: **157**: 141–51.
5. Bishara A, Sherman L, Battat S et al. The probability of finding HLA identical bone marrow donor in Jewish and Arab families referred to Hadassah Medical Center. *Hum Immunol* 1995: **44** (Suppl. 1): 105 [abstract].
6. Aversa F, Tabilio A, Velardi A et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N Engl J Med* 1998: **339**: 1186–93.
7. Ruggeri L, Capanni M, Casucci M et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* 1999: **94**: 333–9.
8. Ruggeri L, Capanni M, Urbani E et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 2002: **295**: 2097–100.
9. Cudkowitcs G, Bennet M. Peculiar immunobiology of bone marrow allografts. II. Rejection of parental grafts by F1 hybrid mice. *J Exp Med* 1971: **135**: 1513–28.
10. Murphy WJ, Longo DL. The potential role of NK cells in the separation of graft versus tumor effect from graft versus host disease after allogeneic bone marrow transplantation. *Immunol Rev* 1997: **157**: 167–701.
11. Moretta A, Moretta L. HLA class-I specific inhibitory receptors. *Curr Opin Immunol* 1997: **9**: 694–701.
12. Lanier L. NK cell receptors. *Annu Rev Immunol* 1998: **16**: 359–93.
13. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* 1995: **268**: 405–8.
14. Biassoni R, Falco M, Cambiaggi A et al. Amino acid substitution can influence the natural killer (NK) mediated recognition of HLA molecules, role of serine-77 and lysin 80 in the target protection from lysis mediated by 'group 2' or 'group 1' NK clones. *J Exp Med* 1995: **182**: 605–9.
15. Pende D, Biassoni R, Cantoni C et al. The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/70 family of inhibitory receptors that is characterized by three immunoglobulin like domains and is expressed as a 140-kD disulfide-linked dimmer. *J Exp Med* 1996: **84**: 505–18.
16. Bishara A, Amar A, Brautbar C, Condiotti R, Lazarovitz V, Nagler A. The putative role of HLA-C recognition in graft versus host disease (GVHD) and graft rejection after unrelated bone marrow transplantation. *Exp Hematol* 1995: **23**: 1667–75.
17. Nagler A, Slavin S, Brautbar C, Shapira M, Bishara A. Killer inhibitory receptor (KIR) mismatches correlates with high incidence of transplant related complication post-haploidentical stem cell transplantation (HaploSCT). *Bone Marrow Transplant* 2001: **27**: 510 [abstract].

18. Davies MS, Ruggieri L, DeFor T et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. *Blood* 2002: **100**: 385–7.
19. Gagne K, Brizard G, Gueglio B et al. Relevance of KIR gene polymorphisms in bone marrow transplantation outcome. *Hum Immunol* 2002: **63**: 271–80.
20. Velardi A, Ruggeri L, Moretta A, Moretta L. NK cells: a lesson from mismatched hematopoietic transplantation. *Trends Immunol* 2002: **23**: 438–44.
21. Terasaki PI, Bernoco D, Park MS, Ozturk G, Iwaki Y. Microdroplet testing for HLA-A, -B, -C and -D antigens. *Am J Clin Pathol* 1978: **69**: 103–20.
22. Petersdorf EW, Smith AG, Haase AM, Martin PJ, Hansen JA. Polymorphism of HLA-DRw52 associated DRB1 genes as defined by sequence-specific oligonucleotide probe hybridization. *Tissue Antigens* 1991: **38**: 169–77.
23. Ben-Yosef R, Or R, Varadi G, Slavin S, Nagler A. long toxicity following single dose total body irradiation in patients undergoing haploidentical stem cell transplantation. *Blood* 1999: **94**: 3349 [abstract].
24. Martin-henao GA, Picon M, Amill B et al. Combined positive and negative cell selection from allogeneic peripheral blood progenitor cells (PBPC) by use of immunomagnetic methods. *Bone Marrow Transplant* 2001: **27**: 683–7.
25. Watts MJ, Somervaille TC, Ings SJ et al. Variable product purity and functional capacity after CD34 selection: a direct comparison of the CliniMACS (v2.1) and Isolex 300i (v2.5) clinical scale devices. *Br J Haematol* 2002: **118**: 117–23.
26. Leclair B, Fregeau CJ, Aye MT, Fourney RM. DNA typing for bone marrow engraftment follow-up after allogeneic transplant: a comparative study of current technologies. *Bone Marrow Transplant* 1995: **16**: 43–55.
27. Przepiorka D, Weisdorf D, Martin P et al. 1994 consensus conference on acute GVHD grading. *Bone Marrow Transplant* 1995: **15**: 825–8.
28. Uhrberg M, Valiante NM, Shum BP et al. Human diversity in killer inhibitory receptor genes. *Immunity* 1997: **7**: 753–63.
29. Gomez-Lozano N, Gardiner CM, Parham P, Vilches C. Some human KIR haplotypes contain two KIR2DL5 genes: KIR2DL5A and KIR2DL5B. *Immunogenetics* 2002: **54**: 314–9.
30. Fisher RA. *Statistical Methods for Research Workers*. 5th edn. Edinburgh: Oliver and Boyd, 1981, 19.
31. Dixon WJ, Massey FG Jr. *Introduction to Statistical Analysis*. 4th edn. New York: McGraw hill, 1967, 121.
32. Kaplan E, Meier P. Non parametric estimation from incomplete observations. *J Am Stat Assoc* 1959: **53**: 457.
33. Asai O, Longo DL, Tian Z et al. Suppression of graft-versus-host disease and amplification of graft-versus-tumor effect by activated natural killer cells after allogeneic bone marrow transplantation. *J Clin Invest* 1998: **101**: 1835–42.
34. Weiss L, Abdul-Hai A, Shufaro Y, Reich S, Slavin S. Linomide administration following bone marrow transplantation in mice. *Cancer Immunol Immunother* 2002: **51**: 596–602.
35. Vourka-Karussis U, Ackerstein A, Pugatsch T, Slavin S. Allogeneic cell mediated immunotherapy fro eradication of minimal residual disease: comparison of T-cell and IL-2 activated killer (LAK) cell-mediated adoptive immunotherapy in murine models. *Exp Hematol* 1999: **27**: 461–9.
36. Nash RA, Storb R. Graft-versus-host effect after allogeneic hematopoietic stem cell transplantation. GVHD and GVL. *Curr Opin Immunol* 1996: **8**: 674–80.
37. Giebel S, Locatelli F, Lamparelli T et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donor. *Blood* 2003: **102**: 814–9.

CHAPTER 5: NATURAL KILLER CELL HLA-C EPITOPES AND KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS BOTH INFLUENCE OUTCOME OF MISMATCHED UNRELATED DONOR BONE MARROW TRANSPLANTS.

De Santis D, Bishara A, Witt CS, Nagler A, Brautbar C, Slavin S, Christiansen FT

The findings from our haploidentical study (Chapter 4) and that of Davies et al (Davies et al. 2002) in unrelated bone marrow transplants lead us to examine the influence of NK alloreactivity in the matched unrelated donor setting. This study was performed in collaboration with the transplant group at the Tissue Typing laboratory at the Hadassah University, Jerusalem, Israel. The study was performed to determine the role of NK epitope incompatibility and the KIR gene repertoire in unrelated bone marrow transplants. A cohort of 104 unrelated bone marrow transplants, in which there was considerable mismatching at the HLA-C locus in donor and recipient, were analysed. The HLA typing on donor and recipients was performed in Jerusalem and DNA samples for KIR genotyping was sent to the Perth laboratory.

The findings were published in;

Tissue Antigens. 2005 Jun;65(6):519-28.

Contributions:

DDS performed the KIR PCR-SSP genotyping of donor-recipient pairs, collated HLA and KIR genotype data, performed statistical analysis and prepared the manuscript.

AB performed the HLA typing of donor-recipient pairs and assisted in the preparation of the manuscript.

CW assisted in the study design, performed statistical analysis and assisted in the preparation of the manuscript.

FTC, CB, RO, AN and SS assisted in the study design and in the preparation of the manuscript.

D. De Santis
A. Bishara
C.S. Witt
A. Nagler
C. Brautbar
S. Slavin
F.T. Christiansen

Natural killer cell HLA-C epitopes and killer cell immunoglobulin-like receptors both influence outcome of mismatched unrelated donor bone marrow transplants

Key words:
engraftment; graft-*vs*-host disease; KIR epitopes; KIR receptors; natural killer cells; rejection; stem-cell transplantation

Acknowledgments:
This work was supported in part by a grant given to Prof. Chaim Brautbar from the Concern Foundation of Los-Angeles, California.

Abstract: Matching of donor and recipient for the class I human leukocyte antigen-C (HLA-C)-encoded natural killer (NK) epitopes has been reported to influence stem-cell (SC) graft outcome, but a consistent picture has not yet emerged. We have analyzed transplant outcome in 104 unrelated SC grafts in relation to NK epitope (C1 and C2) matching and donor killer cell immunoglobulin-like receptor (KIR) genotype. NK epitope mismatching in the rejection direction was strongly associated with an increased probability of rejection subsequent to engraftment. The prevalence of grades III–IV acute graft-*vs*-host disease (GVHD) was significantly higher and occurred significantly earlier when there was NK epitope mismatching in the GVH direction. Higher transplant-related mortality and lower disease-free survival rates were associated with epitope mismatching regardless of the mismatch direction. A greater number of KIR receptors, both activating and inhibitory, in the donor protected against grades III–IV GVHD and improved survival.

Authors' affiliation:

D. De Santis[1,2],
A. Bishara[3],
C.S. Witt[1,2],
A. Nagler[4],
C. Brautbar[3,5],
S. Slavin[4],
F.T. Christiansen[1,2]

[1]Department of Clinical Immunology and Biochemical Genetics, Royal Perth Hospital, Perth, Australia

[2]School of Surgery and Pathology, University of Western Australia, Perth, Australia

[3]Tissue Typing Unit, Hadassah – Hebrew University Medical Center, Jerusalem, Israel

[4]Department of Bone Marrow Transplantation Cancer Immunotherapy, Hadassah Medical Center, Jerusalem, Israel

[5]The Lautenberg Center for General and Tumor Immunology, Hebrew University, Jerusalem, Israel

Correspondence to:
Campbell Witt
Department Clinical Immunology
Royal Perth Hospital
Wellington St, Perth
Australia
Tel.: +61 8 9224 2899
Fax: +61 8 9224 2920
e-mail: campbell.witt@health.wa.gov.au

Human leukocyte antigen (HLA) matching for bone marrow transplantation (BMT) has traditionally been analyzed from the point of view of T-cell alloreactivity. Recent basic research has revealed that natural killer (NK) cells are also capable of alloreactivity (1–4). Furthermore, there is evidence that incompatibility resulting in potential for NK cell alloreactivity (NK epitope mismatching) may be exploited to produce a beneficial graft-*vs*-leukemia effect and to reduce the likelihood of acute graft-*vs*-host disease (aGVHD) and rejection (5–7). However, other publications have shown deleterious effects of NK epitope incompatibility (8–11). The reason for these contradictory findings is not clear but may relate to the differences in transplant protocol including T-cell depletion (10). Although there may be differences between transplants using siblings matched for one haplotype (haploidentical) and HLA-matched unrelated donor transplants, there are contradictory reports in both haploidentical (5, 10) and unrelated donor (7, 8) settings. As there are important implications for donor selection, there is a need for additional transplant cohorts to be analyzed.

Received 8 June 2004, revised 2 December 2004, accepted for publication 7 February 2005

Copyright © Blackwell Munksgaard 2005
doi: 10.1111/j.1399-0039.2005.00396.x

Tissue Antigens 2005: **65:** 519–528
Printed in Singapore. All rights reserved

NK epitope compatibility has generally been analyzed in terms of the 'missing self' hypothesis (12, 13) in which NK cells react against cells that lack one of the three known HLA-C and HLA-B epitopes. These epitopes are ligands for the killer cell immunoglobulin-like receptors (KIRs) on NK cells (1, 14–17). The presence of these epitopes is recognized by inhibitory KIR on the NK cell (13). Although there is considerable genetic variability in the inhibitory KIR repertoire (18, 19), most individuals have inhibitory receptors capable of detecting the absence of the three known inhibitory epitopes (18). It is therefore possible to analyze donor–recipient NK epitope compatibility in most instances without reference to the donor or recipient's inhibitory receptor repertoire (5, 8, 9). There is greater genetic variability in the activating KIR repertoire (19, 20). Sequence homology between various activating and inhibitory KIR suggests that the ligands are similar and there is *in vitro* evidence that NK cells expressing the activating KIR respond to target cells expressing the predicted epitopes (20, 21, 22). However, the ligands for the activating KIR are not known with certainty because the activating KIRs have much lower affinity than the inhibitory receptors for HLA alleles with the three known NK epitopes (23). There have been few reports attempting to relate the activating KIR repertoire to transplant outcome. Cook et al. (24) have reported poor survival in HLA identical sibling transplants when the donor has KIR2DS2 and the recipient does not have the predicted ligand (C1 epitope) for KIR2DS2.

In this report, we analyze the influence of NK epitope compatibility and donor KIR repertoire in 104 unrelated donor bone marrow transplants in which there was considerable mismatching of donor and recipient at the HLA-C locus. We find that mismatching for NK epitopes for inhibitory KIR increases the probability of acute GVHD (aGVHD). Mismatching in the GVHD direction is also associated with higher transplant-related mortality (TRM), while mismatching in the rejection direction increases the probability of rejection. Additionally, we report that the presence in the donor of a greater number of KIRs, both activating and inhibitory, protects against grades III–IV aGVHD and improves survival.

Patients and methods

Patients and donors

A total of 104 consecutive patients (63 males, 41 females, mean age 24, range from 1 to 57 years) who had a hematopoietic stem-cell transplantation between January 1990 and December 2002, at the Department of Bone Marrow Transplantation and Cancer Immunotherapy, Hadassah Medical Center, Jerusalem, Israel, were included in this study. Seventeen patients were diagnosed with acute lymphoblastic leukemia (ALL) – five in second and five in third complete remission (CR) and seven in relapse and advanced disease – three with acute myeloid leukemia (AML) (all in relapse), 22 with chronic myeloid leukemia (CML) – seven in first, two in second, and one in third chronic phase (CP), one in second CR, and 11 in accelerated disease – 14 with acute-nonlymphocytic leukemia (ANLL) – two in first, three in second, two in third CR, and seven in second relapse – 17 with myelodysplastic syndrome (MDS) – two in first CR and 15 with refractory anemia with excess blasts – five with Hodgkin's disease (all in relapse and resistance disease), three with severe aplastic anemia (SAA), five with Fanconi's anemia, five with solid tumors, three with immunodeficiency, two with non-Hodgkin's lymphoma, two with mixed lymphoma, two with lymphoma, one with familial histiophagocytic lymphohistiocytosis, two with multiple myeloma, and one with thalassemia. Sixty-five patients received bone marrow and 39 mobilized peripheral blood from unrelated donors. Forty-nine and 55 recipients were transplanted from sex-matched and sex-mismatched donors, respectively. Twenty-six female recipients were transplanted from male donors and 29 male recipients from female donors.

HLA typing

All recipients were typed for HLA class I by serology (and since 1998 by low to medium resolution using molecular methods) and by molecular methods for high resolution of HLA-DRB1 and -DQB1 alleles. Donors were selected from the local and international registries on the basis of matching for HLA-A and -B by serology and for HLA-DRB1 and -DQB1 by polymerase chain reaction (PCR)-sequence-specific oligonucleotide probe (SSOP) or PCR-sequence-specific priming (SSP). Subsequently, for the purpose of this study, HLA-C typing was performed in all cases by PCR-SSP. Each individual was assigned as having a C1 epitope (HLA-C^{asn80} allele), or C2 epitope (HLA-C^{lys80} allele) on the basis of PCR-SSP results, or the Bw4 epitope on the basis of serological typing.

KIR PCR-SSP typing

All recipients and donors were typed for the presence or absence of 11 KIR genes by PCR-SSP as described previously (26) with modification to primers for KIR2DS4. Primers for the amplification of the expressed and nonexpressed KIR2DS4 were used (25). Only the expressed alleles of KIR2DS4 (KIR2DS4*001 and KIR2DS4*002) were considered in analyses. It was not possible to successfully type all KIR genes in all cases due to either insufficient quantity or quality of DNA. Donors were typed successfully for KIR2DL1, 2DL2, 2DL3, 2DL5, 3DL1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 3DS1 in 92,

92, 97, 83, 96, 96, 88, 91, 94, 80, and 100 cases, respectively. When analysing the influence of individual KIR, all cases with valid data were included. When analysing the influence of the total number of KIRs, only cases with results for all relevant KIR were included. Valid results for all KIR genes were obtained for 59 donors, valid results for all activating KIRs were obtained for 64 donors, and valid results for all inhibitory receptors were obtained for 75 donors.

Conditioning and transplantation protocols

Bone marrow transplantation

Of the 65 recipients who underwent BMT, 56 received unmodified bone marrow cells and standard post-BMT GVHD prophylaxis consisting of cyclosporine A and a short course of methotrexate. The mean CD34 and CD3 counts for these grafts were 3.5×10^6/kg (range $0.5–50 \times 10^6$) and 3.7×10^7/kg (range $7.7–436 \times 10^6$), respectively. Nine recipients received T-cell-depleted marrow as previously described (10).

The patients with acute leukemia and MDS were conditioned with fractionated total body irradiation (TBI) (1200 cGY), cyclophosphamide (CY) 60 mg/kg for 1 day, or busulfan 4 mg/kg for 4 days, CY 60 mg/kg for 4 days. Total nodal irradiation (600 cGY) was given to patients who received T-cell-depleted grafts. The patients with CML were conditioned with TBI (1200 cGY) and CY 60 mg/kg for 2 days. The patients with SAA were conditioned with nodal irradiation (600 cGY) and CY 50 mg/kg for 4 days.

Peripheral blood stem cells (PBSC)

Thirty-nine recipients were infused with granulocyte colony-stimulating factor (G–CSF)-mobilized peripheral blood stem cells (PBSC). Median CD34 and CD3 cell counts were 5.6×10^6/kg (range $2–25 \times 10^6$) and 4.3×10^8/kg (range $2.5–600 \times 10^6$), respectively. No recipients received cyclosporine A or hematopoietic growth factors prior to or following stem-cell transplantation.

Assessment of engraftment, rejection, and aGVHD

Engraftment was defined as an absolute neutrophil count of $>0.5 \times 10^9$/l, a white blood cell count of $>1 \times 10^9$/l for three consecutive days, and unsupported platelet count of $>20 \times 10^9$/l for three consecutive days, or detection of donor DNA by PCR-variable number tandem repeats (VNTR) or PCR-short tandem repeat (STR) (27). Rejection was defined as loss of the allograft as determined by cytogenetic analysis or by VNTR-PCR or PCR-STR (27). Acute GVHD was defined according to the Seattle criteria (28).

Data analysis

Analysis of engraftment was performed on 100 patients who survived more than 28 days ($n = 87$) or engrafted before death ($n = 13$). Analysis of rejection was performed on the 83 patients who engrafted and survived more than 28 days ($n = 82$) and those who rejected and died before day 28 ($n = 1$). Analysis of aGVHD was performed on the 91 patients who engrafted and survived more than 28 days ($n = 82$) and on those who engrafted and developed aGVHD even if they survived less than 28 days ($n = 9$). Analysis of relapse was performed on 79 patients with hematological malignancies who either survived more than 28 days ($n = 77$) or relapsed before day 28 ($n = 2$). TRM was defined as death without relapse less than 3 months post-transplantation ($n = 28$). Twenty-eight patients relapsed. Disease-free survival (DFS) was defined as survival without relapse. The relationship between NK epitope matching and aGVHD, engraftment, rejection, and relapse were tested using Fisher's exact test or Chi-square test. The relationship between epitope mismatching and survival was determined by Kaplan–Meier analysis using the log-rank statistic or Cox regression analysis for multivariate analyses. When testing the relationship between survival and number of KIR receptors, Kaplan–Meier analysis was used, and the significance of the linear trend associated with increasing numbers of KIR was tested. The relationship between number of activating KIR and aGVHD was determined by the Chi-square test for 2×2 contingency tables using the cut-offs for the number of KIR receptors as described in the *Results*. All analyses were performed with SPSS 10.0 (SPSS, Chicago, IL).

Results

Classification of donor–recipient pairs according to potential NK alloreactivity

All transplants were matched at HLA-B. Recipients and donors were assigned as having C1 and C2 epitopes based on their HLA-C typing and then classified with respect to the vector of mismatch 'GVH', 'Rejection', or 'Either' for the purpose of analyses as previously described (10).

Engraftment

NK epitope mismatching in the GVH, rejection, or either direction was not associated with the probability of engraftment (Table 1). Likewise, the total number of inhibitory KIRs, total number of activating KIRs, and the presence of any particular KIR in the donor were not associated with failure to engraft (data not shown).

Frequency of engraftment and rejection in transplants with NK epitope mismatching in the GVH direction, rejection direction, and either direction

	NK epitope mismatching					
	Either direction		GVH direction		Rejection direction	
	Yes $n = 24$[a]	No $n = 76$	Yes $n = 15$	No $n = 85$	Yes $n = 11$	No $n = 89$
Engraftment						
Yes	23 (96)	72 (95)	14 (93)	81 (95)	11 (100)	84 (94)
No	1 (4)	4 (5)	1 (7)	4 (5)	0	5 (6)
P-value	NS		NS		NS	
	$n = 18$	$n = 65$	$n = 11$	$n = 72$	$n = 9$	$n = 74$
Rejection						
Yes	6 (33)	6 (8)	1 (9)	11 (15)	5 (56)	7 (9)
No	12 (67)	59 (92)	10 (91)	61 (85)	4 (44)	67 (91)
RR	3.5		0.50		6.1	
P-value	0.01		NS		<0.005	

GVH, graft-*vs*-host; NK, natural killer; RR, relative risk; NS, not significant.
The values are expressed as *n* (%).
[a]15 patients were mismatched in the GVH direction and 11 were mismatched in the rejection direction, but as two patients were mismatched in both the GVH and the rejection directions, the number of patients mismatched in either direction is only 24.

Table 1

Rejection

The frequency of rejection was significantly higher in transplants with NK epitope mismatching in either direction ($P = 0.01$), and this was entirely accounted for by transplants with NK epitope mismatching in the rejection direction ($P < 0.005$; Table 1). Rejection occurred in five of nine (56%) transplants with NK epitope mismatching in the rejection direction compared with seven of 74 (9%) transplants that were matched in the rejection direction. This association was not due to a coincidental association between NK epitope mismatching and T-cell depletion. Although three of nine T-cell-depleted grafts rejected, only one of these was mismatched in the rejection direction. There was no association between rejection and the total number of donor inhibitory or activating KIRs nor the presence of any particular KIR (data not shown).

Acute GVHD

NK epitope mismatching was associated with a higher incidence of severe aGVHD (grades III–IV) (Table 2).

The effect was significant in transplants with NK epitope mismatching in the GVH direction [relative risk (RR) = 3.1, $P = 0.02$] and almost significant in transplants with NK epitope mismatching

The incidence of severe acute GVHD in transplants with NK epitope mismatching in the GVH direction, rejection direction, and either direction

	NK epitope mismatching					
	Either direction		GVH direction		Rejection direction	
	Yes $n = 23$	No $n = 68$	Yes $n = 14$	No $n = 77$	Yes $n = 11$	No $n = 80$
GVHD						
0–II	13 (56%)	56 (82%)	7 (50%)	62 (81%)	6 (55%)	63 (79%)
III–IV	10 (44%)	12 (18%)	7 (50%)	15 (19%)	5 (45%)	17 (21%)
RR	2.4		3.1		2.6	
P-value	0.02		0.02		0.09	

GVHD, graft-*vs*-host disease; NK, natural killer; RR, relative risk.

Table 2

in the rejection direction (RR = 2.6, $P = 0.09$). The onset of aGVHD in transplants with NK epitope mismatching in the GVH direction was also faster (mean of 11 days) than for transplants that were matched in the GVH direction (mean of 21 days) ($P < 0.05$).

The total number of KIR genes and the presence or absence of each KIR gene in the donor were analyzed with respect to aGVHD in those patients who were evaluable for aGVHD and on whom KIR typing was successful. The incidence of aGVHD was compared in donors with different total numbers of KIR genes. As shown in Fig. 1(A), a greater number of KIR genes were associated with protection against aGVHD. When donors had less than five KIR genes, grades III–IV aGVHD occurred in 11 of 20 transplants compared to only one of 32 when the donor had at least five KIR genes ($P < 0.00002$). However, there did not appear to be a decreasing incidence of aGVHD with each additional KIR gene. Rather, it appeared that grades III–IV aGVHD was equally high among all donors with less than five KIR genes and equally low among all donors with at least five KIR genes. In order to determine whether inhibitory or activatory KIR might be more important, the incidence of aGVHD was compared in donors with different numbers of activating or inhibitory KIR genes. As shown in Fig. 1(B,C), both inhibitory and activating KIR appeared important as the incidence of grades III–IV aGVHD was greater in donors with less than four inhibitory KIRs ($P < 0.00002$) or less than three activating KIRs ($P < 0.0001$).

Inspection of the KIR repertoire of those donors with low numbers of KIR revealed that they were mostly homozygous for the common A haplotype (2DL1, 2DL3, 3DL1, with or without the expressed allele of 2DS4) (29). One interpretation would therefore be that the protective effect of a high number of KIR genes is due to a gene on the B haplotype. In order to further investigate this possibility, the effect of each individual KIR gene was analyzed. The presence of some individual KIR genes in the donor had weakly significant protective effects against grades III–IV aGVHD (Table 3). However, for every KIR gene, the RR of aGVHD was lower if the gene was present in the donor. Arguing against the simple interpretation that the main effect is due to a gene on the 'B' haplotype was the fact that the presence of KIR2DL3 and KIR3DL1, which are found on the 'A' haplotype, were almost significantly protective against aGVHD ($P = 0.11$ and $P = 0.08$, respectively).

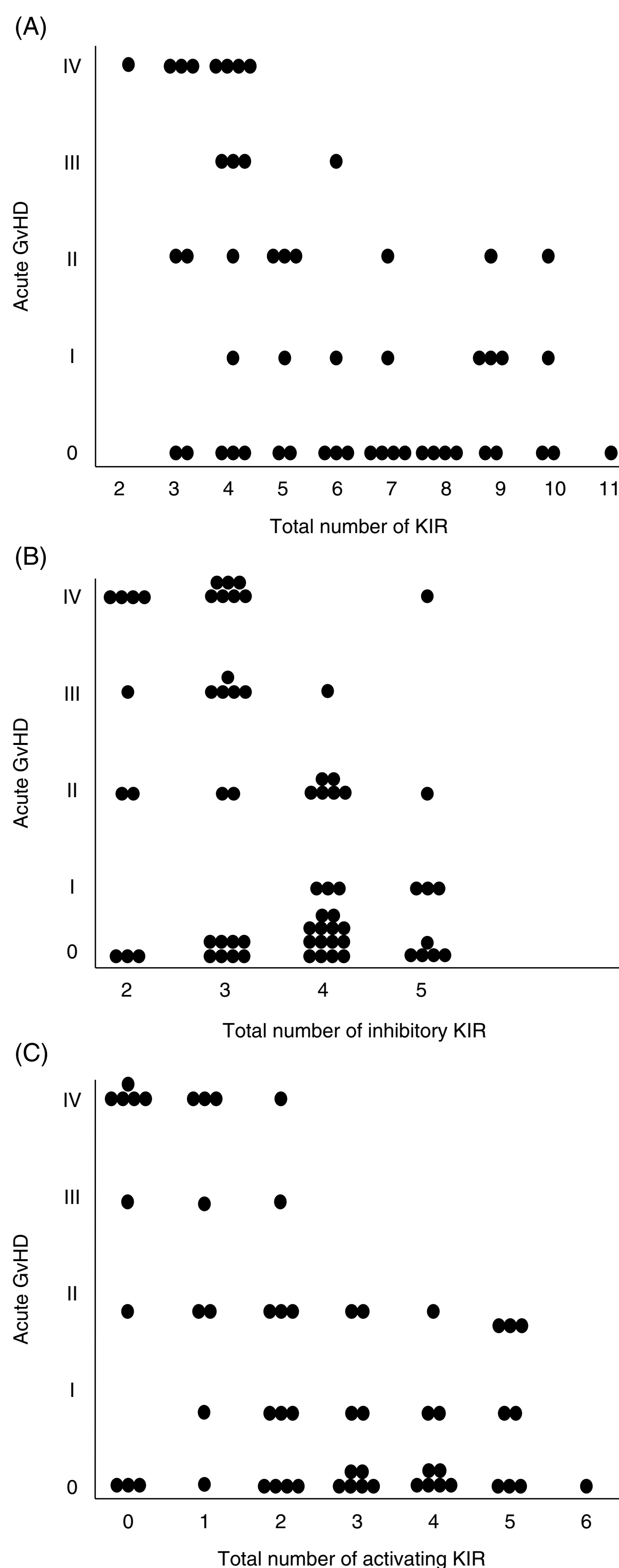


Fig. 1. **Severity of acute graft-*vs*-host disease (aGVHD) is inversely related to the number of donor killer cell immunoglobulin-like receptor (KIR) genes.** (A) The total number of donor KIR genes (inhibitory and activating) ($P < 0.00002$). (B) The total number of donor inhibitory genes ($P < 0.00002$). (C) The total number of donor activating genes ($P < 0.0001$).

Relapse

There was no significant association of NK epitope mismatching, regardless of direction, with relapse. Given the previous report that NK epitope mismatching protects against relapse only in myelogenous leukemias (5), we analyzed AML, CML, and MDS cases as a

Relative hazards and P-values for grades II–IV acute GVHD associated with the presence of each KIR gene in the donor

KIR	Relative risk	*P*-value
KIR2DL1	0.83	0.25
KIR2DL2	0.86	0.16
KIR2DL3	0.69	0.11
KIR2DL5	0.87	0.22
KIR3DL1	0.74	0.08
KIR2DS1	0.75	0.01
KIR2DS2	0.76	0.03
KIR2DS3	0.86	0.23
KIR2DS4	0.91	0.32
KIR2DS5	0.70	0.007
KIR3DS1	0.86	0.15

Table 3

separate subgroup. However, as the overall relapse rate among the 20 CML, three AML, and 15 MDS patients that could be evaluated for relapse was 20%, it would be difficult to detect a lower relapse rate among the five patients with NK epitope mismatching in the GVH direction. One of five patients (18%) with NK epitope mismatching in the GVH direction relapsed, and six of 33 patients (20%) without NK epitope mismatching in the GVH direction relapsed. There was also no correlation between the number of inhibitory KIRs, the number of activating KIRs, and the presence of any particular KIR in the donor and relapse.

Survival

Survival was analyzed in terms of TRM, DFS, and overall survival (OAS). TRM was significantly associated with NK epitope mismatching in either direction (RR = 2.5, $P < 0.01$; Table 4). NK epitope mismatching in the GVH direction alone (RR = 2.7, $P = 0.03$; Table 4) appeared to be slightly more important than mismatching in the rejection direction alone (RR = 1.6, P = not significant; Table 4).

Although NK epitope mismatching was associated with an increased incidence of grades III–IV aGVHD and rejection, the increase in TRM did not appear to be explained by death due to aGVHD and rejection. The incidence of death due to aGVHD or rejection was similar in those who had NK epitope mismatching (4/11) and in those without (2/13), with the remaining deaths being related to infection ($n = 8$), progressive disease ($n = 4$), and respiratory and organ failure ($n = 6$).

NK epitope mismatching in either direction was significantly associated with poorer DFS [relative hazard (RH) = 1.9, $P < 0.01$; Fig. 2A] and was still significant ($P = 0.03$) after taking into account T-depletion, stem-cell source, 'hematological malignancy *vs* other', and 'pediatric *vs* adult' in a multivariate analysis. Although NK epitope mismatching in the rejection direction alone (RH = 1.7, $P = 0.14$; Fig. 2B) and GVHD direction alone (RH = 1.3, $P = 0.27$; Fig. 2C) were not significantly associated with poorer outcome, the RH were greater than 1.0. The nonsignificant *P*-values may be more a reflection of the fact that there were only half as many patients mismatched in the GVHD or rejection directions alone compared to those mismatched in either direction.

A higher number of donor KIRs was associated with improved DFS (Fig. 3A, P for trend <0.02) and with improved OAS (data not shown; P for trend <0.02). In order to determine whether the number of inhibitory or activating KIR was more important, survival was analyzed with respect to the number of inhibitory or activating KIR. A higher number of either inhibitory KIR or activating KIR was associated with improved DFS (Fig. 3B, P for trend = 0.03, and 3C, P for trend = 0.002) and improved OAS (P for trend = 0.02 and 0.003, respectively, data not shown).

In order to determine whether particular KIRs were responsible for improved survival, the influence of each KIR on DFS and OAS

TRM in transplants with NK epitope mismatching in the GVH, rejection, and either direction

	NK epitope mismatching					
	Either direction		GVH direction		Rejection direction	
	Yes	No	Yes	No	Yes	No
TRM						
Yes	12 (48%)	16 (20%)	8 (50%)	20 (23%)	4 (36%)	24 (24%)
No	13 (52%)	63 (80%)	8 (50%)	68 (77%)	7 (64%)	69 (76%)
Relative risk	2.5		2.7		1.6	
P-value	<0.01		0.03		NS	

TRM, transplant-related mortality; NK, natural killer; GVH, graft-*vs*-host; NS, not significant.
The incidence of transplant-related mortality was higher in transplants with NK epitope mismatching in either direction compared to those without NK epitope mismatching.

Table 4

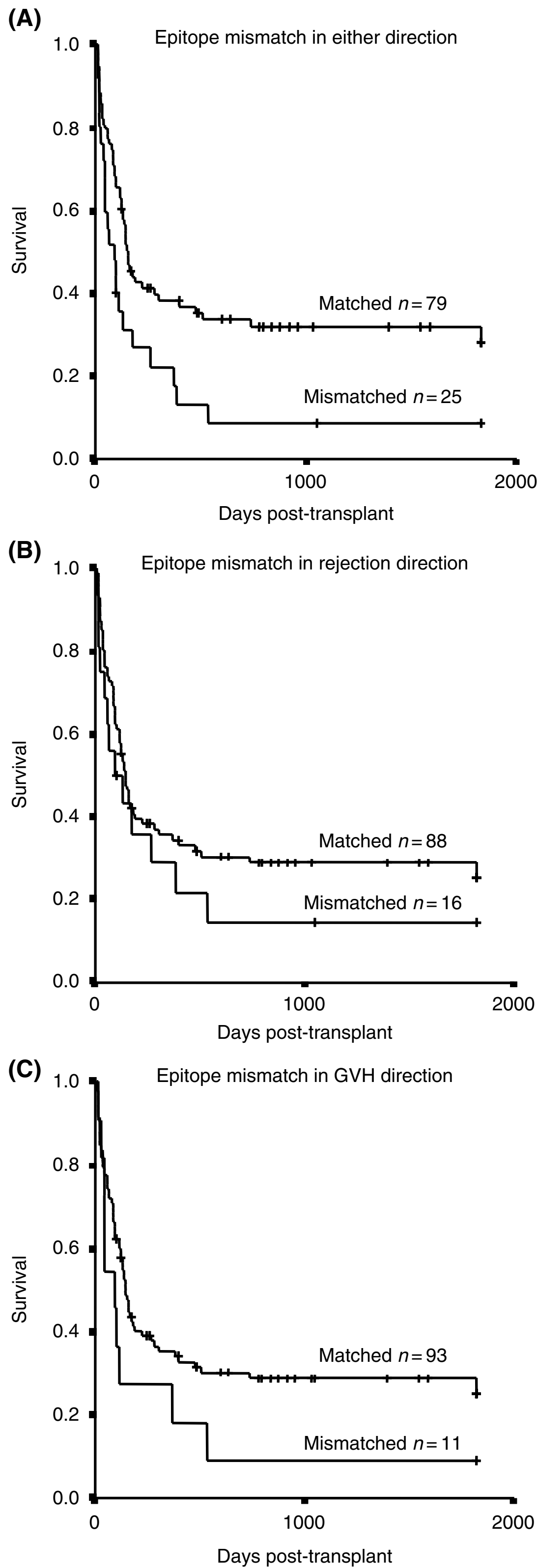


Fig. 2. **Natural killer (NK) epitope mismatching is associated with poorer disease-free survival.** (A) Epitope mismatching in either direction ($P < 0.01$). (B) Epitope mismatching in the rejection direction (P = NS). (C) Epitope mismatching in the graft-*vs*-host (GVH) direction (P = not significant).

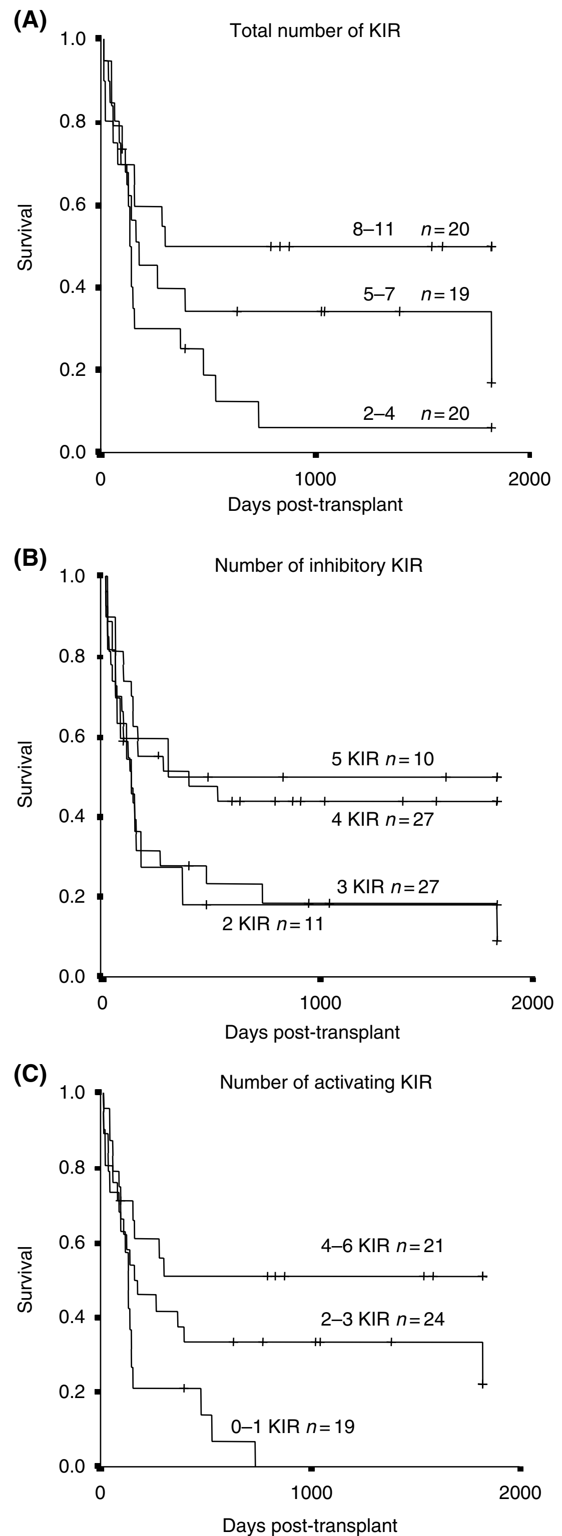


Fig. 3. **Increasing number of donor killer cell immunoglobulin-like receptor (KIR) genes is associated with improved disease-free survival.** (A) Transplants stratified by total number of donor inhibitory and activating genes (P for trend <0.02). (B) Transplants stratified by the total number of donor inhibitory KIR (P for trend $= 0.03$). (C) Transplants stratified by the total number of donor activating KIR genes (P for trend $= 0.002$).

Relative hazards and Kaplan–Meier P-values for the null hypothesis that overall survival (OAS) and disease-free survival (DFS) are not associated with the presence of individual killer cell immunoglobulin-like receptor (KIR) genes

	OAS		DFS	
KIR	Relative hazard	*P*-value	Relative hazard	*P*-value
KIR2DL1	0.54	0.06	0.55	0.08
KIR2DL2	0.56	0.06	0.58	0.05
KIR2DL3	0.5	0.04	0.46	0.03
KIR2DL5	0.69	0.20	0.72	0.25
KIR3DL1	0.67	0.18	0.71	0.25
KIR2DS1	0.5	0.009	0.51	0.008
KIR2DS2	0.61	0.08	0.63	0.08
KIR2DS3	0.88	0.71	1.1	0.77
KIR2DS4	0.78	0.36	0.85	0.52
KIR2DS5	0.67	0.17	0.65	0.12
KIR3DS1	0.54	0.02	0.59	0.04

For each KIR gene, including those with nonsignificant *P*-values, survival was greater when the KIR gene was present in the donor.

Table 5

was analyzed. As summarized in Table 5, several individual KIR had significant or nearly significant *P*-values, and in each case, the presence of the KIR gene was associated with improved survival compared to its absence as indicated by the RH of less than 1.0.

Discussion

In this study, we observed several significant associations between NK epitope incompatibilities and between donor KIR genotype and outcome of unrelated hematopoietic stem-cell transplantations.

Rejection, but not failure to engraft, was associated with NK epitope mismatch in the rejection direction, a result consistent with the murine hybrid resistance phenomenon in which recipient F_1 hybrid NK cells reject homozygous parental bone marrow grafts (30). This finding is also consistent with our earlier demonstration, in a case of graft rejection, of the ability of a recipient's NK cells to lyze HLA-C mismatched donor targets (10). Our present findings are in contrast with those of Ruggeri et al. (5) who showed that NK epitope incompatibility in the GVH direction, rather than the rejection direction, correlated with increased engraftment rates in a T-cell-depleted transplant protocol using haploidentical donors. It is perhaps surprising that NK alloreactivity was found to affect rejection but not engraftment. Failure to engraft would be predicted to involve earlier elements of the immune response and therefore NK cells, whereas rejection implies a delayed response and might be thought more likely to involve the adaptive immune system.

Grades III–IV aGVHD was more frequent in transplants with NK epitope mismatching in the GVH direction. This result is consistent with the findings of our previous study involving haploidentical transplants (26), those of Davies et al. (8) in mismatched unrelated transplants, and those of Lowe et al. (9) in HLA nonidentical pediatric-related and -unrelated transplants. This result is also consistent with our earlier finding that donor NK cells are able to lyze recipient HLA-C mismatched targets and that this ability is associated with severe GVHD (10). The finding of increased aGVHD with epitope mismatching in the GVH direction has been consistent in non-T-cell-depleted protocols and is in contrast to findings published by Ruggeri et al. (5) and Giebel et al. (7) who showed that NK epitope incompatibility in the GVH direction reduces aGVHD, but the latter studies involved T-cell-depleted protocols.

We were unable to show any correlation between relapse and NK epitope mismatching in the GVH direction. This result is not surprising as the GVL effect has been reported to be limited to AML, CML, and pediatric ALL patients (5, 6, 7). The current study included only a small number of such patients.

TRM occurs for a variety of reasons including aGVHD and rejection. It was therefore not surprising to find that NK epitope mismatching, which was associated with aGVHD and rejection, was also associated with an increased risk of TRM. However, aGVHD was not listed as the cause of death in many patients with NK epitope mismatching in the GVH direction, and there was no significant difference in TRM attributed to aGVHD in the matched group compared to the mismatched group. It is possible that although aGVHD was not listed as a cause of death, many of these patients may have been experiencing aGVHD which may interfere with the restoration of functional immunity and may itself predispose mucosal surfaces to infection and contribute to organ failure.

A significantly poorer DFS was associated with NK epitope mismatching in either direction. This result is consistent with the majority of studies in non-T-cell-depleted protocols. Transplants that were matched for NK epitopes had improved survival in our previous study of haploidentical transplants ($P = 0.02$) (26), in unrelated transplants reported by Lowe et al. (9) ($P = 0.08$), and in unrelated transplants for myeloid malignancies reported by Davies et al. (8) ($P < 0.01$). This contrasts with T-cell-depleted protocols reported by Ruggeri et al. (5) and Giebel et al. (7) in which improved survival was observed for transplants with NK epitope mismatching in the GVH direction. However, a recent report using *in vivo* T-cell depletion (11) was unable to show a significant survival benefit among transplants without NK alloreactivity ($P = 0.32$). Thus, the parameters that enable the benefits of NK alloreactivity to be realized are still to be clarified.

Classical transplantation immunology would predict that mismatching for epitopes would result in adverse outcome, particularly as many of the transplants that were matched for NK epitopes would also be matched for HLA-C alleles. Similarly, those that were mismatched for NK epitopes would also be mismatched for other allelic epitopes that would constitute targets for T-cell alloreactivity. It is therefore not clear whether the adverse outcome associated with mismatching for NK epitopes is due to the NK epitopes or other allelic epitopes. This can only be determined by comparing outcome in transplants mismatched for both HLA-C alleles and epitopes with outcome in transplants mismatched for HLA-C alleles but matched for epitopes. However, in this study there were insufficient numbers to adequately address this issue.

An influence of donor KIR repertoire on transplant outcome has not been reported previously. Few studies have examined the role of KIR in stem-cell transplantation. The finding of reduced aGVHD and improved survival when the donor has a high number of KIR was surprising in light of our previous report on haploidentical transplants in which a high number of activating KIR was associated with increased aGVHD (26). However, re-analysis of that data has revealed a trend toward improved survival when the donor has a high number of KIR genes ($P = 0.06$, data not shown). In the current study, both inhibitory and activating KIRs appear to contribute to an improved outcome. The strong linkage disequilibrium in the KIR complex makes it difficult to determine whether the protective effect is due to any one particular KIR or whether several KIR genes contribute to the protective effect. The KIR A haplotype contains relatively few genes compared to the B haplotype, and the genes present on the A haplotype are either all inhibitory on the common version of this haplotype (KIR2DL1, KIR2DL3, and KIR3DL1) or, on a minority, include a single functional activating gene (KIR2DS4) (18, 19, 29). The B haplotypes contain a similar number of inhibitory genes to the A haplotype (KIR2DL2 and KIR2DL5) but a greater number of activating genes (usually at least two of KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, or KIR3DS1). Donors with a low number of KIRs are homozygous for the A haplotype and donors with a high number of KIRs are heterozygous for the A and B haplotypes. There were no donors who were homozygous for the B haplotype. The apparent protective effect of a large number of KIR genes is due to better outcome when donors are A/B heterozygotes. This may be due to the high number of KIR genes in heterozygotes, but it is possible that the protective effect of A/B donors is due to a single protective gene on the B haplotype. It is difficult to distinguish between these two models. The aGVHD data are largely consistent with a protective effect of the B haplotype, as there is little evidence of a linear trend of reduced aGVHD with a higher number of KIR genes. However, arguing against this model is the fact that the survival data show a linear trend toward improved survival with increasing genes. In addition, both the presence of KIR2DL1, a gene common to both the A and B haplotypes, and the presence of KIR2DL3, a gene unique to the A haplotype, appear to improve survival (Table 5). These data argue that all KIR genes, whether on the A or B haplotypes, are important. While Gagne et al. (31) did not analyze the influence of the total number of KIR on outcome, they did claim that in related donor transplants, the frequency of severe aGHVD was reduced if the recipient KIR repertoire was included in that of the donor. This would occur most often when the donor had a relatively large number of KIR genes and would thus be consistent with our findings. However, in unrelated donor transplants Gagne et al. found more severe aGVHD when the recipient's KIR repertoire was included in that of the donor – the opposite of what they found in related transplants and the opposite to what we report in our current analysis. Clearly, it is too early to draw any definite conclusions in relation to these variables. Nevertheless, if the data described in the current study can be reproduced, it suggests that selecting donors with a large number of KIR genes may result in major benefits to transplant outcome.

References

1. Colonna M, Spies T, Strominger JL et al. Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. *Proc Natl Acad Sci USA* 1992: **89**: 7983–85.
2. Ciccone E, Viale O, Pende D et al. Specific lysis of allogeneic cells after activation of CD3-lymphocytes in mixed lymphocyte culture. *J Exp Med* 1988: **168**: 2403–8.
3. Moretta A, Bottino C, Pende D et al. Identification of four subsets of human CD3-CD16+ natural killer (NK) cells by the expression of clonally distributed functional surface molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. *J Exp Med* 1990: **172**: 1589–98.
4. Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. *Science* 1993: **260**: 1121–24.

5. Ruggeri L, Capanni M, Urbani E et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 2002: **295**: 2097–100.
6. Leung W, Iyengar R, Turner V et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol* 2004: **172**: 644–50.
7. Giebel S, Locatelli F, Lamparelli T et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood* 2003: **102**: 814–19.
8. Davies SM, Ruggieri L, DeFor T et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. *Blood* 2002: **100**: 3825–27.
9. Lowe EJ, Turner V, Handgretinger R et al. T-cell alloreactivity dominates natural killer cell alloreactivity in minimally T-cell-depleted HLA-non-identical paediatric bone marrow transplantation. *Br J Haematol* 2003: **123**: 323–26.
10. Bishara A, Amar A, Brautbar C, Condiotti R, Lazarovitz V, Nagler A. The putative role of HLA-C recognition in graft versus host disease (GVHD) and graft rejection after unrelated bone marrow transplantation (BMT). *Exp Hematol* 1995: **23**: 1667–75.
11. Bornhauser M, Schwerdtfeger R, Martin H et al. Role of KIR ligand incompatibility in hematopoietic stem cell transplantation using unrelated donors. *Blood* 2004: **103**: 2860–61.
12. Karre K. How to recognize a foreign submarine. *Immunol Rev* 1997: **155**: 5–9.
13. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK recognition. *Immunol Today* 1990: **11**: 237–44.
14. Wagtmann N, Rojo S, Eichler E, Mohrenweiser H, Long EO. A new human gene complex encoding the killer cell inhibitory receptors and related monocyte/macrophage receptors. *Curr Biol* 1997: **7**: 615–18.
15. Wagtmann N, Rajagopalan S, Winter CC, Peruzzi M, Long EO. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity* 1995: **3**: 801–9.
16. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* 1995: **268**: 405–8.
17. Dohring C, Scheidegger D, Samaridis J, Cella M, Colonna M. A human killer inhibitory receptor specific for HLA-A. *J Immunol* 1996: **156**: 3098–101.
18. Uhrberg M, Valiante NM, Shum BP et al. Human diversity in killer cell inhibitory receptor genes. *Immunity* 1997: **7**: 753–63.
19. Witt CS, Dewing C, Sayer DC, Uhrberg M, Parham P, Christiansen FT. Population frequencies and putative haplotypes of the killer cell immunoglobulin-like receptor sequences and evidence for recombination. *Transplantation* 1999: **68**: 1784–89.
20. Katz G, Markel G, Mizrahi S, Arnon TI, Mandelboim O. Recognition of HLA-Cw4 but not HLA-Cw6 by the NK cell receptor killer cell Ig-like receptor two-domain short tail number 4. *J Immunol* 2001: **166**: 7260–7267.
21. Spaggiari GM, Contini P, Dondero A et al. Soluble HLA class I induces NK cell apoptosis upon the engagement of killer-activating HLA class I receptors through FasL-Fas interaction. *Blood* 2002: **100**: 4098–4107.
22. Mandelboim O, Kent S, Davis DM et al. Natural killer activating receptors trigger interferon gamma secretion from T cells and natural killer cells. *Proc Natl Acad Sci U S A* 1998: **95**: 3798–3803.
23. Vales-Gomez M, Reyburn HT, Erskine RA, Strominger J. Differential binding to HLA-C of p50-activating and p58-inhibitory natural killer cell receptors. *Proc Natl Acad Sci U S A* 1998: **95**: 14326–14331.
24. Cook MA, Milligan DW, Fegan CD et al. The impact of donor KIR and patient HLA-C genotypes on outcome following HLA-identical sibling hematopoietic stem cell transplantation for myeloid leukemia. *Blood* 2004: **103**: 1521–1526.
25. Hsu KC, Liu XR, Selvakumar A, Mickelson E, O'Reilly RJ, Dupont B. Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol* 2002: **169**: 5118–5129.
26. Bishara A, De Santis D, Witt CC et al. The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. *Tissue Antigens* 2004: **63**: 204–11.
27. Leclair B, Fregeau CJ, Aye MT, Fourney RM. DNA typing for bone marrow engraftment follow-up after allogeneic transplant: a comparative study of current technologies. *Bone Marrow Transplant* 1995: **16**: 43–55.
28. Przepiorka D, Weisdorf D, Martin P et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant* 1995: **15**: 825–828.
29. Maxwell LD, Wallace A, Middleton D, Curran MD. A common KIR2DS4 deletion variant in the human that predicts a soluble KIR molecule analogous to the KIR1D molecule observed in the rhesus monkey. *Tissue Antigens* 2002: **60**: 254–258.
30. Yu YYL, Kumar V, Bennett M. Murine natural killer cells and marrow graft rejection. *Annu Rev Immunol* 1992: **10**: 189–213.
31. Gagne K, Brizard G, Gueglio B et al. Relevance of KIR gene polymorphisms in bone marrow transplantation outcome. *Hum Immunol* 2002: **63**: 271–280.

CHAPTER 6: RAPID, FLOW CYTOMETRIC ASSAY FOR NK ALLOREACTIVITY REVEALS EXCEPTIONS TO RULES GOVERNING ALLOREACTIVITY.

De Santis D, Foley BA, John E, Senitzer D, Christiansen FT, Witt CS

If NK cell alloreactivity is to be exploited in stem cell transplants, it will be important to be able to reliably select donors that are alloreactive towards the patient. Prior to stem cell transplants, donor alloreactivity towards the patient is evaluated currently by NK cloning followed by testing of NK clones in the ^{51}Cr-release assay. This approach is laborious and time consuming with final results taking up to 6 weeks. The development of a functional assay of NK alloreactivity without the need for NK cloning was desirable in the routine clinical transplant setting. In this chapter, I describe the development of a flow cytometric cytotoxicity assay utilising CD107a expression on 12-day polyclonally expanded NK cells.

The findings were published in;

Biol Blood Marrow Transplant. 2010 Feb;16(2):179-91.

The supplementary data as been included in this thesis as Appendix IV.

Contributions:

DDS performed HLA typing and KIR genotyping on NK donors, prepared NK polyclonal cells for CD107a assay, established and maintained BLCL used as target cells, developed the CD107a cytotoxicity assay reported, performed KIR phenotyping, analysed the flow cytometric data and undertook the initial preparation of the manuscript.

BAF assisted with sample testing using CD107a assay, performed NKG2A and KIR2DS1 experiments and assisted in the preparation of the manuscript.

EJ performed the KIR phenotyping on the fresh polyclonal NK cells.

DS provided the multiplex PCR-SSP primers used for genotyping the NK donors.

FTC assisted in the study design and in the preparation of the manuscript.

CW assisted in the study design, performed statistical analysis and was responsible for the final preparation of the manuscript.

ASBMT
American Society for Blood and Marrow Transplantation

Rapid, Flow Cytometric Assay for NK Alloreactivity Reveals Exceptions to Rules Governing Alloreactivity

Dianne De Santis,[1,2] Bree A. Foley,[1] Elisabeth John,[2] David Senitzer,[3] Frank T. Christiansen,[1,2] Campbell S. Witt[1,2]

Alloreactive NK cells lyse target cells lacking self-HLA-C or the HLA-B-Bw4 epitope. Prior to haploidentical stem cell transplants, donor alloreactivity toward the patient is evaluated by natural killer (NK) cloning followed by testing of the clones in the ^{51}Cr-release assay. As only a few percent of NK clones are alloreactive, a large number of NK clones must be established and evaluated. This approach is laborious and time consuming, with a complete evaluation taking up to 6 weeks. We developed a flow cytometry-based cytotoxicity assay utilizing CD107a expression on 12-day polyclonally expanded NK cells and showed that NK alloreactivity mediated by inhibitory and activating KIR can be detected by measuring CD107a expression following incubation with targets lacking the appropriate class I epitope. The percentage of alloreactive NK cells varied greatly between individuals and was easily estimated by the CD107a assay. For each epitope (C1, C2, Bw4), donors were found who did not have alloreactivity, although alloreactivity was predicted by the current rules thought to govern alloreactivity. The data emphasize the importance of demonstrating alloreactivity in a functional assay.

Biol Blood Marrow Transplant 16: 179-191 (2010) © 2010 Elsevier Inc. All rights reserved.

KEY WORDS: Human, Natural Killer Cells, Transplantation, MHC

INTRODUCTION

Natural Killer (NK) cell alloreactivity can be exploited in haploidentical hematopoietic stem cell transplantation (HSCT) to prevent leukemia relapse, rejection, and graft-versus-host disease (GVHD) [1], and is also being investigated for treatment of leukemia using nonmyeloablative (NMA) regimens [2]. However, confirming donor alloreactivity is complex and time consuming.

Lysis by NK cells is inhibited when their inhibitory receptors recognize class I HLA molecules on potential target cells. A particular class of inhibitory receptor known as killer cell immunoglobulin-like (KIR) receptors recognizes allelic epitopes on HLA-C and HLA-B molecules [3,4]. All HLA-C alleles can be divided into 2 groups, based on their amino acid at position 80 [5]. C1-group alleles have an asparagine at position 80 and are recognized by the inhibitory KIR receptors KIR2DL2 and KIR2DL3. C2-group alleles have a lysine at position 80 and are recognized by KIR2DL1 [3]. HLA-B alleles can also be divided into 2 groups, Bw4 and Bw6, based on the amino acids 77-83. KIR3DL1 recognizes HLA-B and some HLA-A alleles with the Bw4 epitope [4]. During development in individuals who lack one of these allelic epitopes, NK cells expressing the inhibitory KIR for that epitope as their only inhibitory receptor fail to become armed for cytotoxicity [6,7], thereby preventing the generation of autoreactive NK cells.

To a large extent, donor NK alloreactivity toward a recipient can be predicted if the recipient lacks one of the C1, C2, or Bw4 epitopes that is present in the donor. However, this assumes that (1) all HLA-C and HLA-B alleles interact with the appropriate inhibitory KIR receptors as predicted by the presence of the relevant amino acids, and (2) that all alleles of the inhibitory KIR receptors interact the same way with the HLA epitopes. These assumptions are not always valid. HLA-B13, although known to have the Bw4 epitope, does not interact with KIR3DL1 [8], whereas KIR2DL2 and KIR2DL3 may interact with both C1- and C2-type HLA-C alleles [9-11]. Failure of a particular

From the [1]School of Pathology and Laboratory Medicine, University of Western Australia, Nedlands, Australia; [2]Department of Clinical Immunology and Immunogenetics, PathWest, Royal Perth Hospital, Perth, Australia; and [3]Division of Hematology & Hematopoietic Cell Transplantation, City of Hope National Medical Center, Duarte, California.

Current address for Bree A. Foley: Division of Hematology, Oncology and Transplantation, University of Minnesota Cancer Center, Minneapolis, Minnesota.

Financial disclosure: See Acknowledgments on page 191.

Correspondence and reprint requests to: Campbell Witt, PhD, Department of Clinical Immunology and Immunogenetics, Royal Perth Hospital, GPO Box X2213, Perth, WA 6000, Australia (e-mail: campbell.witt@health.wa.gov.au).

Received May 8, 2009; accepted October 26, 2009

© 2010 Elsevier Inc. All rights reserved.

1083-8791/10/162-0004$36.00/0
doi:10.1016/j.bbmt.2009.10.026

Table 1. HLA Types and KIR Epitopes of BLCL Used as Target Cells Used in the CD107a Assay

	KIR Epitopes					
Identifier	C1	C2	BW4	HLA-A	HLA-B	HLA-C
721.221	–	–	–			
"All"	+	+	+	01, 03	27, 44	01, 04
"C1⁻"	–	+	+	02	57	06
"C2⁻"	+	–	+	33	58	03
"Bw4⁻"	+	+	–	03, 11	07, 35	04, 07

combination of HLA and KIR alleles to interact as predicted will result in failure of the predicted NK alloreactivity to develop in potential donors or may result in a recipient's cells unexpectedly being resistant to lysis by donor NK cells. Moreover, there is increasing evidence that activating receptors, particularly KIR2DS1, interacting with their ligands, may mediate allorecognition and further complicate the rules [11-13]. As there has been no comprehensive study to determine which allelic variants of the KIR alleles interact with the different HLA alleles, it is not possible to reliably predict alloreactivity from HLA types and KIR genotype and, therefore, a functional assay is desirable.

Functional demonstration of donor NK alloreactivity toward patient target cells is currently confirmed by generating donor NK cell clones and testing the ability of these NK clones to lyse a patient's cells in the ^{51}Cr-release cytotoxicity assay [1]. As only a few percent of NK cells are alloreactive [14], it is necessary to generate and test at least 200 NK clones to be sure of detecting low-frequency alloreactive NK cells. This process is labor-intensive, often requiring 4 to 6 weeks to complete, and is thus a significant deterrent for centers that might otherwise exploit alloreactive NK cells. We have therefore developed a flow cytometric, CD107a-based assay capable of detecting NK cell alloreactivity in 14 days. Although the results of this assay correlate well with predictions of alloreactivity based on HLA types and KIR genotype, there were exceptions.

MATERIALS AND METHODS

NK Cells

Blood was obtained from 42 consenting laboratory and volunteer blood donors. All were typed for HLA-A, -B, and -C alleles (Table 1, Supplementary data) and genotyped for the presence or absence of 10 KIR genes (Table 2, Supplementary data). NK cells were purified by ficoll centrifugation with RosetteSep (StemCell Technologies, Vancouver, Canada). Purified polyclonal NK cells were expanded by culturing with irradiated allogeneic feeder cells (peripheral blood mononuclear cells [PBMC] pooled from at least 5 donors) at a 1:10 ratio for 12 days with 200 IU/mL IL-2 (Chiron, Emeryville, CA) replacing the NK medium every 2 to 3 days. Before use in the CD107a assay, 12-day cultured polyclonal NK cells were cultured at 1×10^6/mL for a further 48 hours with 400 IU/mL IL-2. In some experiments, freshly isolated PBMC cultured for 48 hours in the presence of 1000 IU/mL IL-2, but without feeder cells, were used in the CD107a assay.

Target Cells

B lymphoblastoid cell line (BLCL) with various combinations of C1, C2, or Bw4 epitopes, were used as target cells (Table 1). All cells were cultured in RPMI-1640 (Invitrogen Carlsbad, CA), with 10% heat-inactivated fetal calf serum (FCS) (Thermo-Trace, Melbourne Australia).

KIR Genotyping and HLA Typing

HLA-typing of NK donors and target cells was performed by DNA sequencing of exons 2 and 3. KIR genotyping of donors was performed using a multiplex PCR-sequence-specific primers (SSP) [15].

CD107a Cytotoxicity Assay

The expression of CD107a on the membrane has been shown to be a good correlate of target cell lysis in the ^{51}Cr-release assay [16], and was therefore used as the marker for NK-mediated cytotoxicity; 10^5 polyclonally expanded NK cells or 10^6 IL-2 activated PBMC were incubated at 37°C with 10^5 BLCL target cells in a final volume of 200 μL in a 96-well round-bottom plate to which 5 μL of anti-CD107a-FITC (BD Biosciences, Franklin Lakes, NJ) was added. After 1-hour incubation, 6 μg/mL monensin (BD GolgiStop™ BD Biosciences) was added, the cells incubated a further 5 hours, and then resuspended in 50 μL of an antibody cocktail containing anti-CD56-PECy7 (BD Biosciences) and the appropriate conjugated KIR antibodies (Table 2). Duplicate tubes were analyzed on a BD FACSCanto™ flow cytometer using BD

Table 2. Antibodies Used to Identify Potentially Alloreactive NK Cells

Antibody	Label	Clone	Dilution	KIR Reactivity	Supplier
CD158a	APC	EB6B	1/25	2DL1, 2DS1	Beckman Coulter
CD158b	APC	GL183	1/25	2DL2, 2DL3, 2DS2	Beckman Coulter
CD158a	PE	HP-3E4	1/10	2DL1, 2DS1	BD Biosciences
CD158b	PE	CH-L	1/10	2DL2, 2DL3, 2DS2	BD Biosciences
CD158e	PE	DX9	1/10	3DL1	BD Biosciences
CD56	PE-Cy7	B159	1/40		BD Biosciences

FACSDiva software. Events were initially gated on forward and side scatter (SSC) to identify lymphocytes. A bivariate plot of CD56 versus SSC was used to acquire at least 10,000 CD56$^+$ cells. CD56$^+$ cells were further gated to identify cells expressing the KIR phenotype of interest, for example, CD158a$^+$ or CD158a$^+$/158b$^-$. The cells of interest were then analyzed in a bivariate plot showing the receptor of interest (eg, CD158a) on one axis and CD107a on the other axis to determine the percentage of cells with the KIR phenotype of interest that were also CD107a$^+$. Not all donors were tested in all assays. Those tested in each assay are shown in Table 1, Supplementary data. Only donors with KIR3DL1 expression confirmed by flow cytometry were tested for HLA-Bw4-dependent NK cells.

Statistical Analysis

CD107a expression was analyzed as the mean of duplicate wells. The significance of differences in the number of CD107a-positive cells between NK donors of different genotypes in Figures 3 and 4 were tested by Fisher's exact test after setting a cutoff that gave greatest distinction between genotypes.

RESULTS

NK cells identified as potentially alloreactive by virtue of their KIR receptor expression upregulate CD107a in response to targets lacking the relevant HLA epitope

Initially we examined the feasibility of using CD107a expression to detect alloreactive NK cells. NK cells capable of lysing targets lacking the C1 epitope (C1-dependent) would be expected to express KIR2DL2 or KIR2DL3 (CD158b$^+$) in the absence of other inhibitory receptors, particularly KIR2DL1 (CD158a$^-$). Therefore, CD107a expression was examined on such "potentially C1-dependent NK cells." Similarly, NK cells capable of expressing CD107a in the presence of targets lacking the C2 epitope (C2-dependent) or the Bw4 epitope (Bw4-dependent) would be expected to express only KIR2DL1 (CD158a$^+$) in the absence of other inhibitory receptors (Cd158b$^-$) or KIR3DL1 (CD158e$^+$) in the absence of other inhibitory receptors (CD158a$^-$, CD158b$^-$), respectively.

Twelve-day polyclonally expanded NK cells from a donor whose HLA type included the C1, C2, and Bw4 epitopes were incubated with different BLCL targets each lacking just one of the C1, C2, or Bw4 epitopes (see Table 1 for the HLA types of the C1$^-$, C2$^-$, Bw4$^-$ targets) or with a target expressing all epitopes (All) or with the class I-negative 721.221 BLCL (721.221) or without any target present. NK cells that were potentially alloreactive toward targets lacking a single epitope (ie, potentially C2-dependent, C1-dependent, or Bw4-dependent) were gated on the basis of their KIR expression as described earlier. The results of one representative experiment are shown in Figure 1. In the absence of a target cell, ≤1% of all 3 potentially alloreactive NK subsets expressed CD107a (Figure 1A). Only a small proportion (1.6%-2.4%) of potentially alloreactive NK cells were CD107a$^+$ when incubated with the negative control target expressing all (C1, C2, Bw4) epitopes (Figure 1B), whereas the majority (57%-67%) were positive when incubated with the positive control target 721.221. As shown in Figure 1D-F, potentially alloreactive NK cells expressed CD107a when incubated with targets lacking the relevant inhibitory ligand, but few expressed CD107a when incubated with targets lacking irrelevant inhibitory ligand. For example, Figure 1E shows that 10.5% of potentially C2-dependent NK cells (CD158a$^+$, CD158b$^-$) expressed CD107a when incubated with the C2$^-$ (C1/1) target compared to only 4.4% (Figure 1D) and 2.2% (Figure 1F) when incubated with other C2$^+$ targets (C1$^-$ target and Bw4$^-$ target, respectively); proportions that are similar to those when incubated with the target bearing all epitopes (1.6%; Figure 1B). Similar findings are shown for potentially C1-dependent NK cells in the presence of the C1$^-$ (C2/2) target and potentially Bw4-dependent NK cells in the presence of the Bw4$^-$ (Bw6/6) target. Thus, for an individual with all three epitopes, CD107a was expressed on the appropriate population of NK cells when incubated with targets lacking the relevant epitope.

We next examined the influence of the NK donor's HLA epitopes on the presence of alloreactive NK cells. As shown in Figure 2, alloreactive NK cells could only be identified if the missing epitope on the target is a self-epitope. Polyclonally expanded NK cells from a C1$^+$ (C1/1) and a C1$^-$ (C2/2) donor were incubated with a negative control target with all epitopes ("All," Table 1), with a positive control target 721.221, or a target lacking only C1 ("C1$^-$"). Twenty-two percent of potentially C1-dependent NK cells from the C1$^+$ donor expressed CD107a in response to the C1$^-$ target (Figure 2.A3), whereas this was true of only 2.8% of such NK cells from the C1$^-$ donor (Figure 2.A6). Potentially C1-dependent NK cells from both donors expressed CD107a in response to the class I HLA negative 721.221 target cell equally (41.4% versus 41.3%; Figure 2.A2 and A5), indicating that these cells from both donors were armed for cytotoxicity. The inability of these armed cells in the C1$^-$ donor to kill the C1$^-$ (C2/C2) target suggests these potentially C1-dependent cells must express another inhibitory receptor in C1$^-$ donors. Similar data is shown in relation to C2 (Figure 2.B1-6) and Bw4 (Figure 2.C1-6). However, in the case of the Bw4 negative donor (Figure 2 C4-6), it should be noted that whereas a relatively high

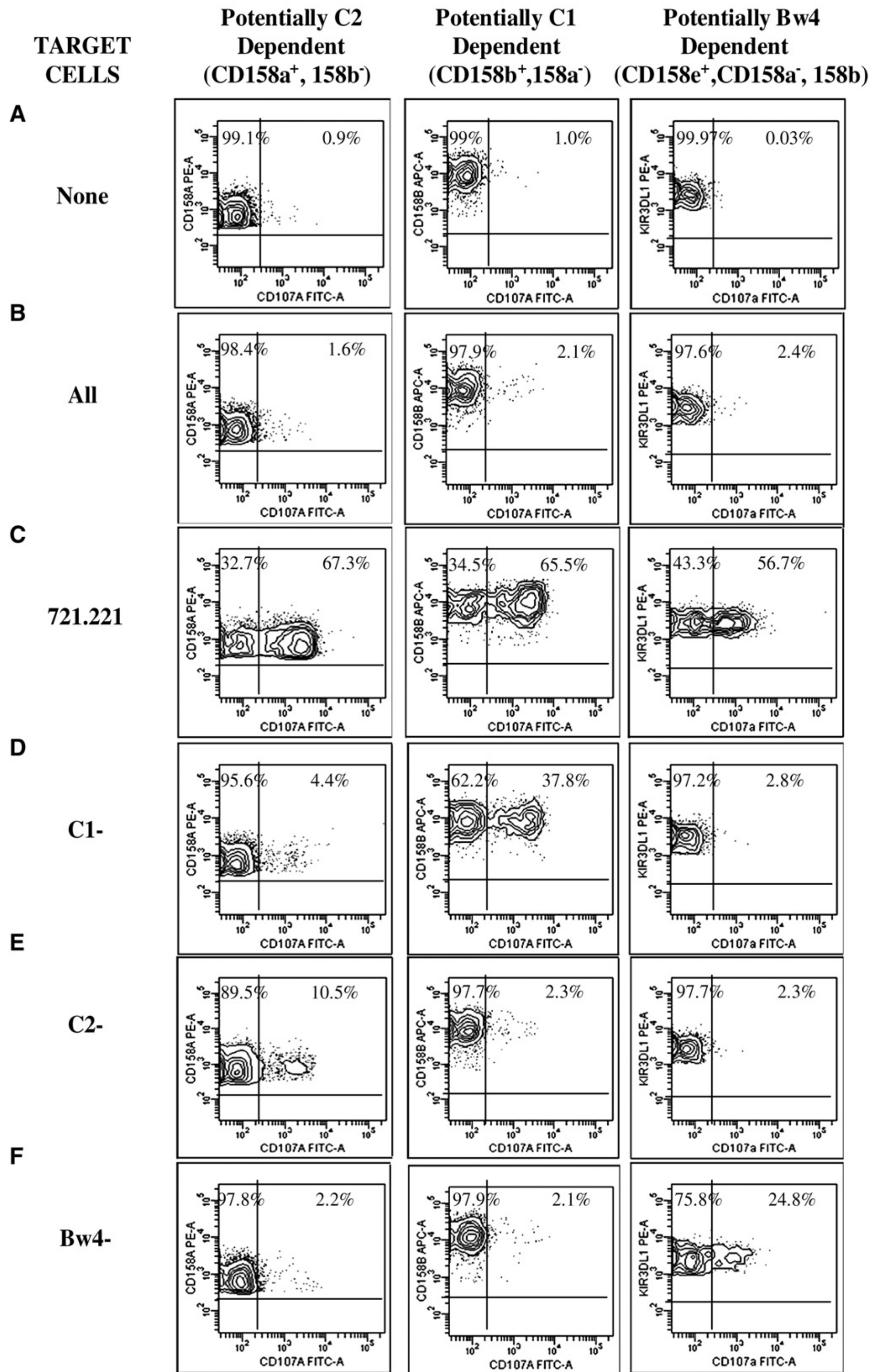


Figure 1. NK cells identified as potentially alloreactive on the basis of KIR receptor expression, express CD107a in response to target cells lacking the appropriate epitope. Day 12, polyclonally expanded NK cells from a donor whose HLA type included the C1, C2, and Bw4 epitopes (donor 5, Table 1, Supplementary data) were incubated for 6 hours with (A) no target cells (None); (B) a target expressing the C1, C2, Bw4 epitopes (All); (C) the class I HLA bare cell line 721.221 (721.221); (D) a target expressing all epitopes except C1 ($C1^-$); (E) a target expressing all epitopes except C2 ($C2^-$); (F) a target expressing all epitopes except Bw4 ($Bw4^-$) (see Table 1 for target cell details). NK cells were then stained with anti-KIR antibodies enabling the identification of NK cells potentially alloreactive toward one or other epitope negative target and CD107a expression assessed. Cells were initially gated for CD56 positivity and a second gate applied to include only those cells with the potentially alloreactive KIR phenotype. Potentially C2-dependent NK cells ($CD158a^+$, $CD158b^-$) (left hand column of graphs) expressed CD107a in the presence of 721.221 and the $C1^-$ target. Potentially C1-dependent NK cells ($CD158a^-$, $CD158b^+$) (center column of graphs) expressed CD107a in the presence of 721.221 and the $C2^-$ target. Potentially Bw4-dependent NK cells ($KIR3DL1(CD158e)^+$, $CD158a^-$, $CD158b^-$) (right-hand column of graphs) expressed CD107a in the presence of 721.221 and the $Bw4^-$ target.

percentage (4.5%) of potentially KIR3DL1-depdendent NK cells were CD107a^{+} in response to the Bw4^{-} target, the sparse dot plots indicate that Bw4 negative donors have very low absolute numbers of potentially KIR3DL1-dependent NK cells. This fact will become important in subsequent analyses (vide infra).

Detection of NK Alloreactivity Is Critically Dependent on the Analysis Method

To determine whether the CD107a assay could be used to replace NK cloning and the ^{51}Cr-release cytotoxicity assay, we asked whether the CD107a assay could reliably detect the presence of alloreactive NK cells as predicted by an individual's HLA and KIR genotype. The ability to detect NK cells alloreactive against targets lacking a single epitope was examined in a panel of 42 NK donors (HLA and KIR genotypes provided in Tables 1 and 2, Supplementary data). Day-12 polyclonally expanded NK cells isolated from these donors were incubated alone, with the HLA class I-negative 721.221 target, a target expressing all epitopes ("All"), and target cells lacking only C1 ("C1^{-}"), only C2 ("C2^{-}"), or only Bw4 ("Bw4^{-}"). The percent of NK cells expressing CD107a when incubated with each target (minus the percent positive in the absence of a target) was plotted and is shown for the C1^{-}, C2^{-} , and Bw4^{-} targets in Figure 3. If CD107a expression occurs on NK cells only in response to missing epitopes on the target, then a simple analysis of the frequency of CD107a^{+} cells as a percentage of all NK cells (ie all C56^{+} cells) should allow the enumeration of alloreactive NK cells. Such an analysis (Figure 3A) shows that this is only partially true. For example, when incubated with a C2^{-} target, C2^{+} donors (C1/C2 and C2/C2) tended to have more C2-dependent CD107a^{+} cells than C2^{-} donors (C1/C1), but there was considerable overlap between the two types of donor. Similar findings were evident for C1-dependent and Bw4-dependent NK cells using targets lacking the C1 or Bw4 epitope, respectively. The lack of a clear distinction between donors with and without predicted alloreactivity suggests that NK receptors other than the inhibitory KIR may mediate CD107a expression in response to these BLCL targets, thereby preventing detection of CD107a expression only because of the missing HLA epitope.

In an attempt to minimize the effect of irrelevant receptors we restricted the analysis to only NK cells expressing the inhibitory KIR receptor relevant to the missing epitope. As shown in Figure 3B, this adjustment resulted in little improvement in distinction between donors with and without predicted alloreactivity. We then further restricted the analysis of CD107a expression to only those NK cells expressing inhibitory KIR relevant to the missing epitope on the target cell and not expressing the other inhibitory KIR (Figure 3C). This resulted in improved discrimination between donors with and without predicted alloreactivity against C2^{-} and C1^{-} targets, but not for Bw4^{-} targets.

The analysis so far examined the percentage of CD107a expression among cells identified as potentially alloreactive without taking into account the frequency of such cells as a proportion of all NK cells. Figure 3D shows the percentage of all NK cells expressing the receptor phenotype of potentially alloreactive NK cells without reference to CD107a expression. It can be seen that for many individuals, these cells are infrequent. In particular, the percentage of CD158a^{+}, CD158b^{-} cells present in C1 homozygous individuals (Figure 3D, left) and the percentage of CD158e^{+}, CD158a^{-}, CD158b^{-} NK cells in Bw6 homozygous individuals (Figure 3D, right) was very low, and therefore the estimates of the proportion of these cells expressing CD107a, as plotted in Figure 3C, may lack precision. We therefore plotted the number of NK cells that expressed the relevant inhibitory KIR receptor and not other KIR (potentially alloreactive) and that were CD107a^{+} as a percentage of all NK cells (CD56^{+} cells), on a linear (Figure 3E) and log scale (Figure 3F). As shown, for NK donors whose HLA epitopes would predict inability to generate alloreactive NK cells for that particular epitope, these cells were invariably present at a frequency of <1%, whereas in most individuals in whom alloreactivity was predicted, such cells were present at a frequency >1% (P < .0001 for all comparisons of number of CD107a^{+} cells in donors with the ligand versus donors without the ligand).

The CD107a Assay Can Be Used to Evaluate the Rules of NK Allorecognition

Given that the CD107a assay usually detected expected NK alloreactivity when the appropriate method of analysis was used, we examined more closely the apparent exceptions. As shown in Figure 3F (left), it can be seen that all C2^{-} (C1/1) NK donors had <1% (and generally fewer than 0.1%) C2-dependent NK cells when incubated with C2^{-} targets. In contrast most C2^{+} donors (all but two C1/2 donors and all but two C2/2 donors) had >1% such cells. The two C1/2 exceptions are actually replicate estimations for the same individual (donor 4, Table 1, Supplementary data) assayed on different days. By DNA sequencing, this individual was shown to be homozygous for the KIR2DL1*004 allele, which is found on some KIR-B haplotypes, suggesting that the KIR2DL1*004 allele may not interact with the C2 epitope. Although not tested, it is unlikely that any other C2^{+} donors had KIR2DL1*004 as their only KIR2DL1 allele because all other donors had KIR2DL3, which is present

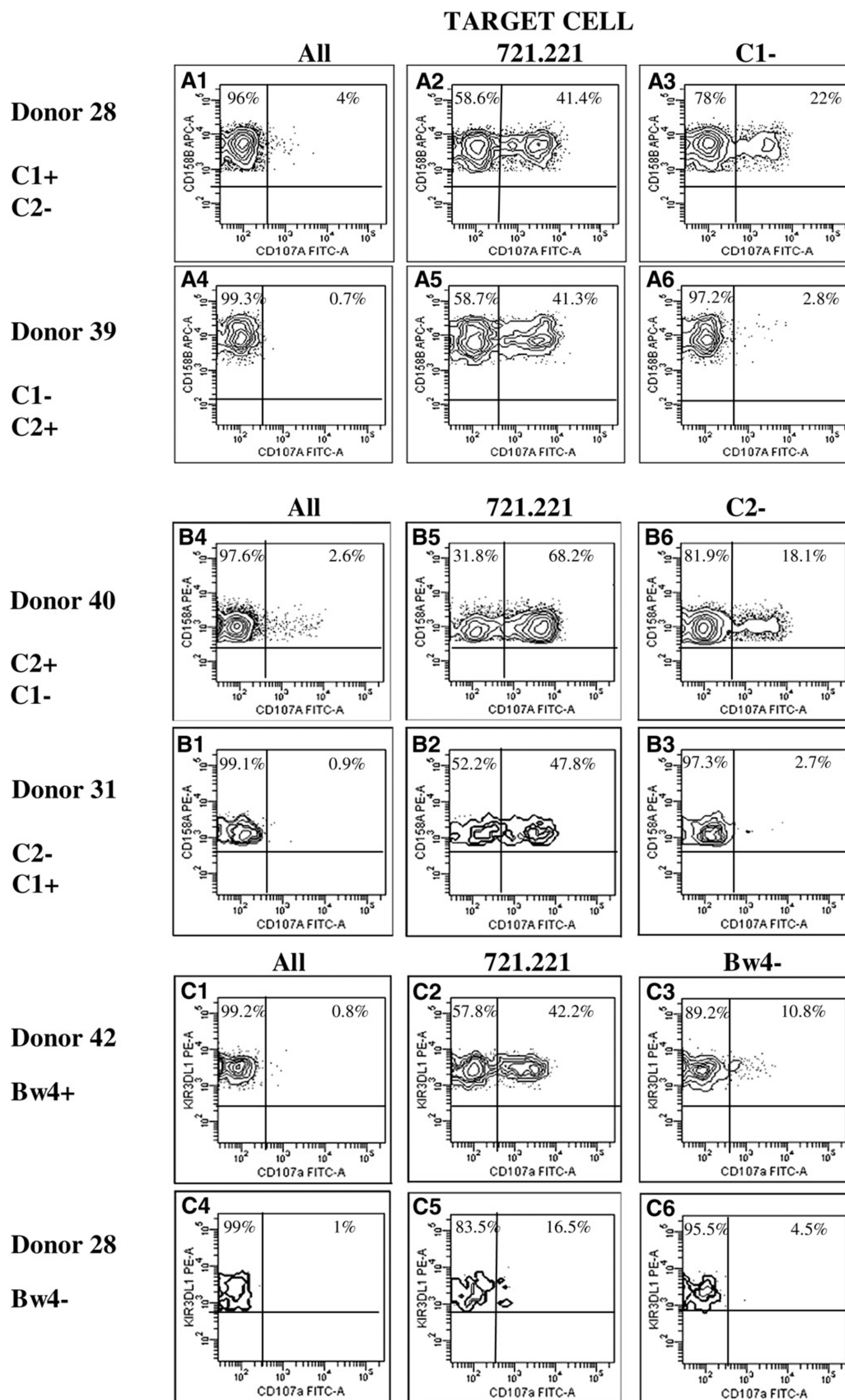


Figure 2. CD107a expression in response to epitope negative targets is dependent on the HLA type of the NK cell donor. Day 12, polyclonally expanded NK cells from a C1$^+$ (C1/1) donor (donor 28 in Table 1, Supplementary data) and a C1$^-$ (C2/2) donor (donor 39 in Table 1, Supplementary data) were incubated with target cells expressing all epitopes ("All" in Table 1) (negative control) (A1 and A4), 721.221 (positive control) (A2 and A5) and a C1$^-$ target (C1$^-$ in Table 1) (A3 and A6) for 6 hours. Potentially C1-dependent (CD158a$^-$, CD158b$^+$) NK cells were then examined for CD107a expression. NK cells from the C1$^+$ (C1/1) donor expressed CD107a in the presence of 721.221 (A2) and the C1-target cell (A3), whereas NK cells from the C1$^-$ (C2/2) donor only expressed CD107a in response to 721.221 (A5) and not the C1$^-$ target (A6). Similar data are shown for C2$^+$ (donor 4) and C2$^-$ (donor 41) donors (B1-B6) and for Bw4$^+$ (donor 42) and Bw4$^-$ (donor 28) donors (C1-C6).

only on KIR-A haplotypes, which almost invariably carry a KIR2DL1 allele other than KIR2DL1*004. The two C1$^-$ donors (C2/2, donors 36, 37 in Table 1, Supplementary data) who had <1% CD107a$^+$ NK cells in the presence of the C2$^-$ target lacked the KIR2DL1 gene and would therefore not be expected to have such alloreactive NK cells.

All C1$^-$ (C2/2) donors had <2% C1-dependent NK cells when incubated with C1$^-$ targets (Figure 3F, middle) whereas, with one exception, all C1$^+$ (C1/2 and C1/1) donors had >4% such cells. The single exception (donor 10, Table 1, Supplementary data) cannot be explained by any of the current rules for alloreactivity. This donor is homozygous for KIR2DL3 gene and has both the KIR2DL3*001 and KIR2DL3*002 alleles. The donor's C1 ligand is HLA-Cw*07, which has been reported to be an effective KIR2DL2/3 ligand [17]. For reasons that are not clear, this donor's alloreactivity was heavily biased towards the use of KIR2DL1 (data not shown).

All Bw4$^-$ (Bw6/6) NK donors had <1% Bw4-dependent NK cells when incubated with the Bw4$^-$ target, whereas most Bw4$^+$ donors had >1% such cells (Figure 3F, right). However, six Bw4$^+$ donors had <1% Bw4-dependent NK cells. The lack of such cells in these 6 donors could not be explained by homozygosity for the nonexpressed allele of KIR3DL1 (KIR3DL1*004), as all donors were shown by flow cytometry prior to testing to have NK cells expressing KIR3DL1. The only Bw4$^+$ allele in three of these donors was HLA-B13, consistent with our recent report that HLA-B13, does not act as a ligand for KIR3DL1. In a fourth donor, HLA-A24 was the only Bw4$^+$ allele, suggesting that HLA-A24 may not always be an effective ligand for the arming of Bw4-dependent NK cells. The reason for the low number of Bw4-dependent NK cells in the remaining 2 donors is unclear. One had both HLA-B57 and HLA-A32 as Bw4$^+$ alleles and, although one of this donor's KIR3DL1 alleles was the non-membrane expressed KIR3DL1*004, the other allele was KIR3DL1*005, which is known to interact with HLA-B57 [8]. The other donor had HLA-B44 (Bw4$^+$) and the KIR3DL1 alleles *001 and *007 and it seems unlikely that neither of these alleles would interact with HLA-B44. Although the frequency of Bw4-dependent NK cells was low in these 6 exceptions, 5 of the 6 donors had between 0.4% and 1% Bw4-dependent NK cells. This frequency was higher than for any of the Bw4$^-$ (Bw6/6) donors, suggesting that these individuals are capable of arming Bw4-dependent NK cells, but at very low frequencies and that the CD107a assay can detect these low frequency cells.

Detection of KIR2DS1-Mediated Alloreactivity

We next tested whether the CD107a assay could detect KIR2DS1-activated alloreactive NK cells. We [13] and others [11,12] have shown that NK cells mediating alloreactivity through KIR2DS1 can be identified among CD158a$^+$ (KIR2DS1$^+$), CD158b$^-$ (KIR2DL2$^-$, KIR2DL3$^-$) cells, but only in C2$^-$ donors [13]. Although the frequency of NK cells with this KIR phenotype varies greatly between donors, it is worth noting that they are more frequent in C2$^+$ donors; individuals in whom KIR2DS1-dependent NK cells would not be expected (Figure 4A). This probably reflects the fact that the majority of CD158a$^+$ cells in such individuals are KIR2DL1$^+$ rather than KIR2DS1$^+$. As the percentage of such cells is so much greater in C2$^+$ individuals than in C2$^-$ (C1/C1) individuals, any CD107a expression among this subset of cells that is unrelated to KIR2DS1-mediated alloreactivity would therefore be likely to obscure the detection of KIR2DS1-mediated CD107a expression. Nevertheless, we initially used the analysis method that best demonstrated alloreactivity of NK cells controlled by inhibitory receptors. Figure 4B shows the percentage of all CD56$^+$ NK cells that are CD158a$^+$, CD158b$^-$, CD107a$^+$ (the phenotype expected for KIR2DS1 controlled NK cells) after incubation with a C2 homozygous target. C2$^-$ donors had few CD107a$^+$ cells, and if CD107a expression was mediated through KIR2DS1, unexpectedly those with the KIR2DS1 gene did not have higher numbers of CD107a$^+$ cells than those without KIR2DS1 (Figure 4B). Rather, the number of CD107a$^+$ cells was closely correlated with the proportion of all CD56$^+$ NK cells that were CD158a$^+$, CD158b$^-$ (Figure 4A), most of which are probably KIR2DL1$^+$ cells. Any "background" CD107a response among these cells may be sufficiently large to obscure the low frequency KIR2DS1-controlled cells. Figure 4C suggests that this is indeed the case. When the number of CD107a$^+$ cells is taken as a percentage of the total number of CD158a$^+$, CD158b$^-$ cells (Figure 4C), then the influence of KIR2DS1 is evident. At least 14% of such cells were CD107a$^+$ among C2$^-$ (C1/1) donors who had the KIR2DS1 gene (those in whom KIR2DS1-mediated alloreactivity might be expected), whereas all C2$^-$ (C1/1 homozygous) donors without the KIR2DS1 gene and all C2$^+$ donors, with a single exception, had <14% CD107a positive cells ($P < .001$). Similar trends were observed for the C1/2 heterozygous target (data not shown) although the proportion of CD107a$^+$ cells was lower with this target, consistent with our previous report that C2 homozygous targets are more easily killed by KIR2DS1 controlled NK cells than are C1/2 heterozygous targets [13]. Taken together, these data suggest CD107a expression can be used to detect KIR2DS1-mediated NK allorecognition. However, the assay did not distinguish between donors expected to have KIR2DS1 controlled cells and donors not expected to have KIR2DS1 controlled cells as clearly as was the case for alloreactive cells controlled by inhibitory KIR.

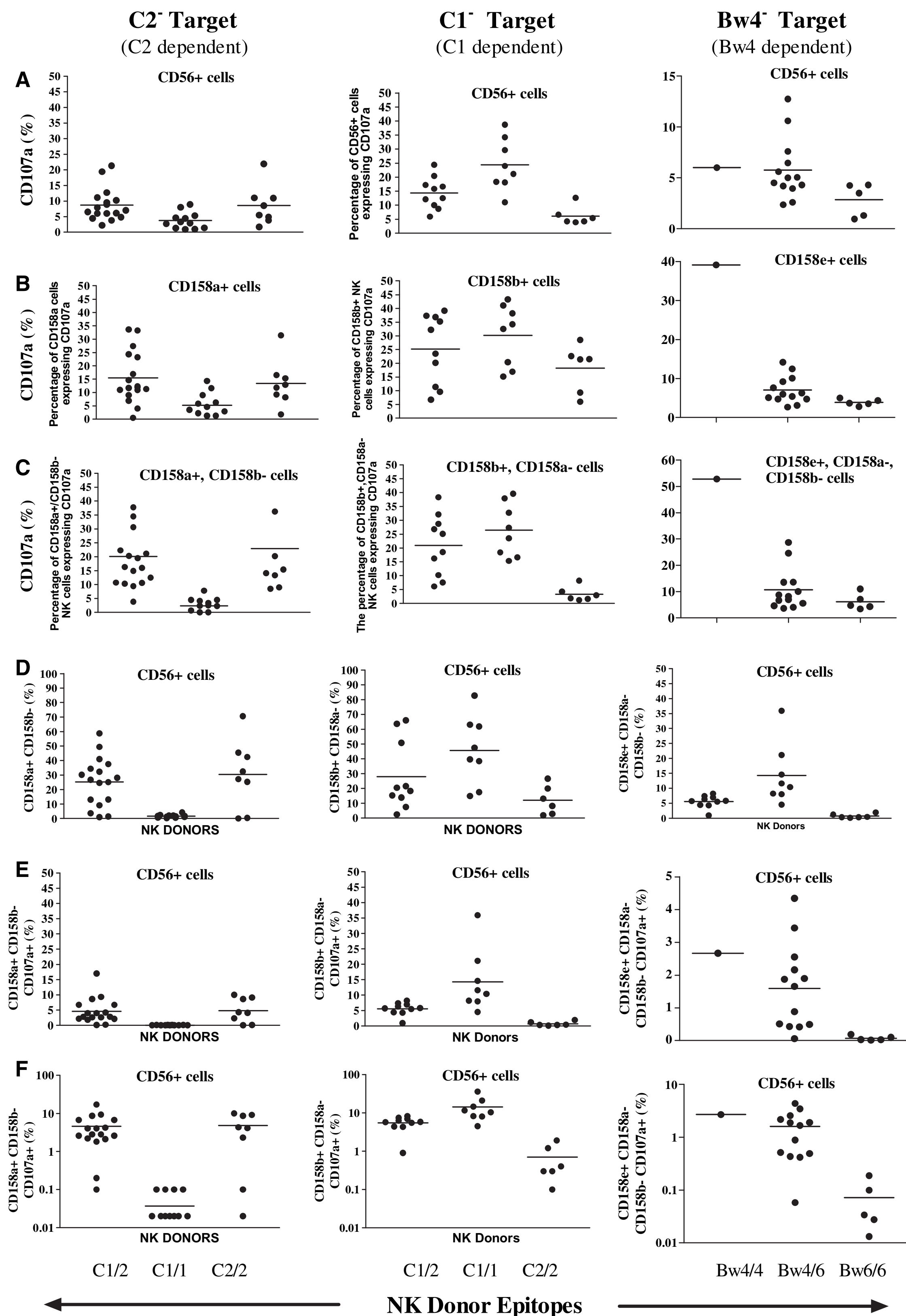


Figure 3. Appropriate analysis of CD107a data reveals alloreactivity that correlates well with alloreactivity predicted by HLA and KIR genetics. Day 12, polyclonally expanded NK cells from different donors were incubated with target cells lacking a single epitope and CD107a expression was measured on the NK cells. The heading at the top of each column indicates the target cell used (see Table 1) for the graphs in that column and, in brackets?, the ligand potentially determining NK allorecognition. The total population of cells considered (denominator) is shown at the top of each graph. Each point represents the number of cells with the phenotype shown on the vertical axis as a percentage of the denominator. The HLA epitopes present in the NK cell donors is shown at the bottom of the figure. The percentage of CD56^{+} cells expressing CD107a (A), percentage of cells with inhibitory receptor relevant to missing epitope on target cells that are expressing CD107a (B), percentage of cells with inhibitory receptor relevant to missing epitope on target cells and lacking other inhibitory KIR that are expressing CD107a (C), percentage of cells with inhibitory receptor relevant to missing epitope on target cells and lacking other inhibitory KIR (D), percentage of CD56^{+} cells with inhibitory receptor relevant to missing epitope on target cells and lacking other inhibitory KIR that are expressing CD107a (E); and the same data as in 3e but with vertical axis on log scale (F). Figure 3F shows that for NK donors whose HLA type would predict inability to generate alloreactive NK cells for that particular epitope, CD107a^{+} cells were invariably present at a frequency of <1%, whereas in most individuals in whom alloreactivity was predicted, such cells were present at a frequency >1% ($P < .0001$ for all comparisons of number of CD107a^{+} cells in donors with the ligand versus donors without the ligand).

Reproducibility

As all assays were performed in duplicate tubes, the CV was calculated for each NK donor whose frequency of alloreactive NK cells was >1%. The mean within-run CV for the estimate of C1-dependent, C2-dependent, and Bw4-dependent alloreactive NK cells was 5.9%, 13.4%, and 6.3%, respectively.

Freshly Isolated and IL-2-Activated NK Cells Are Unsuitable for the CD107a Assay

The data before show that 12-day polyclonally expanded NK cells can be used to detect NK cell alloreactivity. We also assessed whether freshly isolated NK cells or PBMC activated with IL-2 in the absence of feeder cells for 48 hours could be used to detect NK cell alloreactivity in the CD107a assay. Very few freshly isolated NK cells expressed CD107a in response to BLCL targets, so these cells were not pursued further (data not shown). PBMC from 4 donors (donors 8, 18, 28, and 42) were activated with IL-2 for 48 hours and compared with 12-day cultured polyclonal NK cells from the same donors. Donors 8 and 28 and donors 18 and 42 were used to generate C1 and Bw4-dependent NK cells, respectively, and the proportion of each as a percentage of total $CD56^+$ NK cells was determined as described in Figure 3C. Whereas 12-day cultured NK cells were used at an effector to target ratio of 1:1, the IL-2 activated PBMC were used at an effector target ratio of 10:1 as NK cells generally represent only 10% of PBMC.

The percentage of NK cells expressing CD107a in the presence of BLCL targets was markedly decreased in the IL-2 activated PBMC compared to 12-day cultured NK cells (data not shown). For the two C1-dependent NK cell donors, in the presence of the class I bare target (721.221), 12% and 19% of NK cells in the IL-2 activated PBMC expressed CD107a compared to 59% and 31% of cultured NK cells. In the presence of the $C1^-$ target, only 2% and 0.6% of IL-2 activated NK cells in the PBMC were $CD158b^+$, $CD158a^-$, and $CD107a^+$ compared to 6% and 5.3% of cultured NK cells. A similar trend was seen for the two Bw4-dependent NK cell donors. In the presence of 721.221, 17% and 15% of IL-2 activated NK cells in the PBMC were $CD107a^+$ compared to 73% and 20% of cultured NK cells. In the presence of the $Bw4^-$ target, only 0.3% and 0.15% of IL-2 activated NK cells in the PBMC were $CD158e^+$, $CD158a^-$, $CD158b^-$, $CD107a^+$ compared to 3.4% and 4.3% of cultured NK cells. The low percentage of IL-2 activated NK cells in the PBMC expressing CD107a suggested that it would be difficult to establish a reproducible assay using such cells.

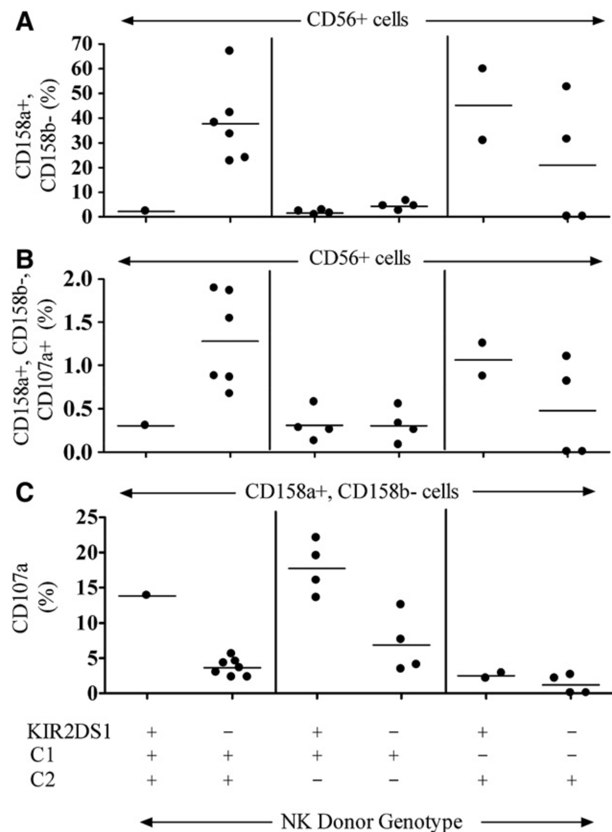


Figure 4. Appropriate analysis of CD107a data results in good correlation with KIR2DS1-mediated alloreactivity predicted by HLA and KIR genetics. Day 12, polyclonally expanded NK cells from different donors were incubated with target cells and CD107a expression was measured on the NK cells. The heading at the top of each graph indicates the membrane phenotype of NK cells analyzed (denominator). The HLA epitope phenotype and presence or absence of the KIR2DS1 gene in the NK cell donors is shown at the bottom of the figure. Each point represents for each donor the number of cells with the phenotype shown on the vertical axis as a percentage of the denominator. (A) Percentage of NK cells $CD158a^+$, $CD158b^-$ without reference to CD107a expression and in the absence of any target. (B) percentage of $CD56^+$ cells that are $CD158a^+$, $CD158b^-$, and $CD107a^+$ after incubation with a $C1^-$ ($C2^+$, $Bw4^+$) target. (C) Percentage of $CD158a^+$, $CD158b^-$ cells that are $CD107a^+$ after incubation with a $C1^-$ ($C2^+$, $Bw4^+$) target. Graph (C) shows that the expected CD107a positivity is restricted to only $KIR2DS1^+$, $C2^-$ donors ($P < .001$ for $\%CD107a^+$ cells in the $KIR2DS1^+$, $C2^-$ versus others).

Role of NKG2A+ NK Cells in the CD107a Assay

Because the ligand for NKG2A is the non-polymorphic HLA-E and because BLCL express HLA-E, it might be expected that NK cells expressing NKG2A would not be alloreactive. However, as we have found alloreactive NK clones that express NKG2A using the ^{51}Cr-release assay (unpublished data), we wondered whether such cells contributed to the alloreactivity detected in the CD107a assay. We therefore tested 2 donors for alloreactivity against BLCL targets lacking only C2 (Figure 5A) or C1 (Figure 5B) and estimated the frequency of CD107a cells with and without gating out $NKG2A^+$ cells to determine whether any alloreactive cells detected in this

assay expressed NKG2A. As shown in Figure 5, the frequency of alloreactive cells was the same in 12-day polyclonally expanded NK cultures whether NKG2A$^+$ cells were gated out or not, suggesting that the alloreactive cells detected in this assay do not express NKG2A.

Influence of 12-Day Culture on Frequency of Alloreactive NK Cells

As freshly isolated NK cells did not up regulate CD107a in response to BLCL targets, it was not possible to directly compare the frequency of functionally alloreactive clones at day 0 and day 12 using the CD107a assay. To determine whether 12 days in culture altered the frequency of potentially alloreactive NK cells, the frequency of NK cells with the potentially alloreactive KIR phenotypes analyzed in the CD107a assay was compared at day 0 and day 12. Figure 6 shows that the frequency of cells with potential alloreactivity tended to increase modestly if the donor was positive for the relevant epitope and decrease if the donor lacked the epitope. The percentage of CD158a$^+$ (Figure 6A) and CD158a$^+$,158b$^-$ (Figure 6C) NK cells increased in C2$^+$ donors (C1/2 heterozygotes) and decreased in C2$^-$ donors (C1/1 homozygotes). The single C2 homozygous donor did not have the KIR2DL1 gene and could not therefore be assessed. A tendency for CD158b$^+$ (Figure 6B) and CD158b$^+$,158a$^-$ (Figure 6D) cells to increase in C1$^+$ donors was also apparent, although two C1/2 donors showed modest decreases, and one C1/1 homozygous donor showed a marked decrease. The donor displaying the marked decrease in CD158b$^+$ cells was the same donor that displayed a marked increase in CD158a$^+$ cells.

DISCUSSION

With potentially increasing use of alloreactive NK cells in clinical practice, the ability to identify suitable donors is becoming more important. Our incomplete knowledge of KIR receptor interaction with HLA ligands mandates that donor alloreactivity toward the intended recipient be demonstrated in a functional assay. NK cloning and the ^{51}Cr-release assays are time consuming and beyond the capacity of many transplant centers. The flow cytometric assay described here is relatively simple to perform, reproducible, and can be completed in two weeks. Results obtained using purified NK cells activated by polyclonal expansion for 12 days were largely concordant with expectation based on HLA epitopes. Attempts to perform the assay using freshly isolated NK cells or PBMC cultured for 48 hours in IL-2 were largely unsuccessful, perhaps because the NK cells were insufficiently activated to kill BLCL targets efficiently. However, we did not test NK cells that had been polyclonally expanded for <12 days. Therefore, it may be possible to further shorten the assay.

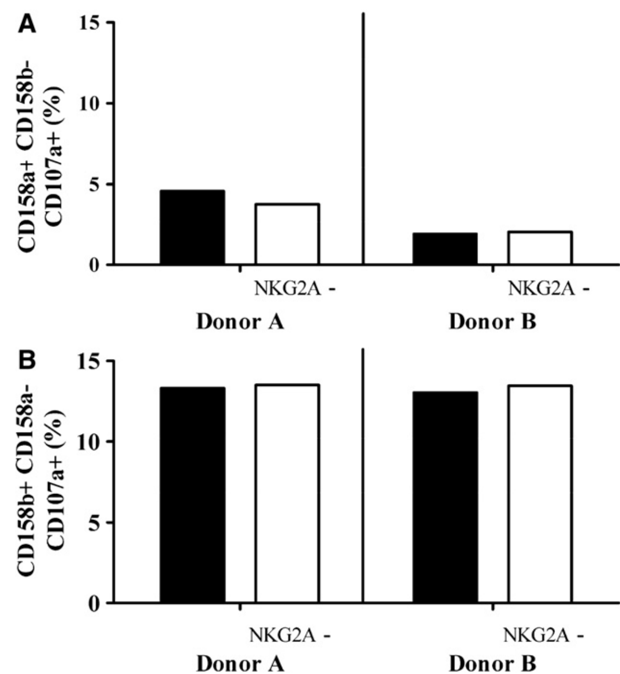


Figure 5. NKG2A+ cells do not contribute significantly to the alloreactive subsets. Twelve -day polyclonally expanded NK cells from 2 donors were incubated with target cells and CD107a expression assessed either without (solid bars) or with (open bars) gating of NKG2A positive cells. (A) The number of cells that were CD158a$^+$, CD158b$^-$, CD107a$^+$ after incubation with a target lacking only ,C2 and (B) the number of cells that were CD158b$^+$, CD158a$^-$, CD107a$^+$ after incubation with a target lacking only C1. The frequency of CD107a$^+$ cells was unchanged by NKG2A gating.

Twelve days in culture increased the percentage of NK cells with the potentially alloreactive phenotype in donors whose HLA type would predict alloreactivity, but not in other donors. In C2$^+$ donors, CD158a$^+$ NK cells tended to increase in number during culture, whereas they decreased in number when from a C2$^-$ donor. This is consistent with recent data showing that that C2$^-$ feeder cells drive proliferation of alloreactive CD158a$^+$ cells from C2$^+$ donors [18], whereas all CD158a$^+$ cells from C2$^-$ donors should be unresponsive to C2$^-$ feeder cells, and therefore would not be expected to expand. Figure 3D shows that the disappearance of these cells is almost complete in the case of CD158a$^+$, 158b$^-$ cells in C1 homozygous (C2$^-$) donors and CD158e$^+$, 158a$^-$, 158b$^-$ cells in Bw6 (Bw4$^-$) homozygous donors. The expansion of CD158b$^+$ cells in C1$^+$ donors was not as consistent as for CD158a$^+$ cells in C2$^+$ donors. This may reflect the fact that a greater proportion (34%) of random donors constituting the feeder cell pool would lack the C2 ligand, thereby supporting proliferation of CD158a$^+$ cells, than would lack the C1 epitope (19%). It could also reflect the promiscuity of KIR2DL2 and KIR2DL3, which permits these receptors to interact weakly with C2 alleles [11]. Presumably, cloning of NK cells using pooled feeder cells would result in a similar preferential growth of alloreactive clones.

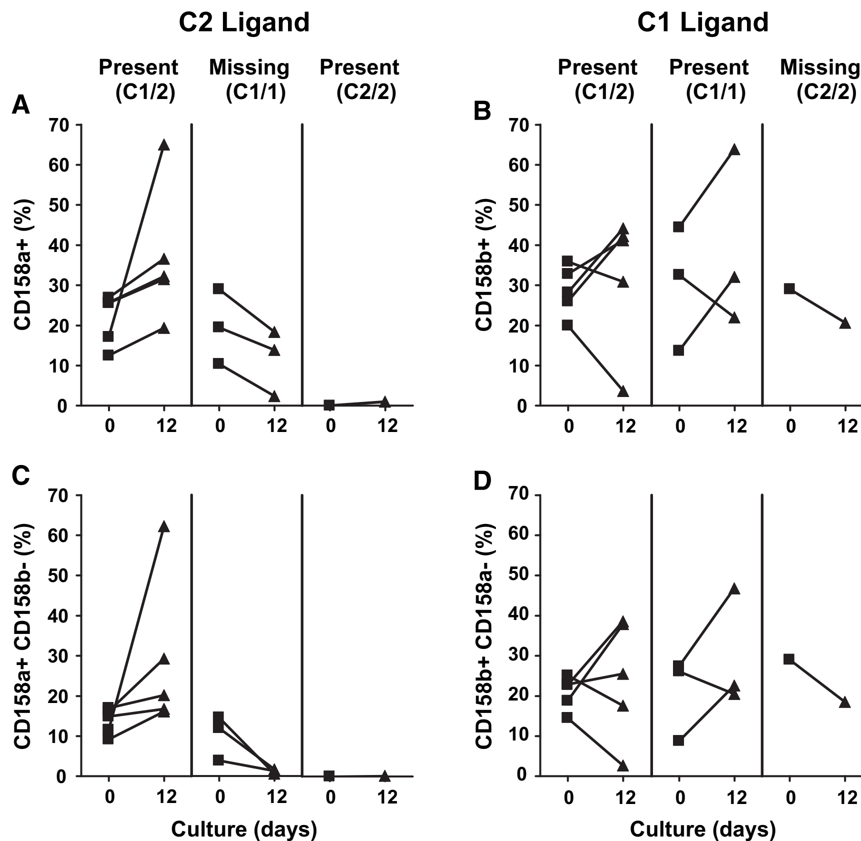


Figure 6. Twelve-day culture expands the alloreactive subset of NK cells. The percentage of NK cells from 9 donors with various C1, C2 genotypes that were CD158a$^+$ (A); or CD158a$^+$b$^-$ (C); or CD158b$^+$ (B); or CD158b$^+$,158a$^-$ (D) was estimated before and after 12 days culture with pooled, irradiated feeder cells. NK cells expressing CD158a (A,C) tended to increase in number if the NK donor had the relevant C2 ligand (C1/2 donors) and decrease in number if the donor lacks the relevant C2 ligand (C1/1 donors). The single C2 homozygous donor (C2/2) lacked the KIR2DL1 gene and thus could not be assessed. A similar, though not as pronounced, effect was seen for NK cells expressing CD158b (B,D).

Other groups have published methods that indirectly estimate the alloreactive subsets of NK cells [11,19]. However, reliable estimation of functional alloreactive NK cells cannot be achieved by simply enumerating cells with a particular KIR phenotype because, as we have shown, these cells may not be armed. Similarly, functional assays using class I HLA bare targets or HLA bare cells transfected with single HLA alleles cannot be used to accurately estimate alloreactive NK cells. Such targets will stimulate all NK cells to express CD107a except those with the relevant inhibitory KIR, only a subset of which are alloreactive. Detection of functionally alloreactive NK cells requires targets that express all HLA epitopes for inhibitory KIR except the epitope of interest as used in our method.

Our results show that the method of analyzing the flow cytometric data is critically important. We found a small percentage of NK cells express CD107a in the presence of a target that expresses all 3 epitopes (C1, C2, and Bw4). This response must be unrelated to KIR2DL1, KIR2DL2/3, KIR3DL1, and KIR2DS1, and is unlikely to represent KIR3DL2 activity, as the target with all epitopes was also HLA-A3 positive. This response interferes with the detection of C1, C2, or Bw4-dependent allo-responses unless it is minimized by restricting the analysis to only those NK cells expressing the inhibitory KIR relevant to the missing epitope. In the case of KIR2DL1 and KIR2DL2/3-mediated alloreactivity, we found it necessary only to gate out cells coexpressing these receptors, whereas in the case of KIR3DL1-mediated alloreactivity, we found it necessary to gate out cells coexpressing either KIR2DL1 and/or KIR2DL2/3 with KIR3DL1. After focusing on the relevant NK subset, it was found that the number of these cells that expressed CD107a in the presence of a target lacking the appropriate epitope, expressed as a percentage of all NK cells, correlated well with predictions of alloreactivity based on the missing ligand model. Moreover, the frequency of alloreactive cells as a percentage of all NK cells correlates well with the frequency reported by NK cloning and ^{51}Cr-release. For example, in the CD107a assay, we found C2-, C1-, and Bw4-dependent NK cells to be present at an average frequency of 6%, 10%, and 2%, which is similar to the frequencies reported by NK cloning (9%, 5%, 3%, respectively) [14].

We [8] and others [17] have recently reported that HLA-B13, despite reacting with anti-Bw4 antisera, is not a ligand for KIR3DL1. The inability of NK cells

in HLA-B13-positive donors to express CD107a in the presence of Bw4⁻ targets shown in our study is consistent with the earlier reports. We [8] and others [17,20,21] have also reported that the $Bw4^+$ HLA-A alleles, HLA-A24, A32, and to a lesser extent, HLA-A23, are ligands for KIR3DL1, and in our earlier study, we provided evidence that HLA-A24 can also induce arming of KIR3DL1-dependent NK cells [8]. In our current study, it was therefore surprising to find one individual, in whom HLA-A24 was the only $Bw4^+$ allele, but who had very low numbers of KIR3DL1-dependent NK cells. Thus, although HLA-A24 inhibits cytotoxicity, it may not always arm significant numbers of NK cells for Bw4-dependent cytotoxicity. Further studies are required to clarify this point. Although the frequency of Bw4-dependent NK cells was very low in 3 of the 4 donors who had HLA-B13 or HLA-A24 as their only $Bw4^+$ alleles, the frequency was intermediate (0.4%-0.9%) between that of donors with other $Bw4^+$ alleles and those lacking Bw4 altogether, suggesting the flow cytometric assay can detect alloreactive NK cells present at frequencies <1%. It is not clear whether such a low frequency of alloreactive clones would be efficacious in HSCTs.

In two individuals, the absence of HLA-C-mediated alloreactivity could not be explained by our current understanding of rules governing alloreactivity. One of these individuals, whose NK cells failed to express CD107a in response to a $C2^-$ target, was homozygous for KIR2DL1*004, suggesting that KIR2DL1*004 may not interact with C2, and therefore does not arm NK cells expressing this as the only inhibitory receptor. This is consistent with the recent report that this allele only weakly arms NK cells in $C2^+$ individuals as assessed by their ability to secrete IFNγ in response to class I bare 721.221 targets [17].

There are several reports implying that KIR2DL2 and KIR2DL3 distinguish poorly between the C1 and C2 epitopes [9-12]. KIR2DL3-Fc and KIR2DL2-Fc fusion proteins have been shown to bind almost equally to both C1 and C2 alleles [10]. It has even been suggested that $KIR2DL2/3^+$ NK cells generally require coexpression of KIR2DS1 to mediate cytotoxicity toward $C1^-$ (C2/C2) targets [11,12]. Chewning et al. [12], using polyclonal NK cells, reported that the alloresponse to missing self when C1 is a self-epitope, is rarely observed in NK donors lacking KIR2DS1. Pende et al. [11] also concluded that $KIR2DL2/3^+$ NK cells can barely distinguish between $C1^+$ or $C2^+$ target cells and their alloreactivity is, in most cases, dependent on the coexpression of KIR2DS1. In the ^{51}chromium-release assay employed by Chewning et al. [12], a significant alloresponse was interpreted as cytotoxicity significantly above background levels. However, there was a considerable background cytotoxicity in their system and, as we have shown, in polyclonal systems, alloresponses can be hidden among the background responses unless the analysis is restricted to those NK cells with a phenotype relevant to the missing ligand. Using this approach, we found alloresponses to $C1^-$ (C2/C2) targets (but not $C2^-$ targets) mediated by $KIR2DL2/3^+$,$KIR2DS1^-$ NK cells in all but one $C1^+$ donor. Such responses were not observed in $C1^-$ donors. Thus, as assessed by our CD107a assay, KIR2DL2/3-dependent NK cells are present in almost all $C1^+$ donors and demonstrate functional distinction between C1 and C2 epitopes without the need for KIR2DS1 expression. In the study by Pende et al. [11], in one post-transplant patient, the majority of polyclonal NK cells expressing only KIR2DL2/3 (and not KIR2DS1) were unable to kill $C1^-$ targets (or $C2^-$ targets), indicating that such cells are not always alloreactive. However, in the same patient, cytotoxic $KIR2DL2/3^+$ NK clones that did not express KIR2DS1, but did discriminate between $C1^+$ and $C2^+$ targets, were identified. Our study does not exclude the possibility that $KIR2DS1^+$ donors have $KIR2DL2/3^+$,$KIR2DS1^+$ cells, in addition to $KIR2DL2/3^+$,$KIR2DS1^-$ cells, that lyse $C1^-$ (and thus $C2^+$) targets. Thus, among C1 homozygous donors, those with KIR2DS1 may well have a greater total alloresponse to $C1^-$ targets than those without KIR2DS1. However, it is clear from our data that KIR2DS1 is not necessary for a KIR2DL2/3-mediated alloresponse.

Similar frequencies of alloreactive NK cells were achieved in different ways in different donors. For example, the single individual who was homozygous for Bw4 had relatively few (8%) KIR3DL1-positive NK cells compared to the Bw4 heterozygous donors (range: 15%-65%), a finding previously noted [22]. However, in the Bw4 homozygote, a relatively large proportion (30%) of the $KIR3DL1^+$ NK cells were Bw4-dependent, resulting in 3% of all NK cells being Bw4-dependent. In contrast, the Bw4 heterozygous individuals, although they had a higher percentage of $KIR3DL1^+$ cells, had a comparatively smaller proportion of these cells that were Bw4-dependent, resulting in a similar proportion (2%-7%) of all NK cells being Bw4-dependent.

Therapeutic approaches employing alloreactive NK cells hold considerable promise for diseases like acute myelogenous leukemia (AML). However, despite our improving knowledge of the rules governing alloreactivity, there is a continuing need for a functional assay of alloreactivity. The CD107a assay described here is simple, robust, and avoids the need for expensive and time-consuming cloning. In addition, it has proved useful in the dissection of the rules governing NK alloreactivity.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of the Ray and Bill Dobney Foundation and Western

Australian Institute for Medical Research. The authors also acknowledge the help of the HLA typing laboratory of PathWest, Royal Perth Hospital, for typing of NK donors and BLCL targets and Tena Causerano for transformation and maintenance of BLCL. The authors gratefully acknowledge the advice and suggestions of Dr. Gerald Waneck.

Financial disclosure: The authors have nothing to disclose.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbmt.2009.10.026.

REFERENCES

1. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097-2100.
2. Davies SM, Ruggieri L, DeForT, et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. *Blood*. 2002;100:3825-3827.
3. Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. *Science*. 1993;260:1121-1124.
4. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med*. 1995;181:1133-1144.
5. Mandelboim O, Reyburn HT, Vales-Gomez M, et al. Protection from lysis by natural killer cells of group 1 and 2 specificity is mediated by residue 80 in human histocompatibility leukocyte antigen C alleles and also occurs with empty major histocompatibility complex molecules. *J Exp Med*. 1996;184:913-922.
6. Raulet DH, Vance RE, McMahon CW. Regulation of the natural killer cell receptor repertoire. *Annu Rev Immunol*. 2001;19:291-330.
7. Kim S, Poursine-Laurent J, Truscott SM, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*. 2005;436:709-713.
8. Foley BA, De Santis D, Van Beelen E, Lathbury LJ, Christiansen FT, Witt CS. The reactivity of Bw4+ HLA-B and HLA-A alleles with KIR3DL1: implications for patient and donor suitability for haploidentical stem cell transplantations. *Blood*. 2008;112:435-443.
9. Winter CC, Gumperz JE, Parham P, Long EO, Wagtmann N. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J Immunol*. 1998;161:571-577.
10. Moesta AK, Norman PJ, Yawata M, Yawata N, Gleimer M, Parham P. Synergistic polymorphism at 2 positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *J Immunol*. 2008;180:3969-3979.
11. Pende D, Marcenaro S, Falco M, et al. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and re-definition of inhibitory KIR specificity. *Blood*. 2008;113:3119-3129.
12. Chewning JH, Gudme CN, Hsu KC, Selvakumar A, Dupont B. KIR2DS1-positive NK cells mediate alloresponse against the C2 HLA-KIR ligand group in vitro. *J Immunol*. 2007;179:854-868.
13. Foley B, De Santis D, Lathbury L, Christiansen F, Witt C. KIR2DS1-mediated activation overrides NKG2A-mediated inhibition in HLA-C C2-negative individuals. *Int Immunol*. 2008;20:555-563.
14. Ruggeri L, Mancusi A, Capanni M, Martelli MF, Velardi A. Exploitation of alloreactive NK cells in adoptive immunotherapy of cancer. *Curr Opin Immunol*. 2005;17:211-217.
15. Sun JY, Gaidulis L, Miller MM, et al. Development of a multiplex PCR-SSP method for Killer-cell immunoglobulin-like receptor genotyping. *Tissue Antigens*. 2004;64:462-468.
16. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods*. 2004;294:15-22.
17. Yawata M, Yawata N, Draghi M, Partheniou F, Little AM, Parham P. MHC class I-specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response. *Blood*. 2008;112:2369-2380.
18. Rose MJ, Brooks AG, Stewart LA, Nguyen TH, Schwarer AP. Killer Ig-like receptor ligand mismatch directs NK cell expansion in vitro. *J Immunol*. 2009;183:4502-4508.
19. Fauriat C, Andersson S, Bjorklund AT, et al. Estimation of the size of the alloreactive NK cell repertoire: studies in individuals homozygous for the group A KIR haplotype. *J Immunol*. 2008;181:6010-6019.
20. Stern M, Ruggeri L, Capanni M, Mancusi A, Velardi A. Human leukocyte antigens A23, A24, and A32 but not A25 are ligands for KIR3DL1. *Blood*. 2008;112:708-710.
21. Sakai H. Lymphocyte function in IgA nephropathy. In: Clarkson AR, editor. *IgA Nephropathy*. Boston, MA: Martinus Nijhoss; 1987 p. 176-187.
22. Yawata M, Yawata N, Draghi M, Little AM, Partheniou F, Parham P. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. *J Exp Med*. 2006;203:633-645.

CHAPTER 7: KIR2DS1-MEDIATED ACTIVATION OVERRIDES NKG2A MEDIATED INHIBITION IN HLA-C C2-NEGATIVE INDIVIDUALS.

Foley BA, De Santis D, Lathbury LJ, Christiansen FT, Witt CS

As part of a study performed by BAF (first author) to determine the ability of common HLA-C alleles to inhibit KIR2DL receptors, we performed a systematic analysis of the ability of common HLA-C alleles to inhibit KIR2DL2 and KIR2DL3. With the use of HLA homozygous BLCL and NK cloning and chromium release assay we identified NK clones, most clones of which expressed the CD158b marker (KIR2DL2/3), that lysed C1 negative (C2 homozygous) targets. However, two clones of the 122 clones tested had an unexpected receptor profile in that they did not express CD158b (KIR2LD2/3) and were positive for CD158a. This suggested that another receptor was responsible for the alloreactivity observed in these two clones. A new study to determine the identity of this receptor was initiated. We demonstrated that the receptor responsible was the activating receptor KIR2DS1. These findings may have important implications in donor selection in bone marrow transplantation.
The findings were published in;
International Immunology, Vol. 20 (4), 555-563.

Contribution:

BAF performed most of the bench work, including maintenance of cell lines, flow cytometry, NK cloning, and chromium release assays, and helped draft the manuscript.

DDS developed the flow cytometric assays for CD107a which was used to detect KIR2DS1-dependent cells, maintained the cell lines used as target cells

in the CD107a assay and helped with NK cloning and chromium release assays.

LJL assisted with assay development, laboratory management and design of experiments

FTC assisted with the design and analysis of the experiments and assisted with manuscript preparation

CSW designed and analysed experiments, assisted with manuscript preparation, and had overall responsibility for the research program.

International Immunology Advance Access published February 27, 2008

International Immunology 2008; 1 of 9
doi:10.1093/intimm/dxn013

© *The Japanese Society for Immunology. 2008. All rights reserved.*
For permissions, please e-mail: journals.permissions@oxfordjournals.org

KIR2DS1-mediated activation overrides NKG2A-mediated inhibition in HLA-C C2-negative individuals

Bree Foley[1,2], Dianne De Santis[1,2], Louise Lathbury[1,2], Frank Christiansen[1,2] and **Campbell Witt[1]**

[1]Department of Clinical Immunology and Immunogenetics, PathWest Laboratory Medicine, Royal Perth Hospital, Perth, Western Australia, Australia
[2]Department of Surgery and Pathology, University of Western Australia, Perth, Western Australia, Australia

Keywords: activating receptor, alloreactivity, KIR, NK cell

Abstract

NK cell cytotoxicity is controlled through a balance of both activating and inhibitory signals. The HLA specificity of alloreactive NK cells has been previously shown to be controlled by inhibitory killer immunoglobulin-like receptors (KIRs). Alloreactive NK cells lyse targets that lack the HLA ligand for their inhibitory KIR. We have characterized in detail an alloreactive NK clone in which the specificity is controlled by an activating receptor, KIR2DS1. Only target cells expressing the HLA-C group 2 (C2) epitope were lysed by this clone and homozygous C2 targets were lysed more strongly than heterozygous C1/C2 targets. Anti-CD158a (KIR2DS1) blocked lysis of targets confirming KIR2DS1 was responsible. Although this NK clone expressed NKG2A, an inhibitory receptor whose ligand is HLA-E, targets with ligands for both KIR2DS1 and NKG2A were lysed by this clone indicating that the KIR2DS1-mediated activation signal overrides the NKG2A-mediated inhibitory signal. KIR2DS1 activated NK clones in polyclonally expanded NK cultures from a donor that lacked the C2 epitope accounted for ~1% of all NK cells. This study highlights a potential role for NK cells controlled by activating KIR in mediating NK alloreactivity.

Introduction

NK cell cytotoxicity is controlled by a balance of both activating and inhibitory signals. To prevent autoreactivity, NK cells do not become competent for cytotoxicity (armed or licenced) unless they express at least one inhibitory receptor recognizing self-ligands (1, 2). As the ligands for some NK inhibitory receptors are allelic epitopes on class I HLA molecules, NK cell clones that depend on such receptors for inhibition exhibit alloreactivity. It is thought that the inhibitory signals mediated by these receptors under normal conditions are dominant over signals generated by activating receptors (3, 4).

The killer immunoglobulin-like receptors (KIRs) recognize allelic epitopes present on HLA-A, HLA-B or HLA-C molecules. HLA-C alleles can be divided into two groups, HLA-C group 1 (C1) and HLA-C group 2 (C2), based on their amino acid sequences in the α1 helix. C1-group alleles have the amino acid serine (Ser) at residue 77 and asparagine (Asn) at residue 80, whereas C2-group alleles have the amino acid Asn at residue 77 and lysine (Lys) at residue 80 (5). The inhibitory receptor KIR2DL1 recognizes C2-group alleles while KIR2DL2 and KIR2DL3 (CD158b) recognize C1-group alleles. Although the amino acid sequences of activating KIR (KIR2DS1, KIR2DS2 and KIR3DS1) suggest that they might bind to similar ligands as their inhibitory counterparts (KIR2DL1, KIR2DL2/3 and KIR3DL1), it has been difficult to show that activating receptors bind to these ligands.

Alloreactive NK clones from C1 homozygous individuals lyse C2 homozygous EBV-transformed B lymphoblastoid cell lines (BLCLs). These clones have been shown to be alloreactive due to expression of the inhibitory receptor KIR2DL2 or KIR2DL3 (CD158b) for which the C2 homozygous targets cannot supply the C1 self-ligand that is they recognize 'missing self' (6). However, it has been shown that some NK clones that lyse C2-expressing targets lack CD158b and react with the anti-CD158a antibody which recognizes KIR2DL1 or KIR2DS1. Moreover, activation of these NK clones by C2 homozygous targets was abolished by the addition of anti-CD158a, suggesting that these NK clones mediate alloreactivity by recognition of C2 ligands by the activating receptor KIR2DS1 (6). Despite this early data, there has been little additional evidence of activation of NK cells through KIR2DS1 recognition of the C2 ligand. Stewart *et al.* (7) used KIR2DL1 and KIR2DS1 tetramers to determine whether these receptors bound to C2. KIR2DL1 tetramers bound to C2-positive uninfected B cells and EBV-infected

Correspondence to: C. Witt; E-mail: Campbell.Witt@health.wa.gov.au

Transmitting editor: E. Vivier

Received 20 November 2007, *accepted* 23 January 2008

B cells. In contrast, KIR2DS1 tetramers were found to bind only to C2-positive B cells after EBV infection. It was postulated that this difference in KIR2DS1 binding was due to increased HLA-C density on the surface of EBV-infected cells.

In 2007, Chewning *et al.* (8) provided evidence to support the earlier work of Moretta *et al.* that KIR2DS1 binding could be responsible for NK cell lysis of C2-positive BLCL target cells showing that such lysis could be blocked by anti-CD158a antibody.

Here, we provide further evidence supporting the findings of Chewning *et al.* that KIR2DS1-positive NK clones are activated by recognition of C2 on BLCL targets. In addition, we show that the activation signal generated by engagement of KIR2DS1 overrides the inhibitory signal generated by engagement of CD94/NKG2A in these clones. Furthermore, we report that C2 homozygous targets undergo greater cell lysis than C1/C2 heterozygous targets consistent with the idea that activation through KIR2DS1 is dependent on class I density at the cell surface. We also demonstrate that KIR2DS1-activated cells can lyse PHA blasts in addition to EBV-transformed cells from the same individual, indicating that non-transformed cells express enough HLA-C to activate NK cells through KIR2DS1 highlighting a potential role for these NK clones in mediating NK alloreactivity.

Methods

EBV cell lines

EBV-transformed BLCLs were either 10th International Histocompatibility Workshop cells or generated by the Department of Clinical Immunology, Royal Perth Hospital (Table 1). The 721.221 class I-negative cell line was a gift from J. McCluskey (University of Melbourne, Australia). The RPMI-8866 cell line was obtained from the American Type Cell Collection (Manassas, VA, USA). All cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) with 10% heat-inactivated FCS (ThermoTrace, Melbourne, Australia) at 37°C in 5% CO_2.

NK cells

Blood was obtained from laboratory volunteers and blood donors from the Australian Red Cross Blood Service with informed consent. HLA-A, -B and -C typing of all donors was performed by DNA sequencing (Table 2). NK cells were purified from blood by ficoll density gradient centrifugation with RosetteSep (StemCell Technologies, Vancouver, Canada). Purified NK cells were plated out at limiting dilutions into 96-well round-bottom plates in NK medium [RPMI-1640, 10% FCS, 0.1 mM MEM, non-essential amino acids and 1 mM sodium pyruvate (Invitrogen)]. Irradiated, allogeneic, ficoll separated PBMC feeder cells (pooled from 10 donors) were added to each well at a concentration of 1×10^5 cells per 100 μl and then cultured at 37°C in 5% CO_2. On day 1, 100 μl of medium was removed and replaced with fresh NK medium containing 400 IU ml^{-1} IL-2 (Chiron, Emeryville, CA, USA). On day 4, 100 μl of medium was removed and replaced with irradiated PBMC feeder cells added to each well at a concentration of 8×10^4 cells per 100 μl. On day 11, 100 μl of medium was removed and replaced with 100 μl fresh NK medium containing 200 IU ml^{-1} IL-2. On day 14 onwards cell growth was monitored by light microscopy and cells were split if necessary and fed with 1×10^5 per ml irradiated RPMI-8866 cells every 3–4 days. NK clones were screened for NK receptor expression and alloreactivity by flow cytometry and ^{51}Cr release, respectively.

Ficoll separated purified polyclonal NK cells were expanded by culturing with irradiated allogeneic feeder cells at a 1:10 concentration for 12 days with 200 IU ml^{-1} IL-2 replacing with fresh NK medium every 2–3 days. Before use in the CD107a assay, these NK cells were cultured with irradiated RPMI-8866 at a 1:10 concentration for 7 days with 200 IU ml^{-1} IL-2 replacing the medium containing every 2–3 days.

Generation of PHA blasts

PHA blasts were generated from ficoll-purified PBMC; 10-μg ml^{-1} PHA (Sigma–Aldrich, St Louis, MO, USA) was added to 1×10^6 cells ml^{-1} and cultured for 4 days. Cells were then diluted to 2×10^5 cells ml^{-1} in RPMI-1640 containing 10% FCS and 20 IU ml^{-1} IL-2 and grown for an additional 3 days at which point they were stored at −80°C in 1 ml of 90% FCS and 10% dimethyl sulfoxide (BDH Chemicals, Poole, UK). Before use as cytotoxicity targets, PHA blasts were cultured for 48 h with 20 IU ml^{-1} IL-2.

Table 1. Class I HLA typing and NK epitopes of BLCL target cell panel

Cell identifier	HLA-A	HLA-B	HLA-C	NK epitopes
721.221	—	—	—	—
IHW9034	A*0301	B*0702	C*0702	C1
Q94056412J	A*0201, 1101	B*0702	C*0702	C1
IHW9065	A*0301	B*0702	C*0702	C1
IHW9157	A*3301	B*5801	C*0302	C1, Bw4
R050307566X	A*1101, 3101	B*5101	C*1402	C1, Bw4
R020349726A	A*0301, 1101	B*07, 35	C*0401, 0702	C1, C2
R020358799H	A*0201	B*4402, 5101	C*0501, 1402	C1, C2, Bw4
Q9454288T	A*01, 0301	B*2705, 4403	C*04, 0102	C1, C2, Bw4
R97349692S	A*0301, 1101	B*4001, 5701	C*0304, 0602	C1, C2, Bw4
IHW9019	A*3002	B*180101	C*0501	C2
IHW9084	A*0201	B*4002	C*0202	C2
IHW9090	A*0201	B*4402	C*0501	C2, Bw4
IHW9016	A*0201	B*5101	C*1502	C2, Bw4
Q95050308M	A*0201	B*4402	C*0501	C2, Bw4

Table 2. HLA typing NK epitopes and KIR typing of NK cell donors

Donor	HLA-A	HLA-B	HLA-C	NK epitopes	KIR2DL1	KIR2DL2	KIR2DL3	KIR3DL1	KIR2DS1
1	A*24, 32	B*18, 51	C*07, 1402	C1, Bw4	+	+	+	+	+
2	A*03, 68	B*07, 14	C*07, 08	C1	+	–	+	–	+
3	A*1102, 2410	B*1802, 4601	C*01, 07	C1	+	+	+	+	+
4	A*01, 03	B*08	C*07	C1	+	+	+	+	–
5	A*02, 1101	B*1501, 35	C*0303, 0401	C1, C2	+	+	–	+	+

Flow cytometry

KIR receptor expression was identified on each NK clone using PE-conjugated antibodies to NKG2A/B, CD158a, CD158b (Beckman Coulter, Fullerton, CA, USA) and NKB1 (BD Biosciences, Franklin Lakes, NJ, USA). KIR3DL2 expression was determined using Q66 mAb (gift from D. Pende, University of Genoa, Italy) and anti-human PE-conjugated IgM (Beckman Coulter). Flow cytometry was performed on a Coulter EPICS XL-MCL™ flow cytometer and the BD Biosciences FACSCanto™ flow cytometer. Class I HLA expression on BLCL and PHA blasts was determined using the pan class I mAb W6/32 (a gift from J. McCluskey, University of Melbourne, Australia). FITC-conjugated anti-human IgG (Chemicon, Temecula, CA, USA) was used as the secondary antibody.

^{51}Cr release cytotoxicity assay

NK cell-mediated killing was measured by the standard 4-h ^{51}Cr release assay (9). All assays were carried out at an effector to target ratio of 2:1. For blocking experiments, NK cells were pre-incubated with anti-CD158a (BD Biosciences), anti-NKG2A (clone z199, gift from M. Lopez-Botet, University Pompeu Fabra, Spain), IgM isotype control for CD158a and IgG_{2b} isotype control for anti-NKG2A (eBiosciences, San Diego, CA, USA) at concentrations of 10 $\mu g\ ml^{-1}$ for 20 min at 37°C. Assays were performed in triplicate and standard mean error was calculated for each target.

CD107a cytotoxicity assay

NK cell expression of CD107a was used to measure NK cytotoxicity by cultured polyclonal NK cells. Culture polyclonal NK cells were added to BLCL target cells at a 1:1 ratio in a 96-well round-bottom plate. Five microlitres of CD107a–FITC (BD Biosciences) was added to each well. After 1 h of incubation at 37°C, 6 $\mu g\ ml^{-1}$ monensin (BD GolgiStop™, BD Biosciences) was added and the cells were incubated for a further 5 h. NK cells were then stained with anti-CD56–PECy7, anti-CD158a–PE (BD Biosciences) and anti-CD158b–allophycocyanin (Beckman Coulter) and analysed on the BD Biosciences FACSCanto™ flow cytometer using FACSDiva™ software.

KIR2DS1 sequencing and KIR genotyping

mRNA was extracted from NK clone C9 using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from 1 μg of mRNA using oligo(dT) (Promega, Madison, WI, USA) and Omniscript (Qiagen). A genomic fragment of KIR2DS1 including exons 4 and 5 was amplified from cDNA and genomic DNA using KIR2DS1 sequence-specific primers as described (10). To exclude the possibility that NK clones were expressing KIR2DL1, a KIR2DL1-specific PCR reaction was performed using primers as described (10). Full-length KIR2DS1 cDNA was amplified from NK clone C9 using the following primers: KIR2DS1F, 5′TGTAAAACGACGGCCAGTATGTCGCTCAYGGTCGTC3′ and KIR2DS1R, CAGGAAACAGCTATGACCGTGAAAACA-CAGTGATCCAA.

Thermocycling conditions were 95°C for 5 min, 5 cycles of 97°C for 20 s, 65°C for 45 s, 72°C for 90 s, 25 cycles of 95°C for 20 s, 61°C for 45 s, 72°C for 90s and a final extension of 72°C for 10 min. Both KIR2DS1 PCR products were sequenced using BigDye Terminator technology (Applied Biosystems, Foster City, CA, USA) and analysed using SeqScape (Applied Biosystems). KIR genotyping of each donor (Table 2) was performed using a multiplex PCR-Sequence Specific Priming developed by Sun *et al.* (11).

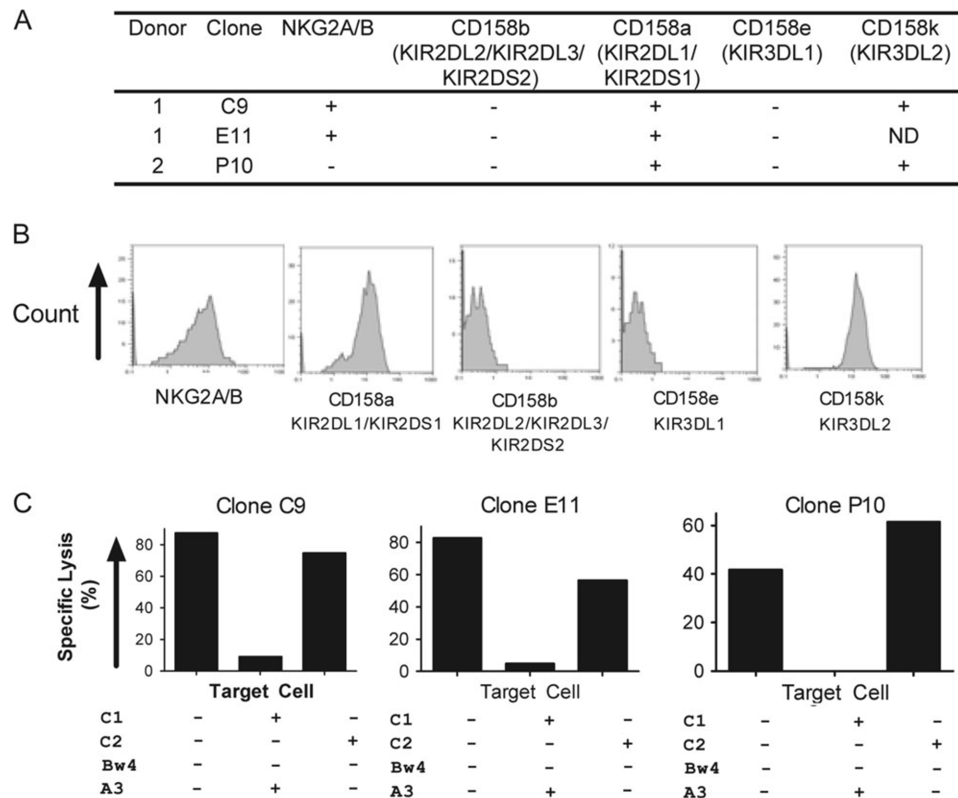
Results

Alloreactive NK clones expressing CD158a lyse C2-expressing targets

As expected, we found that individuals who express the C1 epitope have alloreactive NK clones expressing KIR2DL2 or KIR2DL3 (CD158b) which are inhibited by BLCL targets expressing C1 and lyse targets which lack C1. Of the 122 NK clones tested from a C1 homozygous donor (Donor 1), 28 NK clones lysed C1-negative (C2 homozygous) targets. 26 of these 28 clones expressed CD158b. However, two clones (clone C9 and E11) were CD158b negative (Fig. 1), suggesting that another receptor must explain their alloreactivity. Similarly, 4 of 100 NK clones from another C1 homozygous donor (Donor 2) lysed C1-negative (C2 homozygous) targets. Of these, three were CD158b positive but one (clone P10) was CD158b negative, suggesting that another receptor must explain its alloreactivity. All three CD158b-negative NK clones expressed CD158a (KIR2DL1 or KIR2DS1). Clones C9 and E11 also expressed NKG2A and clones C9 and P10 also expressed CD158k (KIR3DL2).

The allospecificity of clone C9 cannot by explained by KIR3DL2

It has been reported that KIR3DL2 recognizes HLA-A*0301 and HLA-A*1101 but not HLA-A*0201 (12,13). As the C1 homozygous target (C2 negative) used for screening NK clones also expressed HLA-A3 and the C2 homozygous target (C1 negative) did not, we considered the possibility that the lack of cytotoxicity against the C1 homozygous target



Donor	Clone	NKG2A/B	CD158b (KIR2DL2/KIR2DL3/ KIR2DS2)	CD158a (KIR2DL1/ KIR2DS1)	CD158e (KIR3DL1)	CD158k (KIR3DL2)
1	C9	+	-	+	-	+
1	E11	+	-	+	-	ND
2	P10	-	-	+	-	+

Fig. 1. Receptor expression and cytotoxicity pattern of CD158b-negative clones showing specificity for C2-positive targets. (A) Receptor expression on NK clones C9, E11 and P10. (B) Flow cytometry histograms showing NK receptor expression on representative NK clone C9 showing expression of NKG2A and KIR3DL2. (C) Initial screen cytotoxicity patterns of the three CD158a-positive (CD158b negative) clones. Each NK clone lysed the class I HLA-negative target (721.221 cell line) and the C2 homozygous BLCL target (IHW9019) but not the C1 homozygous BLCL target (IHW9034) (Table 1).

was due to HLA-A3-mediated inhibition through KIR3DL2. NK clone C9 was therefore tested against six HLA-A3 homozygous and six HLA-A2 homozygous (i.e. HLA-A3 negative) BLCL targets (Fig. 2). All these targets lacked the C2 epitope. While clone C9 lysed the HLA class I-negative cell line, 721.221, it was inhibited by all A*0301 and A*0201 homozygous cell lines, indicating that KIR3DL2-mediated inhibition was unlikely to explain this clone's allospecificity.

The allospecificity of clone C9 is determined by the presence of the C2 epitope in a dose-dependent manner

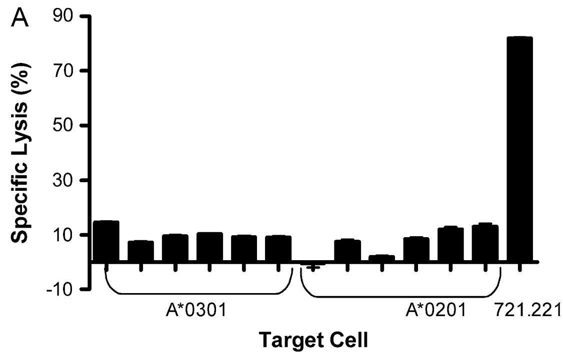
To further characterize the allospecificity of clone C9, we tested a more comprehensive panel of BLCL including C1 homozygous, C2 homozygous and C1/C2 heterozygous targets (Table 1). Clone C9 lysed all five C2-positive targets and none of the C2-negative targets (Fig. 3). Interestingly, cytotoxicity was stronger against C2 homozygous targets than the C1/C2 heterozygous targets.

*Clone C9 expresses KIR2DS1*002*

We postulated that the alloreactivity of clone C9 was conferred by the activating receptor KIR2DS1 (CD158a) interacting with the C2 epitope. In order to confirm that clone C9 transcribes a functional KIR2DS1 gene, cDNA was prepared from clone C9 and KIR2DS1 was amplified using KIR2DS1 sequence-specific primers (10) (Fig. 4) and primers that would amplify the full-length KIR2DS1 cDNA (data not shown). The sequence-specific primers detected KIR2DS1 transcript. The full-length KIR2DS1 PCR product was also amplified and sequenced. The sequence was identical to KIR2DS1*002. Since CD158a stains both KIR2DS1 and its inhibitory counterpart KIR2DL1, we also tested the mRNA using KIR2DL1-specific primers. Clone C9 did not transcribe KIR2DL1 although a KIR2DL1 transcript could be amplified from a positive control (data not shown).

Activation by C2-positive targets is mediated by KIR2DS1 and overrides NKG2A-mediated inhibition

In order to prove that lysis of C2-positive targets by the CD158b-negative and CD158a-positive clone C9 was mediated by KIR2DS1, we determined the effect on cytotoxicity of blocking KIR2DS1 using anti-CD158a mAb. The addition of anti-CD158a completely abolished lysis of C2-positive targets and had no effect on the lysis of C2-negative targets confirming the role of KIR2DS1 (Fig. 5). Clone C9 also expressed the NK inhibitory receptor NKG2A. As all the BLCL target cells used in these experiments express HLA class I alleles that provide the necessary leader peptides for expression of HLA-E, NKG2A on clone C9 might be expected to bind to its ligand HLA-E and inhibit lysis of the



B Class I HLA Alleles of BLCL Target Panel

Identifier	HLA-A	HLA-B	HLA-C
R8612350C	A*0301	B*0702	C*0702
R8612340F	A*0301	B*1402	C*0802
R8612319W	A*0301	B*0702	C*0702
R8612298B	A*0301	B*0702	C*0702
R8612318Y	A*0301	B*0702	C*0702
R8612302Q	A*0301	B*0702	C*0702
R8612343Z	A*0201	B*4501	C*1601
R8612289C	A*0201	B*2705	C*0102
R8612317A	A*0201	B*1501	C*0304
R8612347R	A*0201	B*3801	C*1203
R8612346T	A*0201	B*1801	C*0702
R8612323F	A*0201	B*1801	C*0701

Fig. 2. Despite expression of KIR3DL2, clone C9 was inhibited equally well by HLA-A*0301 and HLA-A*0201 homozygous BLCL target. (A) NK clone C9 was tested against a target cell panel of 12 BLCL including six targets homozygous for HLA-A*0301 and six targets homozygous for HLA-A*0201. All targets were homozygous for the C1 epitope (i.e. lacked the C2 epitope). (B) HLA alleles of the BLCL panel.

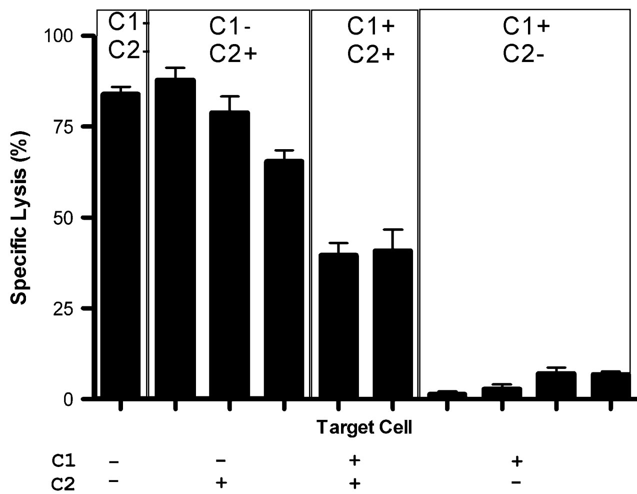


Fig. 3. NK clone C9 only lyses C2-positive BLCL targets and shows a C2 dose effect. NK clone C9 was tested against a panel of BLCL target cells (IHW9090, IHW9019, Q95050308M, R020358799H, R020349726A, IHW9034, R050307566X, IHW9157 and Q94056412J) in the 4-h 51Cr release assay at an E:T ratio of 2:1. NK clone C9 strongly lysed the HLA class I-negative 721.221 cell line (positive control) and the BLCL targets homozygous for C2. C1/C2 heterozygous targets were moderately lysed whereas C1 homozygous targets were essentially negative.

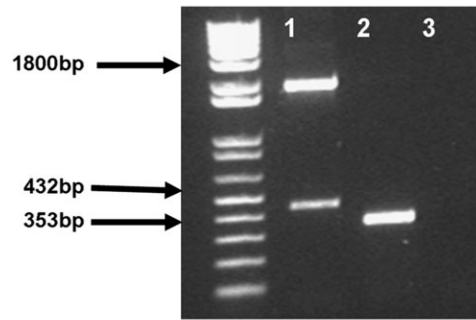


Fig. 4. NK clone C9 transcribes KIR2DS1*002. Genomic DNA from donor 1 (lane 1), cDNA from NK clone c9 (lane 2) and cDNA from a CD158a-negative NK clone (Donor 2) (lane 3) were amplified with KIR2DS1-specific primers (10). The genomic DNA resulted in an expected 1800 bp product (growth hormone amplification control shown as a 433-bp product), confirming the presence of KIR2DS1. The cDNA resulted in a 353-bp product as expected.

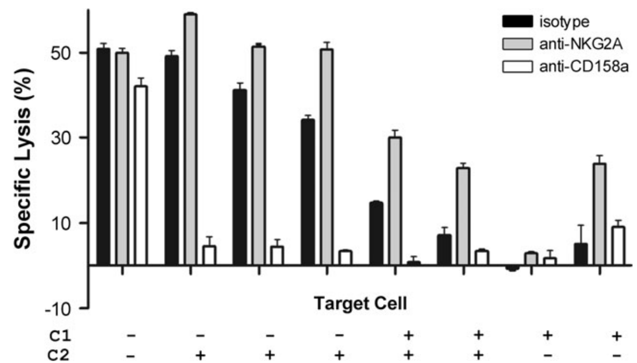


Fig. 5. Target cell lysis by NK clone C9 is enhanced by anti-NKG2A antibody and inhibited by anti-CD158a (anti-KIR2DL1/KIR2DS1). NK clone C9 was tested against the class I-negative control (721.221 cell line) and a panel of BLCL target cells with various C1 and C2 combinations (from left to right IHW9019, IHW9084, IHW9016, R020358799H, R020349726A, IHW9034 and R050307566X). NKG2A and KIR2DS1 receptors were blocked with mAb z199 (NKG2A) (grey bars) and HP-3E4 (CD158a) (open bars), respectively, and isotype control (solid bars). Addition of anti-CD158a blocked lysis of C2-positive targets but had no effect on C2-negative targets. Addition of ant-NKG2A resulted in moderate enhanced lysis of six of the seven targets.

target cell. To determine whether NKG2A was functional in clone C9, we used mAb z199 to block any NKG2A-mediated inhibition. As shown in Fig. 5, the addition of anti-NKG2A had no effect on lysis of the class I-deficient control (721.221 cell line) but moderately enhanced lysis of 6 out of 7 target cells, indicating that NKG2A was functional. This data indicate that in the absence of blocking mAbs, KIR2DS1-mediated activation overrides NKG2A-mediated inhibition. Interestingly, blocking of NKG2A did not result in enhanced lysis of one of the C1 homozygous target cells (IHWS 9034). Blocking with the pan-HLA class I mAb, W6/32 resulted in lysis of this target (data not shown), suggesting that the ligand inhibiting NK cell lysis of this target cell is an HLA class I molecule but not HLA-E.

KIR2DS1-activated NK clones represent 1% of all NK cells in C1 homozygous individuals

To determine the frequency of NK clones that are activated by KIR2DS1 and whether this varies among individuals of different HLA-C and KIR genotype, we determined CD107a expression on CD158a-positive NK cells when incubated with C2-positive BLCL targets. Polyclonal NK cells from a C1 homozygous, KIR2DS1-positive donor (Donor 3), a C1 homozygous, KIR2DS1-negative donor (Donor 4) and a C1/C2 heterozygous, KIR2DS1-positive donor (Donor 5) were incubated with C1 homozygous and C2 homozygous BLCL targets and the percentage of CD107a-positive cells determined. C2-positive targets were predicted to induce CD107a expression only on CD158a-positive NK cells from subjects who have the KIR2DS1 gene and in whom C2 is not a self-epitope (i.e. C1 homozygous donors). As shown in Fig. 6A, C2-positive (either homozygous or heterozygous) targets induced CD107a expression on CD158a-positive NK cells only from the C1 homozygous, KIR2DS1-positive donor. Representative cytofluorograms are shown in Fig. 6 (B1–6). Figure 6 (B1–3) shows that a C1 homozygous target cell induced very little CD107a expression on CD158a-positive cells from a C1 homozygous, KIR2DS1-positive donor (1.9% of CD158a-positive cells Fig. 6, B2), whereas CD107a expression was induced on 33.8% of CD158a-positive cells by a C2 homozygous target (Fig. 6, B3). These represent some 0.8% of all NK cells. Neither C1 homozygous nor C2 homozygous targets induced CD107a expression on CD158a-positive cells from a C1 homozygous KIR2DS1-negative donor (Fig. 6, B4–6). We performed similar

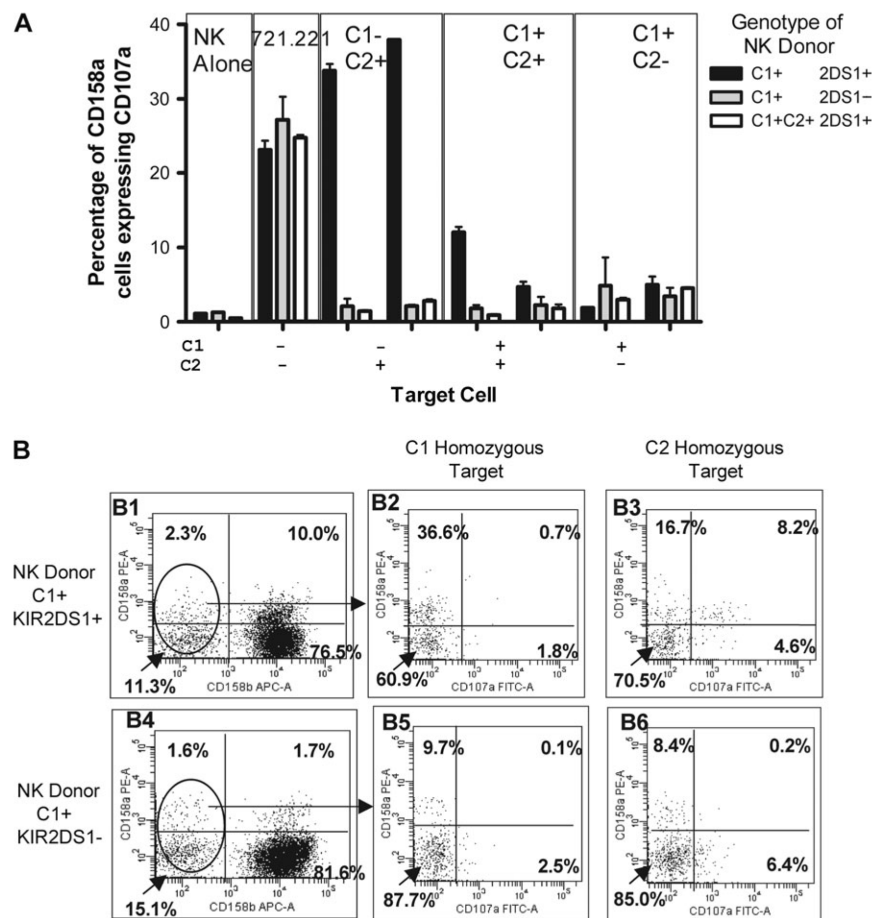


Fig. 6. NK cell activation through KIR2DS1 (CD158a) only occurs in C2 negative, KIR2DS1-positive individuals. (A) NK cells from three donors, Donor 3 (C1+C2− KIR2DS1+: solid bars), Donor 4 (C1+C2− KIR2DS1−: grey bars) and Donor 5 (C1+C2+ KIR2DS1+: open bars) were incubated with 721.221 cell line (positive control), two C2 homozygous cell lines (IHW9019 and IHW9084), two C1/C2 heterozygous cell lines (Q9454288T and R97349692S) and two C1 homozygous cell lines (IHW9034 and IHW9065) and CD107a expression was quantified on the CD158a-positive population. All three NK donors lyse 721.221 cells, but only the C1 homozygous, KIR2DS1-positive donor lyses C2 homozygous BLCLs and to a lesser extent C1/C2 heterozygous BLCLs. NK cells from all donors are inhibited by the C1 homozygous (C2 negative) BLCL targets. (B) Representative flow cytometric plots for the C1 homozygous, KIR2DS1-positive (Fig. 6, B1–3) donor and the C1 homozygous, KIR2DS1-negative (Fig. 6, B4–6) donor with the percentage of cells in each quadrant shown. After incubation with target cells, CD158b-negative cells were identified (B1 and B4). CD107a and CD158a staining of the CD158b-negative NK cells (selected from B1 or B4) are shown after incubation with a C1 homozygous target (B2 and B5) or C2 homozygous target (B3 and B6). CD158a-positive cells from the C1 positive, KIR2DS1-positive donor show CD107a staining (8.2% of the CD158a-positive cells) only after incubation with the C2 homozygous target (B3) but not the C1 homozygous target (0.7% of the CD158a-positive cells). The CD158a-positive cells from the C1 homozygous, KIR2DS1-negative donor do not show CD107a staining when incubated with C1 or C2 homozygous targets (0.1 and 0.2% of CD158a cells, respectively).

experiments using C2 homozygous, KIR2DS2-positive individuals and HLA-Bw6 homozygous, KIR3DS1-positive individuals to determine if NK cells could be activated by the C1 or Bw4 epitopes through KIR2DS1 or KIR3DS1, respectively. We were unable to detect NK cell activation in such experiments (data not shown).

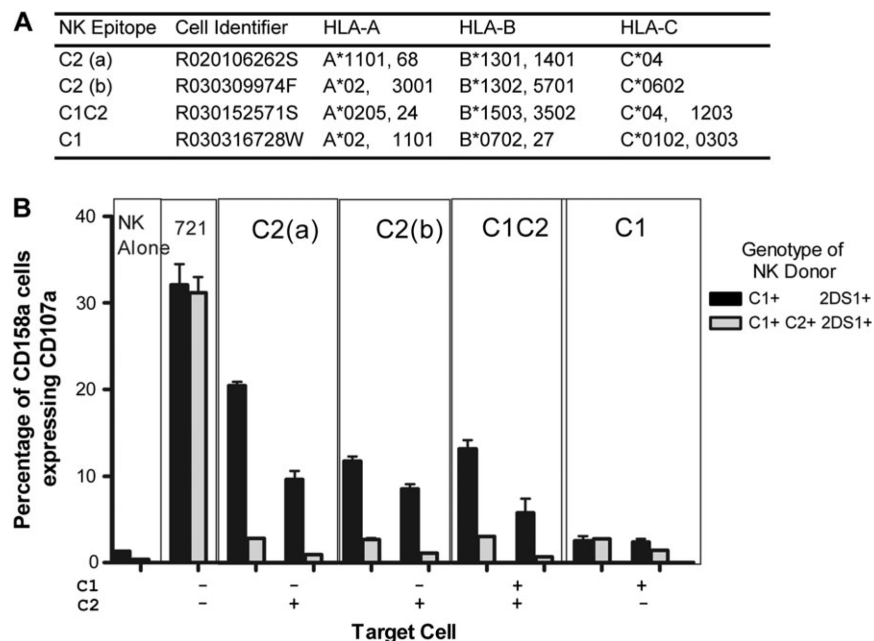
NK cells activated by C2 kill EBV non-infected targets

As it has been shown that KIR2DS1 binds to EBV-infected cells, but not to uninfected cells (7), we tested the ability of KIR2DS1-activated NK cells to kill PHA blasts and EBV BLCL from the same individual. Polyclonally expanded NK cells from a C1 homozygous, KIR2DS1-positive individual (Donor 3) were incubated with C1 homozygous, C2 homozygous and C1/C2 heterozygous PHA blasts and BLCL targets, and CD107a expression measured on CD158a-positive cells. CD107a expression was induced on CD158a-positive NK cells from Donor 3 by both C2-positive BLCL and PHA blasts (Fig. 7), although in all cases, C2-positive BLCL induced CD107a in a higher percentage of NK cells than PHA blasts from the same individual. As expected, CD107a expression was not induced on CD158a-positive NK cells from a KIR2DS1-positive C1/C2 heterozygous donor (Donor 5) by either C2-positive BLCL or PHA target cells (Fig. 7). To determine whether the difference in the ability of BLCL and PHA blasts to induce CD107a expression on NK cells might reflect differences in the level of class I expression, BLCL and PHA blasts were stained using the class I HLA-specific mAb W6/32. The mean fluorescence intensity of class I HLA expression was 20–50% higher on BLCL than PHA blasts (data not shown).

Discussion

We have demonstrated in this study that the specificity of alloreactive NK cells can be determined by the activating receptor KIR2DS1. These results confirm the results of other studies providing evidence that NK cells can be activated by KIR2DS1 recognition of the C2 epitope (6–8). The current data also confirms the finding of Chewning *et al.* that only C1 homozygous (i.e. C2 negative), KIR2DS1-positive individuals are capable of generating such alloreactive NK clones dependent on KIR2DS1. This is intuitively logical as KIR2DS1-activated clones would be autoreactive in individuals whose HLA-C alleles include the C2 epitope. Thus, C1 homozygous individuals produce alloreactive NK clones dependent on KIR2DL2/3 which are activated by the absence of the C1 epitope and also KIR2DS1-positive clones that are activated by the presence of the C2 epitope. The latter population of cells represents ~1% of all NK cells in C1 homozygous individuals. Thus, in addition to detecting missing self, NK cells are capable of detecting 'non-self'.

It is generally believed that under normal conditions, inhibitory signals are dominant over activating signals in controlling NK cell activation. NK cells are only 'licenced' for cytotoxicity if they express an inhibitory receptor for 'self'. The majority of KIR2DS1-expressing NK clones characterized in this study, and in the studies by Moretta *et al.* (6) and Chewning *et al.* (8), co-expressed NKG2A suggesting



A

NK Epitope	Cell Identifier	HLA-A	HLA-B	HLA-C
C2 (a)	R020106262S	A*1101, 68	B*1301, 1401	C*04
C2 (b)	R030309974F	A*02, 3001	B*1302, 5701	C*0602
C1C2	R030152571S	A*0205, 24	B*1503, 3502	C*04, 1203
C1	R030316728W	A*02, 1101	B*0702, 27	C*0102, 0303

Fig. 7. C2-positive EBV-transformed and PHA blast cells both activate CD158a-positive NK cells. Polyclonally expanded NK cells from a C1+ KIR2DS1+ donor (Donor 3) and a C1+C2+ KIR2DS1+ donor (Donor 5) were used in the CD107a assay against a panel of EBV-transformed BLCL and PHA blasts from the same individuals. (A) HLA typing and NK epitopes of the BLCL and PHA blast donors. (B) CD158a-positive (CD158b negative) NK cells from both donors expressed CD107a when incubated with the 721.221 cell line (positive control). Only NK cells from the C1+ KIR2DS1+ donor (Donor 3, solid bars) expressed CD107a in the presence of both the C2 homozygous and C1/C2 heterozygous BLCL and PHA blasts but, as expected, not in the presence of the C1 homozygous BLCL or PHA blasts. By contrast, the CD158a-positive cells from the C1+C2+ KIR2DS1+ donor (Donor 5, open bars) did not express CD107a in the presence of any of the target pairs.

that KIR2DS1-dependent alloreactive NK cells are licensed by NKG2A interaction with self. Our data suggest that the interaction of activating receptors for non-self-ligands, such as NK cells from a KIR2DS1 positive, C2-negative individual interacting with C2, can override NKG2A-mediated inhibition. It has previously been reported that under cellular stress the activating receptor NKG2D can override inhibitory signals to allow NK cells to eliminate tumour or infected cells (14) but our data are the first evidence of an activating KIR behaving in a similar manner. Why the inhibitory signal generated through NKG2A does not suppress the activation signal generated through KIR2DS1 is unclear. It has been speculated that the ability of inhibitory signals to dominate over activating signals is due to the low affinity with which activating receptors bind to their ligand compared with their inhibitory counterparts (15,16). The kinetics of NKG2A binding to HLA-E are similar to those for inhibitory KIR binding to HLA-C (15) so it seems unlikely that our current data showing the inability of NKG2A to override KIR2DS1 can be explained by low affinity binding of NKG2A to HLA-E.

This study only examined one allele of KIR2DS1 in detail, KIR2DS1*002, which is expressed by clone C9 and we do not know the KIR2DS1 alleles expressed by the other donors. Four alleles of KIR2DS1 have been reported (17). KIR2DS1*001 and KIR2DS1*004 differ from KIR2DS1*002 at positions 70 and 90 of the first immunoglobulin domain, respectively. KIR2DS1*003 differs from KIR2DS1*002 only in the leader sequence. Differences in the immunoglobulin domains may result in different binding affinities. Therefore, we cannot be sure that KIR2DS1*001 and KIR2DS1*004 would behave like KIR2DS1*002, whereas it seems likely that KIR2DS1*003 would have similar specificity to KIR2DS1*002 in its interaction with its C2 ligand.

Stewart *et al.* (7) demonstrated that KIR2DS1 tetramers only bound to C2 on EBV-infected cells compared with normal B cells and B cells infected with members of the human herpesvirus family and postulated that this was due to an increase in class I HLA expression induced by EBV infection. In the current study, lysis of C1/C2 heterozygous BLCL was weaker than for C2 homozygous BLCL, suggesting that increased target cell density of HLA-C enhances KIR2DS1-mediated activation. We also found that BLCL were more susceptible to lysis than PHA blasts from the same person and that this correlated with lower class I HLA expression. Nevertheless, KIR2DS1-activated NK cells were able to lyse PHA-activated T cells (non-EBV infected) from both C2 homozygous and heterozygous individuals despite lower class I expression than on BLCL from the same individuals.

It is unclear as to whether other activating KIR (KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1) can mediate NK alloreactivity. While we and others (6–8, 18, 19) have been unable to demonstrate that NK cell activation mediated through KIR2DS2 interacting with C1, or through KIR3DS1 interacting with Bw4 (20), it is possible that this may be due to the fact that these epitopes are simply not the cognate ligands for these receptors. Therefore, further work is required to resolve this issue.

It is not surprising that KIR2DS1-dependent NK clones which only arise in C1 homozygous individuals have been largely overlooked. When considering potential donors for haploidentical stem cell transplant (HSCT), C1 homozygous donors are rarely considered because all haploidentical recipients would also express C1, thereby preventing any inhibitory receptor-mediated NK alloreactivity by such donors. Leukaemia patients with all three ligands (C1, C2 and Bw4) for inhibitory KIR are thought to be unable to benefit from NK-mismatched HSCT, as all inhibitory KIR on haploidentical donor NK cells would be inhibited by HLA ligands on the recipient's cells. The data described in this report suggest that it may be possible for patients with all three epitopes to benefit from the use of alloreactive NK cells. NK cells from a C1 homozygous sibling donor sharing one haplotype with a C1/C2 heterozygous recipient may provide an anti-leukaemic effect [and reduced graft versus host disease (GvHD)] through KIR2DS1-activated NK clones. Such NK cells may also be evident in HSCT using an unrelated donor where the donor has KIR2DS1 and the recipient has C2. Indeed, there is evidence to suggest that C1/C2 heterozygous bone marrow transplant recipients who receive a transplant from a KIR2DS1-positive donor have a lower risk of developing acute GvHD compared with the same transplant in a C1 homozygous recipient (21). These findings have potentially important implications in the selection of HSCT donors.

Acknowledgements

The authors gratefully acknowledge the generous support of The Ray and Bill Dobney Foundation.

Abbreviations

BLCL	B lymphoblastoid cell line
C1	HLA-C group 1
C2	HLA-C group 2
GvHD	graft versus host disease
HSCT	haploidentical stem cell transplant
KIR	killer immunoglobulin-like receptor

References

1 Raulet, D. H., Vance, R. E. and McMahon, C. W. 2001. Regulation of the natural killer cell receptor repertoire. *Annu. Rev. Immunol.* 19:291.

2 Kim, S., Poursine-Laurent, J., Truscott, S. M. *et al.* 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature.* 436:709.

3 Biassoni, R., Pessino, A., Malaspina, A. *et al.* 1997. Role of amino acid position 70 in the binding affinity of p50.1 and p58.1 receptors for HLA-Cw4 molecules. *Eur. J. Immunol.* 27:3095.

4 Vales-Gomez, M., Reyburn, H. T., Erskine, R. A. and Strominger, J. 1998. Differential binding to HLA-C of p50-activating and p58-inhibitory natural killer cell receptors. *Proc. Natl Acad. Sci. USA.* 95:14326.

5 Colonna, M., Borsellino, G., Falco, M., Ferrara, G. B. and Strominger, J. L. 1993. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc. Natl Acad. Sci. USA* 90:12000.

6 Moretta, A., Sivori, S., Vitale, M. *et al.* 1995. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. *J. Exp. Med.* 182:875.

7 Stewart, C. A., Laugier-Anfossi, F., Vely, F. *et al.* 2005. Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proc. Natl Acad. Sci. USA.* 102:13224.

8 Chewning, J. H., Gudme, C. N., Hsu, K. C., Selvakumar, A. and Dupont, B. 2007. KIR2DS1-positive NK cells mediate alloresponse against the C2 HLA-KIR ligand group *in vitro*. *J. Immunol.* 179:854.

9 Warren, H. S., Kinnear, B. F., Phillips, J. H. and Lanier, L. L. 1995. Production of IL-5 by human NK cells and regulation of IL-5 secretion by IL-4, IL-10, and IL-12. *J. Immunol.* 154:5144.
10 Uhrberg, M., Valiante, N. M., Shum, B. P. *et al.* 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity.* 7:753.
11 Sun, J. Y., Gaidulis, L., Miller, M. M. *et al.* 2004. Development of a multiplex PCR-SSP method for killer-cell immunoglobulin-like receptor genotyping. *Tissue Antigens.* 64:462.
12 Pende, D. 1996. The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors which is characterised by three Ig-like domains and is expressed as a 140 kD disulphide-linked dimer. *J. Exp. Med.* 184:505.
13 Gavioli, R., Zhang, Q. J. and Masucci, M. G. 1996. HLA-A11-mediated protection from NK cell-mediated lysis: role of HLA-A11-presented peptides. *Hum. Immunol.* 49:1.
14 Bauer, S., Groh, V., Wu, J. *et al.* 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science.* 285:727.
15 Vales-Gomez, M., Reyburn, H. T., Erskine, R. A., Lopez-Botet, M. and Strominger, J. L. 1999. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. *EMBO J.* 18:4250.
16 Lopez-Botet, M., Bellon, T., Llano, M., Navarro, F., Garcia, P. and de Miguel, M. 2000. Paired inhibitory and triggering NK cell receptors for HLA class I molecules. *Hum. Immunol.* 61:7.
17 Robinson, J., Waller, M. J., Parham, P. *et al.* 2003. IMGT/HLA and IMGT/MHC: sequence databases for the study of the major histocompatibility complex. *Nucleic Acids Res.* 31:311.
18 Winter, C. C., Gumperz, J. E., Parham, P., Long, E. O. and Wagtmann, N. 1998. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J. Immunol.* 161:571.
19 Saulquin, X., Gastinel, L. N. and Vivier, E. 2003. Crystal structure of the human natural killer cell activating receptor KIR2DS2 (CD158j). *J. Exp. Med.* 197:933.
20 O'Connor, G. M., Guinan, K. J., Cunningham, R. T., Middleton, D., Parham, P. and Gardiner, C. M. 2007. Functional polymorphism of the KIR3DL1/S1 receptor on human NK cells. *J. Immunol.* 178:235.
21 McQueen, K. L., Dorighi, K. M., Guethlein, L. A., Wong, R., Sanjanwala, B. and Parham, P. 2007. Donor-recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation. *Hum. Immunol.* 68:309.

CHAPTER 8: THE REACTIVITY OF BW4^{+} HLA-B AND HLA-A ALLELES WITH KIR3DL1: IMPLICATIONS FOR PATIENT AND DONOR SUITABILITY FOR HAPLOIDENTICAL STEM CELL TRANSPLANTATIONS

Foley BA, De Santis D, Van Beelen E, Lathbury LJ, Christiansen FT, Witt CS

NK cells from donors whose HLA-B alleles include the Bw4 epitope lyse cells lacking the Bw4 epitope because such target cells do not express the ligand for the inhibitory receptor KIR3DL1. However, Ruggeri et al (Ruggeri et al. 2006) reported that only two-thirds of donors with HLA-B alleles make NK clones that lyse Bw4^{-} targets. Until the study reported here, there had not been a systematic study of the ability of HLA-B alleles to interact with KIR3DL1.

At the time of commencement of my PhD, while assessing donor NK alloreactivity using NK cloning and the ^{51}chromium release assay to identify a suitable haploidentical donor for a patient with AML, it was observed that NK cells expressing KIR3DL1 from 2 Bw4^{+} donors (siblings) did not lyse a Bw4^{-} BLCL. As both donors had an expressed allele of KIR3DL1 and HLA-B*1301, a Bw4^{+} allele, KIR3DL1-dependent NK cells capable of lysing a Bw4^{-} BLCL were expected. As we were unable to demonstrate KIR3DL1-mediated NK alloreactivity in these donors and since we were inexperienced with NK cloning at that time, we concluded that the unexpected result was due to technical difficulties related to NK cloning and ^{51}chromium release assay.

Subsequently, we undertook a systemic analysis of the ability of common HLA-B alleles and Bw4^{+} HLA-A alleles to inhibit KIR3DL1. We also tested the ability of individuals with Bw4^{+} HLA-A alleles to generate KIR3DL1-dependent NK clones. With the use of HLA homozygous BLCL and by using the CD107a

cytotoxicity assay that I developed in chapter 6, we were able to demonstrate that most Bw4$^+$ HLA-B alleles tested, but not B*1301 or B*1302, are ligands for KIR3DL1. These results demonstrated that our initial observations with the 2 haploidentical donors tested in NK cloning and ^{51}chromium release assay experiments were not related to technical difficulties but to the HLA genotype of these donors.

Bw4$^+$ HLA-A*2402 and HLA-A*3201 were also shown to be ligands for KIR3DL1 but HLA-A*2501 and HLA-A*2301 provided target cells with only weak protection from lysis. Consistent with the finding that HLA-A*2402 on target cells inhibited KIR3DL1-dependent NK clones, KIR3DL1-dependent NK alloreactive clones were shown to be present in donors whose HLA type included HLA-A*2402 as the only Bw4+ allele, but not in donors whose HLA type included the Bw4$^+$HLA-B*1301 or -B*1302 alleles as the only Bw4+ allele.

These findings are published in;

Blood. 2008 Jul 15;112(2):435-43

Contribution:

BAF performed most of the bench work, including maintenance of cell lines, flow cytometry, NK cloning, and chromium release assays, and helped draft the manuscript

DDS developed the flow cytometric assays for CD107a, helped maintain the cell lines and performed the initial NK cloning and ^{51}chromium release assay experiments on the haploidentical donor family study.

EVB performed the NK cloning of the HLA-A24 individual, chromium release assays, and flow cytometry to characterise NK clones from this individual

LJL assisted with assay development, laboratory management and design of experiments

FTC assisted in the design and analysis of the experiments and assisted with manuscript preparation

CSW designed and analysed experiments, assisted with manuscript preparation, and had overall responsibility for the research program.

From www.bloodjournal.org by on December 4, 2009. For personal use only.

The reactivity of Bw4$^+$ HLA-B and HLA-A alleles with KIR3DL1: implications for patient and donor suitability for haploidentical stem cell transplantations

Bree A. Foley,[1] Dianne De Santis,[1,2] Els Van Beelen,[3] Louise J. Lathbury,[1] Frank T. Christiansen,[1,2] and Campbell S. Witt[2]

[1]School of Pathology and Laboratory Medicine, University of Western Australia, Nedlands, Australia; [2]Department of Clinical Immunology and Immunogenetics, PathWest Royal Perth Hospital, Perth, Australia; and [3]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

Natural killer (NK)–cell alloreactivity can be exploited in haploidentical hematopoietic stem cell transplantation (HSCT). NK cells from donors whose HLA type includes Bw4, a public epitope present on a subset of HLA-B alleles, can be alloreactive toward recipients whose cells lack Bw4. Serologically detectable epitopes related to Bw4 also exist on a subset of HLA-A alleles, but the interaction of these alleles with KIR3DL1 is controversial. We therefore undertook a systematic analysis of the ability of most common HLA-B alleles and HLA-A alleles with Bw4 serologic reactivity to protect target cells from lysis by KIR3DL1-dependent NK cells. All Bw4$^-$ HLA-B alleles failed to protect target cells from lysis. All Bw4$^+$ HLA-B alleles with the exception of HLA-B*1301 and -B*1302 protected targets from lysis. HLA-A*2402 and HLA-A*3201 unequivocally protected target cells from lysis, whereas HLA-A*2501 and HLA-A*2301 provided only weak protection from lysis. KIR3DL1-dependent alloreactive NK clones were identified in donors with HLA-A*2402 but not in donors with HLA-B*1301 or -B*1302. These findings clarify the HLA types that donors and recipients need in haploidentical HSCT and other NK allotherapies in order to benefit from NK alloreactivity. (Blood. 2008;112:435-443)

Introduction

Natural killer (NK)–cell alloreactivity can be exploited in haploidentical hematopoietic stem cell transplantation (HSCT) to improve graft survival, reduce graft-versus-host disease and decrease leukemic relapse.[1] Infusion of alloreactive NK cells without stem cells has also been shown to result in hematologic remission in patients with acute myeloid leukemia (AML).[2] NK cells lyse cells that have reduced expression of class I HLA molecules. In an allogeneic setting, donor NK cells are activated by the absence of donor (self) class I HLA molecules on recipient cells[3,4]; the absence of self-epitopes are detected by inhibitory killer immunoglobulin-like receptors (KIRs) on donor NK cells. HLA-C molecules with an asparagine at amino acid 80 provide the C1 epitope for the KIR2DL2 and KIR2DL3 inhibitory receptors and those with a lysine at amino acid 80 provide the self-epitope, C2, for the KIR2DL1 inhibitory receptor. The self-epitope of relevance to the KIR3DL1 receptor is the Bw4 epitope at amino acids 77 to 83 on some HLA-B alleles. All HLA-B alleles have either the Bw4 or the Bw6 epitope. NK cells from donors whose HLA-B alleles include the Bw4 epitope lyse cells lacking the Bw4 epitope because such target cells cannot supply the ligand for the NK inhibitory receptor KIR3DL1. However, it has been reported (reviewed in Ruggeri et al[5]) that only two-thirds of donors with Bw4$^+$ HLA-B alleles make NK clones that lyse Bw4$^-$ targets. Therefore, selecting an NK or stem cell donor for a particular patient currently requires a lengthy, labor-intensive in vitro NK cloning procedure to confirm the donor has NK cells alloreactive toward the patient.[1]

It is not clear why some Bw4$^+$ donors cannot make NK clones that lyse Bw4$^-$ targets. A likely explanation in some cases is that a common allele of KIR3DL1 (KIR3DL1*004) is not expressed at the cell surface.[6] However, a systematic examination of the ability of HLA-B alleles to bind to KIR3DL1 has not been undertaken. Amino acid 80 in the HLA-B protein critically determines binding to KIR3DL1.[7] Alleles with the Bw6 epitope have asparagine (N) at this position and do not bind to KIR3DL1, while alleles with Bw4 have either an isoleucine (I) or threonine (T) and are ligands for KIR3DL1. It has been reported that alleles with an isoleucine (80I) are stronger ligands than alleles with a threonine (80T),[8,9] while other investigators have noted differences within the Bw4 80I family of alleles[10] and peptide-dependent differences in the binding of Bw4 tetramers to different KIR3DL1 variants.[11] Several HLA-A alleles are also known to react with anti-Bw4 antibodies, but their ability to behave as a ligand for KIR3DL1 is controversial.[8,10,11] These studies used HLA-bare Epstein-Barr virus (EBV) cell lines transfected with individual HLA alleles or tetramers loaded with individual peptides to compare the effect of different alleles. A related question that has not been addressed is whether donors with the different forms of Bw4 epitope are all capable of producing NK clones that lyse cells lacking Bw4.

To improve the ability to select donors for haploidentical HSCT or NK-cell infusions based on HLA rather than NK cloning assays, we undertook a systematic analysis of the ability of common HLA-B alleles and Bw4$^+$ HLA-A alleles to inhibit KIR3DL1-dependent NK clones. In addition, we tested the ability of individuals with Bw4$^+$ HLA-A alleles to produce KIR3DL1-dependent clones. Because it is difficult to know whether results from HLA-bare cells transfected with single HLA alleles, or tetramers loaded with single peptides, accurately reflect the complex array of HLA ligands presented to NK cells (in vivo), we

Submitted January 10, 2008; accepted March 17, 2008. Prepublished online as *Blood* First Edition paper, April 2, 2008; DOI 10.1182/blood-2008-01-132902.

An Inside *Blood* analysis of this article appears at the front of this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2008 by The American Society of Hematology

From www.bloodjournal.org by on December 4, 2009. For personal use only.

Table 1. HLA class I and KIR3DL1 allele typing of NK-cell donors

Donor	HLA-A	HLA-B	Bw4/6	HLA-C	C1/2	KIR3DL1 typing	KIR3DL1 expression
1	0201, 2902	4403, 5703	4	0701, 1601	1	01501/017, 008	High
2	0201, 2601	0702, 3801	4,6	0702, 1203	1	002, 00101	High
3	0101, 2402	0801, 5109	4,6	0102, 0701	1	00501-like, 01502	High, low
4	0201, 3303	4601, 5801	4,6	0102, 0302	1	00501, 01502	High, low
5	1101	1301, 1501	4,6	0304, 0401	1,2	007, 01502	High, low
6	1101	1301, 1501	4,6	0304, 0401	1,2	00501, 007	Low
7	0301, 3101	1302, 3501	4,6	0401, 0602	2	001/016, 002	High
8	0101, 0301	0801	6	0701	1	NT	High
9	0201, 1101	1501, 3501	6	0303, 0401	1,2	NT	Low
10	0101, 2402	0801, 5501	6	0303, 0701	1	NT	High

The KIR3DL1*005-like allele in donor 3 differs from KIR3DL1*005 by a valine-to-leucine substitution at amino acid 18 in the D0 domain. In all cases, HLA alleles were assigned on the basis of sequencing of exons 2 and 3. In some cases, the assignment of class I HLA alleles is based on the most common allele consistent with the sequence in exons 2 and 3, and alleles differing in other exons cannot be excluded. As the signal peptide of KIR3DL1 was not sequenced, KIR3DL1*01501 cannot be distinguished from KIR3DL1*017 in donor 1, and KIR*001 cannot be distinguished from KIR3DL1*016 in donor 7.

NT indicates not tested.

chose to use EBV cell lines that express a normal array of HLA antigens but which were homozygous for the HLA alleles of interest. Our results confirm that most, but not all, of the common $Bw4^+$ HLA-B alleles are ligands for KIR3DL1. In addition, the status of the $Bw4^+$ HLA-A alleles is clarified, and we demonstrate that individuals with a $Bw4^+$ HLA-A allele are able to generate KIR3DL1-dependent clones. These results have implications for donor selection for haploidentical HSCT.

Methods

NK cells

Blood was obtained from laboratory volunteers and blood donors from The Australian Red Cross Blood Service, Western Australia, with informed consent obtained in accordance with the Declaration of Helsinki. Written approval to use blood samples was obtained from the Ethics Committee of Royal Perth Hospital. HLA typing was performed by DNA sequencing (Table 1). NK cells were purified by ficoll centrifugation with RosetteSep (StemCell Technologies, Vancouver, BC). Purified NK cells were plated at limiting dilutions into 96-well round-bottom plates in NK medium (RPMI-1640, 10% fetal calf serum [FCS], 0.1 mM modified Eagle medium [MEM], nonessential amino acids, and 1 mM sodium pyruvate; Invitrogen, Carlsbad, CA). Irradiated, allogeneic, ficoll-separated peripheral blood mononuclear cell (PBMC) feeder cells (pooled from 10 donors) were added to each well at a concentration of 10^5 cells/100 µL and cultured at 37°C in 5% CO_2. A total of 100 µL of medium was replaced with fresh NK medium containing 400 IU/mL IL-2 (Chiron, Emeryville, CA) on day 1, with irradiated PBMC feeder cells added to each well at a concentration of 8×10^4 cells/100 µL on day 4, and with fresh NK medium containing 200 IU/mL IL-2 on day 11. After day 13, cell growth was monitored, cells were split if necessary, and cells were fed every 3 to 4 days with 10^5/mL irradiated RPMI-8866 cells.

Purified polyclonal NK cells were expanded by culturing with irradiated allogeneic feeder cells at a 1:10 ratio for 12 days with 200 IU/mL IL-2, replacing the NK medium every 2 to 3 days. Before use in the CD107a assay, NK cells were cultured with irradiated RPMI-8866 at a 1:10 ratio for 7 days with 200 IU/mL IL-2, replacing medium every 2 to 3 days. Alternatively, when determining frequency of KIR3DL1-dependent clones, 12-day culture polyclonal NK cells were cultured at 10^6/mL for a further 48 hours with 400 IU/mL IL-2 before use in the CD107a assay.

EBV cell lines

EBV-transformed B lymphoblastoid cell lines (BLCLs) were either 10th International Histocompatibility Workshop cells, or generated in-house (Table 2). The 721.221 class I–negative cell line was a gift from J. McCluskey (University of Melbourne, Australia). The RPMI-8866 cell line was obtained from ATCC (Manassas, VA). All cells were cultured in RPMI-1640 (Invitrogen) with 10% heat-inactivated FCS (ThermoTrace, Melbourne, Australia).

Flow cytometry

KIR expression was identified on NK clones using PE-conjugated antibodies to NKG2A/B, CD158a, CD158b (Beckman Coulter, Fullerton, CA), and KIR3DL1 (BD Biosciences, Franklin Lakes, NJ). Flow cytometry was performed on a BD Biosciences FACSCanto instrument and analyzed using BD FACSDiva Software (BD Biosciences) and FlowJo Software (TreeStar, Ashland, OR). Bw4 or Bw6 expression on BLCL target cells was determined using a Bw4 or Bw6 monoclonal antibody (mAb; gifts from J. McCluskey). FITC-conjugated anti–human IgG (Chemicon, Temecula, CA) was used as the secondary antibody.

^{51}Cr release cytotoxicity assay

NK cell–mediated killing was measured by the standard 4-hour ^{51}Cr release assay[12] at an effector-target ratio of 2:1. For blocking experiments, NK cells were preincubated with anti-KIR3DL1 antibody (DX9; BD Biosciences) or IgG_1 isotype control (Biolegend, San Diego, CA) at concentrations of 2.5 µg/mL and 10 µg/mL, respectively, for 20 minutes at 37°C. Assays were performed in triplicate and standard errors of the mean were calculated.

CD107a cytotoxicity assay

NK-cell expression of CD107a was used to measure NK cytotoxicity by cultured polyclonal NK cells. These cells were added to BLCL target cells at a 1:1 ratio in a 96-well round-bottom plate. A total of 5 µL of anti-CD107a–FITC (BD Biosciences) was added to each well. After a 1-hour incubation at 37°C, 6 µg/mL monensin (BD GolgiStop; BD Biosciences) was added, and the cells were incubated a further 5 hours. NK cells were then stained with anti-CD56–PECy7, anti-KIR3DL1–PE (BD Biosciences), anti-CD158b–APC, and anti-CD158a–APC (Beckman Coulter) and analyzed by flow cytometry.

KIR3DL1 allele sequencing and KIR genotyping

KIR3DL1 alleles were determined by sequencing genomic DNA. Exons 3 to 9 were amplified as outlined in Norman et al.[13] KIR genotyping of donors was performed using a multiplex polymerase chain reaction with sequence-specific primers (PCR-SSP).[14]

From www.bloodjournal.org by on December 4, 2009. For personal use only.

Table 2. HLA class I typing of BLCL target cell panel

ID	Short ID	HLA-B	AA 80	Bw4/6	HLA-A	HLA-C	C1/2
IHW 9084	B13	1302	T	4	3001	0602	2
IHW 9067	B27	2705	T	4	0201	0102	1
IHW 9009	B37	3701	T	4	0101	0602	2
IHW 9090	B4402	4402	T	4	0201	0501	2
IHW 9027	B4403	4403	T	4	2902	1601	1
IHW 9047	B47	4701	T	4	0301	0602	2
IHW 9062	B38	3801	I	4	0201	1203	1
IHW 9040	B49	4901	I	4	0101	0701	1
IHW 9016	B51	5101	I	4	0204	1502	2
IHW 9011	B52	5201	I	4	0101	1202	1
IHW 9010	B53	5301	I	4	6802	0401	2
IHW 9052	B57	5701	I	4	0201	0602	2
IHW 9157	B58	5801	I	4	33	0302	1
IHW 9029	A23	1402	I	6	2301	0802	1
IHW 9001	A24	0702	I	6	2402	0702	1
IHW 9008	A25	1801	I	6	2501	1203	1
Q94 0055016Y	A32	1501, 3901	I	6,6	3201, 0201	0303, 1203	1,1
IHW 9065	B07	0702	N	6	0301	0702	1
IHW 9088	B08	0801	N	6	0101	0701	1
IHW 9029	B14	1402	N	6	2301	0802	1
IHW 9099	B15	1501	N	6	0217	0303	1
IHW 9019	B18	1801	N	6	3002	0501	2
IHW 9068	B35	3501	N	6	0201	0401	2
Q94 0052722Q	B39	0702, 3901	N	6,6	0201, 0301	0702, 1203	1,1
IHW 9084	B40	4002	N	6	0201	0202	2
IHW 9043	B41	4101	N	6	0101	1701	2
IHW 9021	B42	4201	N	6	3001, 6802	1701	2
IHW 9058	B45	4501	N	6	0201	1601	1
IHW 9076	B46	4601	N	6	0206, 0207	0102, 0801	1,1
R98 0903165W	B50	5001, 0702	N	6,6	0301, 0201	—	—
R04 0901000J	B55	5502, 0801	N	6,6	0101, 0206	0102, 0701	1,1
R97 0330581F	B56	5601, 0801	N	6,6	01, 34	—	—

HLA types of target cells used in flow cytometric and chromium release assays. In all cases, HLA alleles were assigned on the basis of sequencing of exons 2 and 3. In some cases, the assignment of class I HLA alleles is based on the most common allele consistent with the sequence in exons 2 and 3, and alleles differing in other exons cannot be excluded. Targets that were homozygous for HLA-B50, HLA-B55, and HLA-B56 were not available. Therefore, cells that were heterozygous for these antigens and had a common Bw6 antigen as the second antigen were selected. AA80 indicates the amino acid present at residue 80 of the HLA-B alleles.

— indicates HLA-C typing not performed.

Results

KIR3DL1 genotype of NK cells

As different alleles of KIR3DL1 have high or low expression at the cell surface, we determined the KIR3DL1 alleles and receptor expression pattern on 10 NK donors (Table 1). To compare the behavior of high- and low-expressing alleles, 2 donors with only high-expression alleles and 2 donors heterozygous for high- and low-expression alleles (donors 1 and 2 and donors 3 and 4, respectively, in Table 1) were selected. In addition, because the high-expressing allele in both donors 3 and 4 was the same (KIR3DL1*01502), between-donor reproducibility could be assessed. All 4 donors had at least one Bw4^{+} HLA-B allele (Table 1). Interestingly, the low-expression receptors in donors 3 and 4 differed in their level of expression (Figure 1). This difference may be because the low-expression receptor in donor 3 differed from the low-expression receptor encoded by KIR3DL1*005 in donor 4 by a valine to leucine substitution at amino acid 18 in the D0 domain.

KIR3DL1-dependent NK cells lyse cells lacking the Bw4 epitope

KIR expression on polyclonally expanded NK cells has been demonstrated not to alter the frequency of NK cells expressing particular KIR receptors.[9] To detect NK-cell cytotoxicity toward

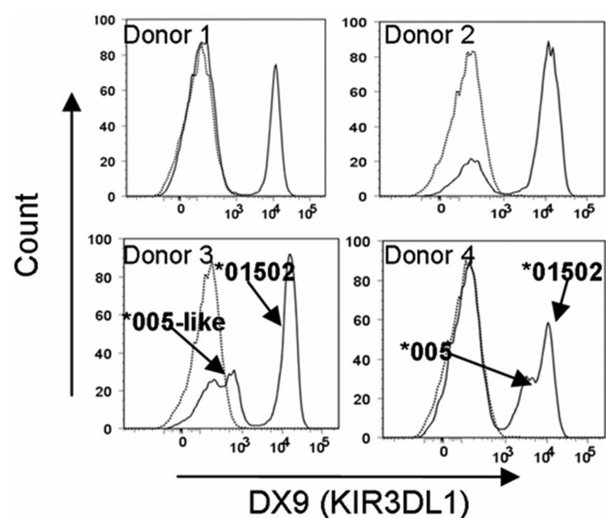


Figure 1. Low-level KIR3DL1 expression differs in 2 donors due to a nonsynonymous mutation at nucleotide position 115 in exon 3 encoding the D0 domain of the KIR3DL1*005 allele. NK cells from each donor were stained with anti-KIR3DL1 antibody (DX9; solid lines) or isotype control (IgG_1; dotted lines). KIR3DL1 surface expression correlated with KIR3DL1 allele typing, with donors 1 and 2 having unimodal high-level expression and donors 3 and 4 having bimodal (high- and low-level) expression. The low-level receptor expression differed between donors 3 and 4. Comparison of both KIR3DL1 sequences from donors 3 and 4 revealed a single nonsynonymous nucleotide mutation at position 115 of the KIR3DL1*005 allele in donor 3.

From www.bloodjournal.org by on December 4, 2009. For personal use only.

Bw4⁻ targets mediated by KIR3DL1-dependent clones, polyclonal NK cells from donors 1 to 4 were expanded by in vitro culture and then incubated with BLCL targets expressing common HLA-B alleles or the $Bw4^+$ HLA-A alleles A*2301, A*2402, A*2501, and A*3201 (Table 2). Cytotoxicity was measured by CD107a expression on $KIR3DL1^+$ ($DX9^+$), $CD158b^-$ ($KIR2DL2^-$, $KIR2DL3^-$, $KIR2DS2^-$) NK cells. Because all 4 NK donors were homozygous for C1-group HLA-C alleles and a significant proportion of $KIR3DL1^+$ NK cells coexpressed CD158b, the inhibitory receptor for the C1-epitope, only $KIR3DL1^+$, $CD158b^-$ cells were examined. $KIR3DL1^+$, $CD158b^-$ NK cells up-regulated CD107a when incubated with either the class I HLA–negative target (721.221^+ control) or targets homozygous for the Bw6 epitope (Figure 2). $Bw4^+$ targets tended to inhibit CD107a expression. Overall, there was little difference between the 4 donors in terms of the target cells that showed greatest inhibition of CD107a expression. CD107a expression was inhibited by all Bw4-expressing targets with 4 exceptions: those expressing HLA-B*1302, HLA-B*5101, HLA-A*2301, and HLA-A*2501. There were some small differences in the ability of the other Bw4-expressing targets to inhibit CD107a expression, but these differences did not relate to the presence of 80I or 80T alleles.

Of the 2 donors with high KIR3DL1 expression, donor 1 had a higher percentage of NK cells expressing CD107a in response to $Bw4^-$ targets and therefore appeared to better discriminate between $Bw4^+$ and $Bw4^-$ targets. However, this may simply reflect the fact that donor 1 had a higher proportion of NK cells dependent on KIR3DL1 for inhibition than did donor 2. The high-expression KIR3DL1 receptor in donors 3 and 4 and the low-expression allele (KIR3DL1*005) in donor 4 behaved in a similar way to NK cells in donor 1. However, in donor 3, NK cells with the low-expression KIR3DL1*005-like variant up-regulated CD107a relatively weakly in response to Bw6 targets and were less completely inhibited by Bw4 targets so that there was relatively poor discrimination between the Bw4 and Bw6 targets. Interestingly, in this donor, those NK cells expressing the KIR3DL1*005-like variant also exhibited less up-regulation of CD107a in response to the class I bare 721.221 cells than the NK cells expressing the KIR3DL1*01502 allele. These results suggest that the poor discrimination between Bw4 and Bw6 targets exhibited by the NK cells expressing the receptor encoded by the KIR3DL1*005-like variant may be due to these cells being relatively weakly armed rather than a smaller proportion being KIR3DL1-dependent for inhibition. Donor 3 was particularly informative in terms of the Bw4 alleles that are KIR3DL1 ligands. For almost all Bw4 targets, NK cells with the high-expression allele KIR3DL1*01502 were more strongly inhibited than NK cells with the weakly expressed KIR3DL1*005-like variant. For each Bw6 target, NK cells with the high-expression allele KIR3DL1*01502 were less inhibited than NK cells expressing the weakly expressed KIR3DL1*005-like variant. In this respect, HLA-B*1302, HLA-A*2301, and HLA-A*2501 all behaved like Bw6 alleles, reinforcing the conclusion that these alleles are not KIR3DL1 ligands. Interestingly, in donor 4, the only target that appeared to discriminate between NK cells expressing KIR3DL1*01502 and KIR3DL1*005 was the HLA-A*23 target. This suggests that some KIR3DL1 alleles may provide exceptions to the more general rules.

The ability of the different targets to inhibit CD107a expression on KIR3DL1-expressing effectors could be related not so much to the affinity of the ligand but rather to differences in the level of expression of the ligand on the various targets. Therefore, the level of Bw4 expression was checked by staining each target with an

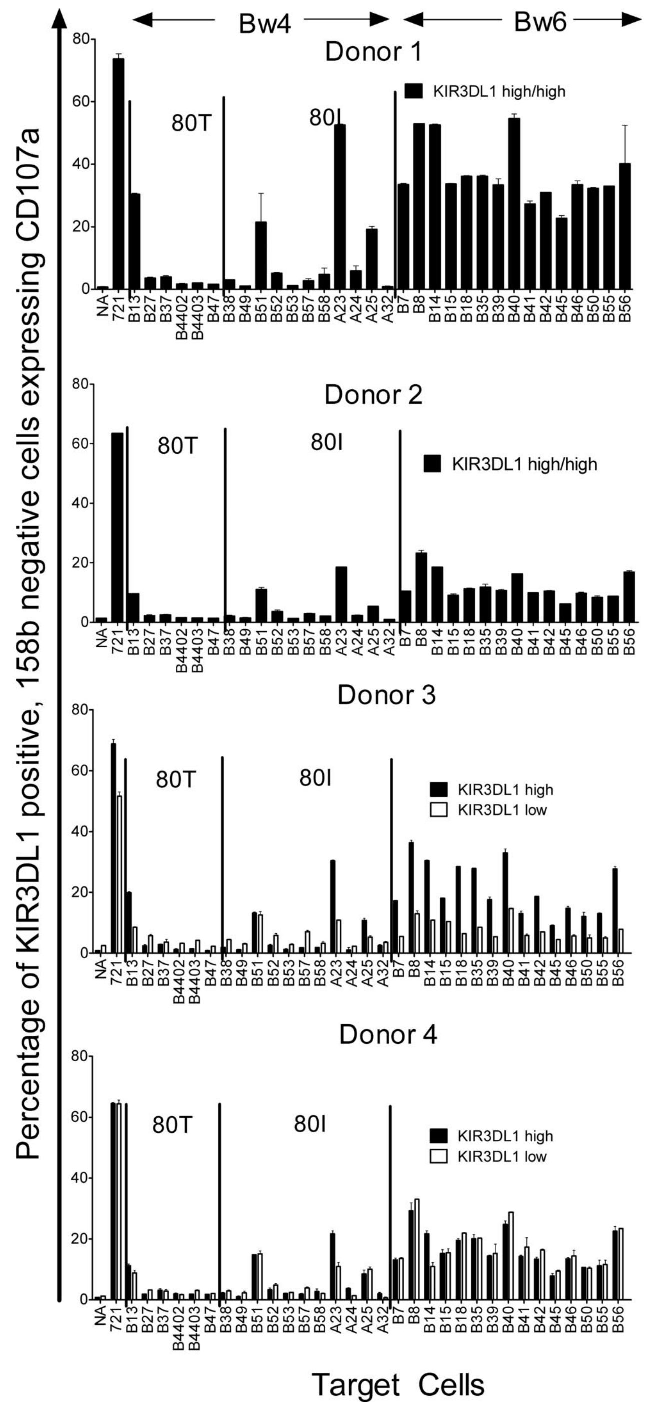


Figure 2. CD107a expression by $KIR3DL1^+$ polyclonal NK cells is inhibited by most $Bw4^+$ targets. Polyclonally expanded NK cells from 4 donors (donors 1-4) were incubated alone (NA), with a positive control, the class I–negative BLCL 721.221 (721), and a range of $Bw4^+$ and $Bw6^+$ targets (Table 2) and CD107a expression were measured on $KIR3DL1^+$, $CD158b^-$ NK cells. Donors 3 and 4 had NK cells that showed high or low expression of DX9, and for each, the percentage of $CD107a^+$ cells among either the NK cells bearing the high level (■) or low level (□) of DX9 expression are shown. All donors lysed the 721.221 cell line and all Bw6-homozygous ($Bw4^-$) targets. All donors were inhibited by most of the $Bw4^+$ targets except for targets B13, B51, A23, and A25. For donor 3, the low-level expression allele was less cytotoxic against Bw6-expressing targets and not as well inhibited by most Bw4-expressing targets compared with the high-expression allele. By contrast for donor 4, the high- and low-level expression alleles behaved similarly. Error bars represent SEM.

From www.bloodjournal.org by on December 4, 2009. For personal use only.

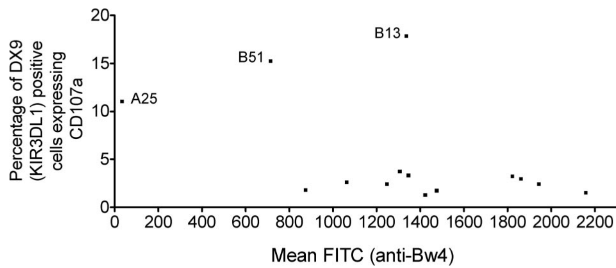


Figure 3. Level of Bw4 expression does not correlate with percentage of CD107a expression induced on KIR3DL1+ NK cells. Each Bw4+ target cell was stained with an anti-Bw4 mAb to detect Bw4 expression. The mean channel fluorescence (MCF) for each target was plotted against the percentage of CD107a+ NK cells induced by the target on NK cells with a high-expression KIR3DL1 receptor, averaged across the 4 NK-cell donors. Target A25 did not stain with the Bw4 mAb and does not inhibit NK-cell cytotoxicity. Target B51, which weakly inhibited NK-cell cytotoxicity, has lower expression of Bw4 than the other Bw4-expressing alleles. However, the B13 target, which also weakly inhibited NK-cell cytotoxicity, expressed levels of Bw4 comparable with most Bw4+ targets. After excluding the A25 and B51 targets, there was no significant correlation between percentage of KIR3DL1+ cells expressing CD107a and Bw4 expression on the target cell ($r = -0.13$; $P = .68$).

anti-Bw4 mAb. As shown in Figure 3, the average CD107a expression induced on KIR3DL1+ NK cells was not related to the level of Bw4 expression on the target. The A23, A24, and A32 targets also stained strongly, whereas the A25 target did not stain with the Bw4 mAb and did not inhibit NK-cell cytotoxicity despite being known to have the Bw4 motif. However, this mAb also did not react with 2 additional cell lines with HLA-A25, indicating that this mAb does not react with the HLA-A25–associated Bw4 epitope. The B51 target did have reduced HLA-B expression, which could explain the poor inhibition mediated by this target. However, the B13 target, which was also poorly inhibitory, had levels of Bw4 expression comparable with the other Bw4-expressing targets. Therefore, reduced HLA-B expression was not responsible for its lack of inhibition. There was no correlation between the level of Bw4 expression and ability to suppress CD107a expression when the HLA-A*2501 and B*5101 targets were excluded ($r = -0.13$; $P = .68$).

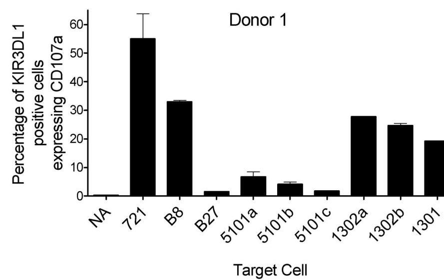
HLA-B*13 is not a KIR3DL1 ligand, but HLA-B*5101 is

To confirm the lack of inhibition seen with the targets bearing HLA-B*1302 and HLA-B*5101, additional examples of BLCL expressing B*1302 and B*5101, and one expressing B*1301 (Figure 4A), were tested in the CD107a assay against NK cells from donor 1. KIR3DL1+ NK cells up-regulated CD107a when incubated with either the positive control (721.221) or the Bw6 control (B8) as expected (Figure 4B). The Bw4 control (B27) inhibited CD107a expression as expected. Both examples of targets expressing HLA-B*1302 and the single example of a target expressing HLA-B*1301 failed to inhibit CD107a expression. In contrast, the original HLA-B*5101 target with reduced HLA-B expression (5101a) inhibited CD107a expression less strongly than the 2 additional examples of B*5101 targets. The additional B*13 and B*5101 targets had HLA-B expression comparable with the other Bw4+ targets as determined by staining with the Bw4 mAb (data not shown). These data suggest that the observation in relation to the original B51 target was due to reduced expression of HLA-B*5101 on that target, whereas HLA-B*13 appears to be a poor ligand for KIR3DL1. Lack of inhibition of cytotoxicity by HLA-B*1302 was confirmed using NK clones from donors 3 and 4 in a 4-hour ^{51}Cr release assay (Figure 4C). A total of 2 KIR3DL1+ clones (clone C3 with low KIR3DL1 expression from donor 4 and clone D2 with high KIR3DL1 expression from donor 3) were tested. As shown in Figure 4C, cytotoxicity of clones C3 and D2 against the class I–negative cell line (721.221) and a Bw6 control (B35; Table 2) was not affected by blocking KIR3DL1 with DX9 antibody, whereas inhibition of cytotoxicity by a Bw4 control (B57; Table 2) was reversed by blocking with DX9 (Figure 4C).

A

Lab Number	Short ID	HLA-B	AA 80	HLA-A	HLA-C
IHW 9016	5101a	5101	I	0204	1502
R05 0307566X	5101b	5101	I	1101, 3101	1402
IHW 9045	5101c	5101	I	0216, 0301	0704, 1502
IHW 9084	1302a	1302	T	3001	0602
IHW 9093	1302b	1302	T	0201	0602
R03 0242430X	1301	1301, 5501	T	0207, 1101	0102, 0304

B



C

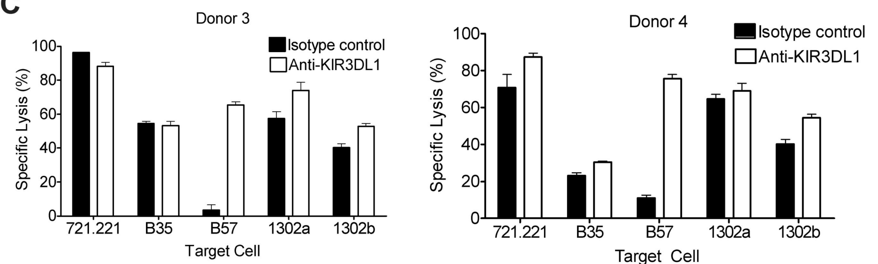


Figure 4. HLA-B*1302 and HLA-B*1301 do not inhibit cytotoxicity of KIR3DL1-dependent NK cells. (A) Polyclonally expanded NK cells from donor 1 were incubated with the class I HLA–negative cell line, 721.221 (positive control), a Bw6 control (B8; Table 2), a Bw4 control (B27; Table 2), 2 HLA-B*1302 targets, 1 HLA-B*1301 target, and 3 HLA-B*5101 targets. (B) CD107a expression was measured on KIR3DL1+, CD158b− NK cells incubated with various target cells or alone (NA). CD107a expression was induced by the 721.221 cell line and the Bw6 control (B8), and not by the Bw4 control (B27) as expected. All targets expressing either HLA-B*1302 or HLA-B*1301 failed to inhibit CD107a expression. HLA-B*5101–expressing targets inhibited CD107a expression. (C) A total of 2 KIR3DL1+ NK clones (clone C3 from donor 4 expressing the low-expression KIR3DL1*005 allele and clone D2 from donor 3 expressing the high-expression KIR3DL1*01502 allele) were used in the 4-hour ^{51}Cr release assay against the class I–negative cell line (721.221), a Bw6 control (B35; Table 2), a Bw4 control (B57; Table 2) and 2 HLA-B*1302 homozygous targets (panel A) to confirm lack of inhibition through KIR3DL1. KIR3DL1 was blocked with anti-DX9 (KIR3DL1; □) and isotype control (IgG_1; ■). Both NK clones lysed 721.221 and the Bw6 control (B35). Both NK clones were inhibited by the Bw4 control (B57), and inhibition was reversed in the presence of anti-KIR3DL1 mAb. Neither clone was inhibited by either HLA-B*1302 target with very little or no reversal of inhibition in the presence of the anti-KIR3DL1 mAb. Error bars represent SEM.

From www.bloodjournal.org by on December 4, 2009. For personal use only.

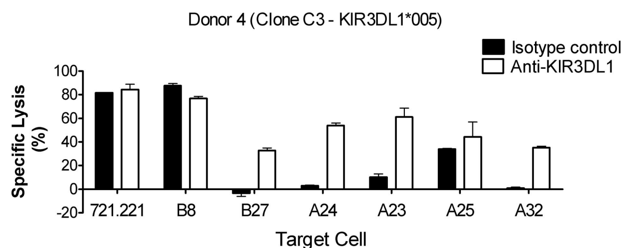


Figure 5. Inhibition through Bw4-expressing HLA-A alleles can be reversed by addition of anti-KIR3DL1. Clone C3 from donor 4 expressing the low allele KIR3DL1*005 was tested in a 4-hour ^{51}Cr release cytotoxicity assay against the class I–negative cell line (721.221), a Bw6 control (HLA-B*0801; Table 2), a Bw4 control (HLA-B*2705; Table 2), and targets expressing one of the 4 Bw4-expressing HLA-A alleles: A23, A24, A25, and A32 (Table 2). KIR3DL1 was blocked with either anti-KIR3DL1 antibody (DX9; □) or isotype control (IgG_1; ■). Clone C3 lysed both 721.221 and the Bw6 control, and inhibition was not enhanced by anti-KIR3DL1 antibody. Clone C3 was inhibited by the Bw4 control and the A24 A23, and A32 targets, and this inhibition was reversed by addition of anti-KIR3DL1 antibody. Clone C3 lysed the A25 target and the anti-KIR3DL1 antibody only weakly enhanced specific lysis. Error bars represent SEM.

Cytotoxicity against the 2 HLA-B*1302 homozygous targets was relatively strong in the absence of blocking antibody and only weakly enhanced in the presence of DX9, confirming that HLA-B*1302 is a poor ligand for KIR3DL1.

HLA-A*2402 and HLA-A*3201 are ligands for KIR3DL1

A total of 4 HLA-A alleles react with anti-Bw4 antibodies: HLA-A*23, HLA-A*24, HLA-A*25, and HLA-A*32. When assessed in terms of the ability of target cells expressing these alleles to inhibit CD107a expression of KIR3DL1$^+$ NK cells (Figure 2), HLA-A*2402 and HLA-A*3201 were clearly KIR3DL1 ligands, while HLA-A*2501 appeared to have some weak inhibitory activity. HLA-A*2301 appeared devoid of inhibitory activity, particularly for NK cells expressing the high level allele (Figure 2 solid bars). Gumperz et al[10] were unable to show reversibility of HLA-A*24–mediated inhibition of cytotoxicity using an anti-KIR3DL1 antibody. We therefore investigated the reversibility of inhibition by HLA-A alleles in a ^{51}Cr release assay with an NK clone from donor 4 expressing the KIR3DL1*005 allele. As shown in Figure 5, the HLA bare (721.221) and Bw6$^-$ control target (B8) were lysed, and lysis was not enhanced by blocking KIR3DL1. The Bw4 control target (B27) completely inhibited lysis, and inhibition was reversed substantially by blocking with mAb. The A24 and A32 targets provided just as effective inhibition as the B27 control and again, inhibition was blocked by the mAb. In contrast, the A25 target inhibited lysis less effectively and showed only slightly enhanced lysis after blocking KIR3DL1, suggesting that HLA-A*2501 may have some weak binding to KIR3DL1. The A23 target provided intermediate inhibition, which was substantially reversed by blocking the receptor. Interestingly, in donor 4, A23 was the only target that showed a difference in CD107a expression between the high- and low-allele–expressing cells, suggesting a difference in the interaction of HLA-A*2301 with these 2 alleles.

Donors who have HLA-B*1301 or B*1302 can make KIR3DL1-dependent NK clones, but these NK clones are infrequent

Because HLA-B*1302 and HLA-B*1301 are poor ligands for KIR3DL1, we asked whether donors who have HLA-B*13 as their only Bw4$^+$ allele can generate KIR3DL1-dependent NK clones capable of lysing Bw4$^-$ targets. Using the CD107a assay, we tested the ability of 4 donors with Bw4$^+$ alleles (donors 1-4; Table 1), 3 donors with B*1301 or B*1302 as their only Bw4$^+$ allele (donors 5-7; Table 1) and 2 donors with only Bw6$^+$ alleles (donors 8 and 9; Table 1) to generate KIR3DL1-dependent alloreactive NK clones. NK cells from each donor were incubated alone, with 721.221, with a target lacking only the Bw4 epitope (C1$^+$, C2$^+$, Bw4$^-$), with a target lacking only the C1 epitope (C1$^-$, C2$^+$, Bw4$^+$), and with a target lacking only the C2 epitope (C1$^+$, C2$^-$, Bw4$^+$). KIR3DL1$^+$, CD158a,b$^-$ cells were selected, and CD107a expression was measured against the range of target cells. Representative cytofluorograms of CD107a expression in the presence of 721.221 and the Bw4$^-$ target are shown in Figure 6A. As shown in Figure 6B, among the donors with Bw4 alleles other than B*13, donors 1 and 4 up-regulated CD107a expression on a large proportion of KIR3DL1$^+$ NK cells in response to the Bw4$^-$ target (KIR3DL1-dependent), whereas for donors 2 and 3, a much lower percentage of KIR3DL1$^+$ NK cells were KIR3DL1 dependent. In fact, donors 2 and 3 had similar proportions of KIR3DL1-dependent NK cells to the 3 HLA-B*13 and the 2 Bw6 homozygous donors. Thus, by this measure, HLA-B*13 donors were not distinguishable from some donors expressing other Bw4 alleles. However, as shown in Figure 6C, the proportion of KIR3DL1$^+$, CD158a,b$^-$ NK cells also differed considerably between donors. When the proportion of KIR3DL1$^+$, CD158a,b$^-$ NK cells that were KIR3DL1 dependent (Figure 6B) was multiplied by the proportion of all NK cells that were KIR3DL1$^+$, CD158a,b$^-$ (Figure 6C) to determine the percentage of all NK cells from each donor that were KIR3DL1 dependent (Figure 6D), HLA-B*13$^+$ donors could be distinguished from other Bw4$^+$ donors and the Bw6 homozygous donors (donors 8 and 9). Donors with Bw4 alleles other than HLA-B*13 had 2% to 3% KIR3DL1-dependent NK clones, whereas donors with HLA-B*13 or homozygous for Bw6 had less than 0.5%. Donors with HLA-B*13 were, however, able to generate C1-dependent and C2-dependent clones (data not shown).

Donors who lack Bw4-expressing HLA-B alleles but express HLA-A*2402 can make KIR3DL1-dependent NK clones

Because HLA-A*2402 appears to be a ligand for KIR3DL1, we asked whether donors who lack Bw4-expressing alleles other than HLA-A*2402 are able to make KIR3DL1-dependent NK clones that could be exploited in haploidentical transplantation. A total of 129 NK clones were generated from a C1-homozygous, Bw6-homozygous, HLA-A*2402$^+$ donor (donor 10; Table 1). Each NK clone was tested against 4 targets: 721.221 (positive control); a C1$^+$, C2$^-$, Bw4$^-$ target (IHW 9065); a C1$^-$, C2$^+$, Bw4$^-$ target (IHW 9019); and a C1$^+$, C2$^-$, Bw4$^+$ target (IHW 9157) in the 4-hour ^{51}Cr release assay. A total of 14% of all NK clones killed the targets in a pattern consistent with KIR2DL2/KIR2DL3-dependent NK clones and expressed CD158b by flow cytometry, indicating that this donor could make KIR2DL2/KIR2DL3-dependent clones. None of the clones killed targets in a KIR2DL1-dependent manner, as would be expected for an individual who does not have a C2$^+$ HLA-C allele. However, a further 12.5% of all NK clones killed the targets in a pattern consistent with KIR3DL1-dependent NK clones (killed both Bw4$^-$ targets but not the Bw4$^+$ target) and expressed KIR3DL1 by flow cytometry. Thus, donors who express HLA-A*2402 and lack Bw4$^+$ HLA-B alleles can generate KIR3DL1-dependent NK clones.

Discussion

Our data confirm that KIR3DL1-dependent NK cells lyse targets that are homozygous for common Bw6 alleles but do not lyse

From www.bloodjournal.org by on December 4, 2009. For personal use only.

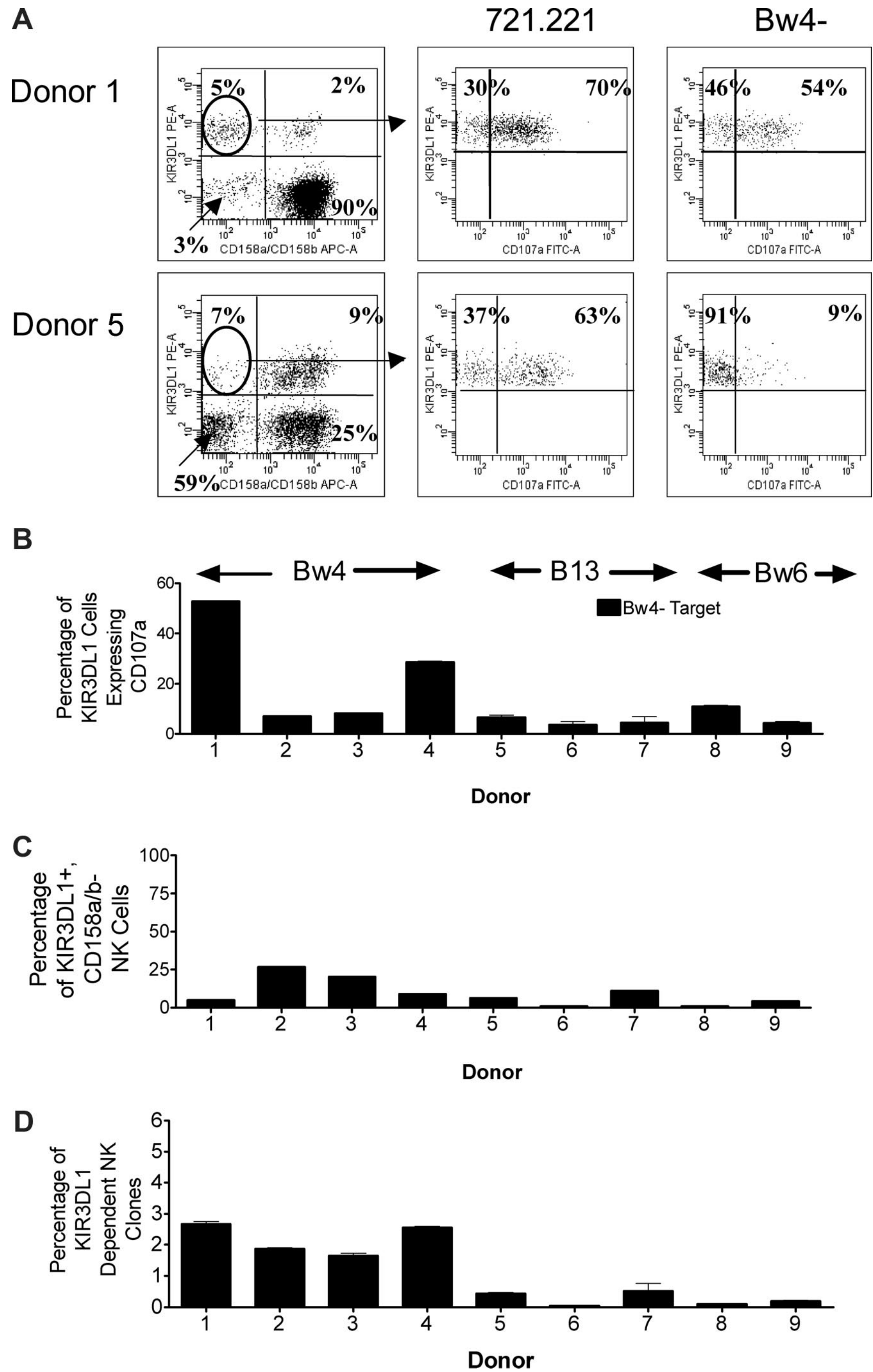


Figure 6. Donors who express HLA-B*1302 or HLA-B*1301 make very few KIR3DL1-dependent NK cells. NK cells from 9 donors (donors 1-9) were tested in the CD107a assay to determine their ability to make KIR3DL1-dependent NK clones. Each donor was incubated with the class I bare target 721.221, targets lacking either the C1 or C2 epitopes (data not shown), or targets lacking only the Bw4 epitope (HLA-A*0301, 1101, B*0702, 3501, C*0401, 0702). (A) Representative examples of flow cytometry showing identification of KIR3DL1$^+$, CD158a,b$^-$ NK cells and their subsequent CD107a expression when incubated with 721.221 or the Bw4$^-$ target. Numbers on plots are percentages of total displayed cells. (B) After incubation with the Bw4$^-$ target, the proportion of KIR3DL1$^+$, CD158a,b$^-$ NK cells that were CD107a$^+$ did not distinguish donors with HLA-B*13 from donors with other Bw4$^+$ alleles and donors homozygous for Bw6. (C) The proportion of KIR3DL1$^+$, CD158a,b$^-$ NK cells does not distinguish HLA-B*13 donors from donors with other Bw4$^+$ alleles and donors homozygous for Bw6. (D) The proportion of KIR3DL1-dependent NK clones of the total NK-cell population distinguishes HLA-B*13 donors from donors with other Bw4$^+$ alleles. A total of 2% to 3% of NK cells from donors with other Bw4$^+$ alleles are KIR3DL1 dependent, whereas this is true of less than 1% of NK cells from HLA-B*13 donors. Less than 0.5% of NK clones from donors homozygous for Bw6 are KIR3DL1 dependent, slightly less than donors with HLA-B13. Error bars represent SEM.

targets with most of the common Bw4 alleles. These findings are consistent with the rule that the amino acid at position 80 of HLA-B molecules has the predominant influence on specificity for KIR3DL1.[7,8] It has been reported that Bw4 alleles with an isoleucine at position 80 (80I) are better inhibitors of KIR3DL1-mediated lysis than those with a threonine at position 80 (80T).[8] In particular, class I HLA-bare cells transfected with HLA-B*5801 (80I) have been reported to be stronger inhibitors of KIR3DL1-dependent clones than cells transfected with HLA-B*2705 and HLA-B*3701 (80T).[15] We were unable to confirm these findings. The interpretation of the data from Cella and colleagues may have been complicated by the fact that most of the targets used in their study were not homozygous at the HLA-B locus. In addition, the difference between 80I and 80T alleles was only apparent when the Bw4-expressing HLA-A alleles were included. It is also possible that the effector cells used in their study had a KIR3DL1 allele that does interact differently with 80I or 80T and was not represented in our study. Nevertheless, our study included 5 high-expression and 2 low-expression alleles of KIR3DL1, including the common KIR3DL1*001, *015, and *005 alleles, and no evidence for an 80I/80T effect was observed. While the hierarchy of different Bw4$^+$ HLA-B alleles reported in earlier publications may well exist if analyzed by the particular read-outs used in those studies, our data suggest that these subtleties may not be significant for target cells expressing a normal array of ligands and polyclonal NK cells.

Among Bw4$^+$ HLA-B alleles, we found only HLA-B*1302 and B*1301 (which is more common in Asian populations) to be poor

From www.bloodjournal.org by on December 4, 2009. For personal use only.

KIR3DL1 ligands. A total of 3 individuals in whom HLA-B*1302 or B*1301 was the only Bw4$^+$ allele had a very low frequency (< 0.5%) of alloreactive NK cells dependent on KIR3DL1 compared with donors with other Bw4$^+$ alleles (2%-3%). These flow cytometric estimates of clonal frequency are comparable with data from Ruggeri and colleagues obtained by direct NK cloning.[5] HLA-B*1301 and HLA-B*1302 are unique among HLA-B and HLA-C alleles in that they have a leucine rather than arginine at position 145, a position that is important in salt bridge formation between HLA-C and KIRs.[16] Mutation of asparagine 135 of KIR2DL2, which forms the salt bridge with arginine 145 of HLA-C, lowered HLA binding affinity by 20-fold relative to the wild-type receptor, suggesting a vital role for proper salt bridge formation in stabilizing KIR/HLA interaction. While the structure of the KIR3DL1/HLA-B complex has not been solved, it is thought that KIR3DL1 interaction with HLA-B may be similar to KIR2DL2 interaction with HLA-C, as many of the positions important in KIR recognition of HLA are conserved among HLA-B and HLA-C alleles.[16] It is possible that the leucine at position 145, which prefers to be buried within the protein hydrophobic core and which also has a nonreactive side-chain, may not form an adequate salt bridge with KIR3DL1.

Conclusions from previous reports addressing the role of Bw4$^+$ HLA-A alleles as ligands for KIR3DL1 have been inconsistent.[8,10,11] In particular, Gumperz et al[10] observed inhibition of a KIR3DL1$^+$ NK clone by HLA-A*2501 and HLA-A*2403, but inhibition was not reversed by blocking with anti-KIR3DL1. Our data agree with the findings of Thananchai et al,[11] which showed that HLA-A*2402 (the common subtype of HLA-A24) is an effective ligand. We show that inhibition by HLA-A24 is reversed by blocking with anti-KIR3DL1 and furthermore show that an individual whose only Bw4$^+$ allele was HLA-A*2402 was able to generate KIR3DL1-dependent NK clones, indicating that, at least in this individual, A*2402 was an effective ligand and capable of "arming" NK cells for effector function. HLA-A*3201 is also an effective ligand. The status of HLA-A*2301 and HLA-A*2501 is less clear. HLA-A*2501–expressing targets exhibited, at best, a weak inhibitory effect in both the CD107a and ^{51}Cr release assays. The interaction of HLA-A*2301 with KIR3DL1 is particularly interesting. The A23 target was virtually ineffective in the CD107a assay with NK cells expressing the KIR3DL1 high allele, but was the only target which clearly showed greater inhibition of NK cells expressing KIR3DL1*005 than those expressing KIR3DL1*01502 in donor 4. The inhibitory capacity of HLA-A*2301 for the KIR3DL1*005 allele was confirmed in the ^{51}Cr release assay using an NK clone expressing KIR3DL1*005 from this same donor. These data suggest that the effectiveness of HLA-A*2301 as a KIR3DL1 ligand may be more dependent on the KIR3DL1 allele than other Bw4 alleles. However, studies using additional A23 targets and further examples of NK cells expressing KIR3DL1*005 and other alleles are required before definitive conclusions can be drawn.

Other groups have reported that different KIR3DL1 alleles interact with Bw4 to differing degrees.[9,15,17,18] For alleles other than HLA-A*2301, we found little difference between the behavior of the high-expression allele KIR3DL1*01502 and the low-expression KIR3DL1*005 allele in donor 4. This result is consistent with the findings of Draghi et al,[9] except they compared KIR3DL1*005 with KIR3DL1*002 rather than KIR3DL1*01502. (KIR3DL1*002 differs from KIR3DL1*01502 by a single amino acid in the D2 domain.) However, for donor 3, there was a clear difference between the NK cells expressing the high-expression and low-expression alleles. The KIR3DL1*005-like variant differs from KIR3DL1*005 by a single nonsynonymous mutation at nucleotide position 115, is weakly expressed, and the mutation appears to alter the ability of this receptor to interact with Bw4. Not only is it poorly inhibited by Bw4 alleles, but NK cells expressing this allele only weakly lyse cells lacking Bw4. Thus, KIR3DL1-dependent clones in donors whose only KIR3DL1 allele is the KIR3DL1*005-like variant may be weakly armed and may not be particularly effective alloreactive NK cells.

One-third of Bw4$^+$ potential stem cell donors cannot make KIR3DL1-dependent clones. Many of these may have the KIR3DL1*004 allele, which is not expressed at the cell surface. However, our data suggest additional explanations. For example, our data suggest that individuals with HLA-B*1301 or HLA-B*1302 as their only Bw4$^+$ allele will make relatively few KIR3DL1-dependent clones and may therefore be a poor choice of donor for a haploidentical transplantation. On the other hand, individuals who have HLA-A*2402 or HLA-A*3201 may well be suitable. The influence of KIR3DL1 polymorphism is also likely to be relevant. Our data suggesting KIR3DL1 polymorphism influences the arming of NK cells on the one hand and the effectiveness of HLA-A*2301 as a ligand on the other hand is consistent with reports of others demonstrating KIR3DL1 allele–dependent variation in strength of interaction with different Bw4 alleles.[9,15,17,18]

The exploitation of alloreactive NK cells in haploidentical transplantations requires both a patient amenable to such therapy and a suitable donor. In addition to Bw4, the HLA-C1 and HLA-C2 epitopes can also mediate NK allorecognition through their corresponding KIR.[5] Only cells from patients who lack either Bw4 and/or the C1 or C2 ligand can be lysed by alloreactive NK cells. Only donors who have the corresponding epitope are capable of NK-mediated alloreactivity. The changed status of HLA-B*13, HLA-A*24, and HLA-A*32 shown in our study means that a number of individuals (patients and donors) will need reclassification. Individuals currently classified as Bw4$^-$ (Bw6 homozygous) would now be classified as Bw4$^+$ if they have HLA-A24 or HLA-A32, while those whose only Bw4$^+$ allele is HLA-B13 would now be classified as Bw4$^-$. Although we do not have a database of HLA-C–typed, haploidentical pairs in which to directly analyze these effects, we can make some predictions from the general population. In a database of 200 white Western Australians typed at HLA-A, HLA-B, and HLA-C by sequencing based on the current definition of Bw4 positivity and the HLA-C alleles present, 28% of individuals would have all 3 NK-cell epitopes (Bw4, C1, C2) and thus not be amenable to NK allotherapy. However, HLA-A24, HLA-A32, and HLA-B13 have frequencies of 20%, 6%, and 6%, respectively, in this population, resulting in 15% of individuals (thus patients and donors) requiring reclassification. The net effect when examined empirically in this population is such that the proportion of individuals (and thus patients) who would now be considered to have all 3 NK-cell epitopes will increase from 28% to 32%. However, for Bw4$^-$ patients, an additional 11% of individuals (and thus donors) would now be expected to be Bw4$^+$ and therefore suitable donors. Such effects will also be evident to various degrees in other ethnic groups depending upon the frequencies of the relevant alleles. HLA-B13, for instance, has a frequency of 25% in some Chinese populations.[19]

Until the rules governing the interaction of HLA alleles and KIR3DL1 alleles are better understood, methods to accurately estimate the frequency of alloreactive clones are required. Our data indicate that the simple flow cytometric assay we have used can do this allowing faster identification of donors suitable for haploidentical transplantations and other NK allotherapeutic applications.

From www.bloodjournal.org by on December 4, 2009. For personal use only.

A formal evaluation of the CD107a assay for detecting C1-, C2-, and Bw4-dependent alloreactive clones is currently under way.

Acknowledgments

The authors gratefully acknowledge the support of the Ray and Bill Dobney Foundation of Royal Perth Hospital and the Child Health Research Foundation of Western Australia.

Authorship

Contribution: B.A.F. performed most of the bench work, including maintenance of cell lines, flow cytography, NK cloning, and chromium release assays, and helped draft the manuscript; D.D.S. helped develop the flow cytometric assays for CD107a, maintained cell lines, and performed NK cloning; E.V.B. performed the NK cloning of the HLA-A24 individual, chromium release assays, and flow cytography to characterize NK clones from this individual; L.J.L. assisted with assay development, laboratory management and design of experiments; F.T.C. designed and analyzed experiments and assisted with manuscript preparation; and C.S.W. designed and analyzed experiments, assisted with manuscript preparation, and had overall responsibility for the research program.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Campbell S. Witt, Department of Clinical Immunology and Immunogenetics, PathWest Royal Perth Hospital, Wellington St, Perth, Western Australia 6000, Australia; e-mail: campbell.witt@health.wa.gov.au.

References

1. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295:2097-2100.
2. Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005;105:3051-3057.
3. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986;319:675-678.
4. Ljunggren HG, Karre K. In search of the “missing self”: MHC molecules and NK recognition. Immunol Today. 1990;11:237-244.
5. Ruggeri L, Mancusi A, Burchielli E, et al. Natural killer cell recognition of missing self and haploidentical hematopoietic transplantation. Semin Cancer Biol. 2006;16:404-411.
6. Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P. The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. J Immunol. 2003;171:6640-6649.
7. D’Andrea A, Chang C, Franz-Bacon K, McClanahan T, Phillips JH, Lanier LL. Molecular cloning of NKB1: a natural killer cell receptor for HLA-B allotypes. J Immunol. 1995;155:2306-2310.
8. Cella M, Longo A, Ferrara GB, Strominger JL, Colonna M. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. J Exp Med. 1994;180:1235-1242.
9. Draghi M, Yawata N, Gleimer M, Yawata M, Valiante NM, Parham P. Single-cell analysis of the human NK cell response to missing self and its inhibition by HLA class I. Blood. 2005;105:2028-2035.
10. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. J Exp Med. 1995;181:1133-1144.
11. Thananchai H, Gillespie G, Martin MP, et al. Cutting edge: allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. J Immunol. 2007;178:33-37.
12. Warren HS, Kinnear BF, Phillips JH, Lanier LL. Production of IL-5 by human NK cells and regulation of IL-5 secretion by IL-4, IL-10, and IL-12. J Immunol. 1995;154:5144-5152.
13. Norman PJ, Abi-Rached L, Gendzekhadze K, et al. Unusual selection on the KIR3DL1/S1 natural killer cell receptor in Africans. Nat Genet. 2007;39:1092-1099.
14. Sun JY, Gaidulis L, Miller MM, et al. Development of a multiplex PCR-SSP method for killer-cell immunoglobulin-like receptor genotyping. Tissue Antigens. 2004;64:462-468.
15. O’Connor GM, Guinan KJ, Cunningham RT, Middleton D, Parham P, Gardiner CM. Functional polymorphism of the KIR3DL1/S1 receptor on human NK cells. J Immunol. 2007;178:235-241.
16. Boyington JC, Motyka SA, Schuck P, Brooks AG, Sun PD. Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. Nature. 2000;405:537-543.
17. Carr WH, Pando MJ, Parham P. KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. J Immunol. 2005;175:5222-5229.
18. Yawata M, Yawata N, Draghi M, Little AM, Partheniou F, Parham P. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. J Exp Med. 2006;203:633-645.
19. Middleton D, Menchaca L, Rood H, et al. New Allele Frequency Database. Tissue Antigens. 2003;61:403-407. Belfast, UK: www.allelefrequencies.net. Accessed February 22, 2008.

CHAPTER 9

FINAL DISCUSSION AND CONCLUSIONS

CHAPTER 9: FINAL DISCUSSION AND CONCLUSIONS

9.1 PCR-SSP SYSTEM FOR KIR GENOTYPING

The increasing number of studies on the role of KIR in transplantation has highlighted the need for a robust and accurate KIR genotyping method. Although the improvements to the PCR-SSP system for KIR genotyping presented in this thesis provided accurate and robust results (as described in chapter 2 and 3), the PCR amplicons were large (~2kb) for the majority of the 11 KIR genes. In addition, each gene was amplified in separate tubes. In a routine diagnostic setting, or when genotyping large cohorts, this method is laborious and the amplification of large amplicons requires high quality DNA to reduce false negative reactions. Since 2004, the PCR-SSP method described in this thesis has been replaced in our laboratory by a multiplex PCR-SSP method described by Sun et al (Sun et al. 2004). This method uses 14 primer pairs used in 4 multiplex reactions for the detection of 15 KIR genes. Primer pairs were designed to amplify shorter amplicons (108-565bp) thereby reducing the likelihood of false negative reactions. The multiplex PCR-SSP system detects all functional KIR genes and the non-expressed KIR2DS4 allele containing the 22-bp deletion. As recent studies including data presented in this thesis (chapter 7) have shown that allelic polymorphism can affect receptor expression and function (Carr et al. 2005,Foley et al. 2008,Yawata et al. 2006), it may be necessary to develop allele-specific typing systems using high throughput methods, such as real-time PCR or sequence based typing, in order to identify allelic polymorphisms influencing function.

9.2 RELEVANCE OF NK ALLOREACTIVITY IN CLINICAL HSCT

9.2.1 KIR Ligand Incompatibility in Haploidentical HSCT

In 2002, Ruggeri et al demonstrated the potential of NK alloreactivity to reduce relapse and allograft rejection. These findings however had not been confirmed by others. Consequently, I studied the role of NK ligand incompatibility and donor/recipient KIR genotypes in an Israeli series of haploidentical donor HSCT (chapter 4). My findings were opposite to those demonstrated by the Italian group. We could not confirm the beneficial effects of NK alloreactivity in the GVH direction. In fact, I found that the potential for NK alloreactivity in the GVH direction was associated with higher incidence of GVHD, rejection and significant decrease in overall survival. Although the transplant protocols were similar between the two studies, the relatively greater number of donor T cells in the Israeli study suggest that thorough T cell depletion may be critical to realising the benefits of NK alloreactivity. Although the increased frequency of GVHD in transplants with potential NK alloreactivity due to HLA-C mismatching was attributed to NK cell alloreactivity, it should be pointed out that there was more HLA-C mismatching in the transplants with potential NK alloreactivity and therefore it is possible that the increased GVHD was due to increased T cell alloreactivity.

Since the initial study by Ruggeri et al (Ruggeri et al. 1999,Ruggeri et al. 2002) and the study presented in this thesis, a number of additional retrospective analyses of haploidentical donor HSCTs have been performed. Both adverse and beneficial effects of potential NK alloreactivity have been reported. In support of the studies reported in this thesis and in contrast to the Perugia study, both Huang et al (Huang et al. 2007) and Zhao et al (Zhao et al. 2007)

reported adverse effects of NK ligand incompatibility in haploidentical transplantation on transplantation outcome.

Huang et al (Huang et al. 2007) studied 76 patients with myeloid leukaemia receiving haploidentical donor HSCT. The transplant protocol differed to that of the Perugia study, in that all patients received T cell replete grafts. Of the 76 patients, 18 patients had KIR ligand incompatible donors and 58 patients had KIR ligand compatible donors. Patients were also categorised as receiving ‘low’ (< 1.48 x 10^8/kg) or ‘high’ (> 1.48 x 10^8/kg) $CD3^+$ T cell doses. The incidence of aGVHD was increased in patients with KIR ligand incompatibility compared to patients who were KIR ligand compatible (p=0.013). The incidence of aGVHD was increased, particularly in patients in the ‘high’ T cell group, compared to patients in the ‘low’ T cell group (p=0.001). There was a highly significant increase in the incidence of aGVHD in patients with a KIR ligand incompatibility who received ‘high’ T cell numbers compared with patients with a KIR ligand incompatibility who received ‘low’ T cell numbers (p<0.00001). These data hint at an interaction between T cells and NK alloreactivity on the occurrence of GVHD. The incidence of relapse was also significantly higher in patients with KIR ligand incompatibility regardless of ‘high’ or ‘low’ T cell numbers and overall survival was inferior compared to KIR ligand compatible patients. It is possible that thorough depletion of T cells may be necessary to achieve the benefits of NK alloreactivity but there were many other protocol differences between the two studies and it is not possible to determine whether T cells are the critical difference.

Zhao et al (Zhao et al. 2007) studied 47 patients receiving T cell replete haploidentical donor HSCT for myeloid leukaemia. This study reported a trend towards inferior leukaemia free survival, overall survival and increased TRM in patients with KIR ligand incompatibility. Consistent with my study in the Israeli cohort and the study of Huang et al (Huang et al. 2007), KIR ligand incompatibility was also associated with increased incidence of aGVHD compared to patients with KIR ligand compatibility (p=0.002).

In a study of 44 patients receiving haploidentical donor HSCT for myeloid leukaemia, Vago et al (Vago et al. 2008) failed to find any effect of NK ligand incompatibility on transplantation outcome. The risk of disease relapse and transplant related mortality were similar in patients with (n=23) or without (n=20) NK ligand incompatibility and no effect was observed in the occurrence of aGVHD or overall survival. The transplant protocol used in this study differed from all the preceding studies in that T-cell add-backs were administered from day 30 after HSCT to protect patients from post-transplant infections. The conditioning regimen was also chemotherapy-based rather than radiation-based. Despite thorough T cell depletion of the graft in this study, the administration of T-cell add backs may have prevented the beneficial effects of NK alloreactivity to be realised.

All the studies mentioned above analysed the effect of potential NK alloreactivity on transplantation outcome based on the KIR ligand incompatibility model. Leung et al (Leung et al. 2004) studied the risk of relapse in 36 paediatric patients transplanted with extensively T cell depleted haploidentical donors for myeloid (n=17) or lymphoid (n=19) leukaemia. Three different

models of NK alloreactivity were used to categorise the risk of relapse for each patient into high and low risk; (1) KIR ligand incompatibility, (2) KIR ligand-receptor incompatibility (at least one inhibitory KIR gene expressed in the donor's NK cell repertoire did not recognise any of the recipient's KIR ligands) and (3) NK cell cytotoxicity model (NK cell cytotoxicity against K562 cells was higher than the median 1 month after transplantation). Under the KIR ligand incompatibility model, 28 of the 36 donor-recipient pairs were KIR ligand compatible and therefore classified as high risk for relapse. Nine of these 28 patients (32%) relapsed compared to 1 out of 8 (13%) classified as low risk (ie KIR ligand incompatible). Although this difference was in the right direction for the model, it was not statistically significant. Under the KIR ligand-receptor model, 13 recipients had the appropriate KIR ligands for all the inhibitory KIRs in the donor repertoire and were therefore classified as high risk of relapse (the KIR ligand incompatibility model classified these recipients as low risk). Seven of these 13 patients (54%) relapsed compared to 3 of 23 (13%) patients who had KIR ligand-receptor incompatibility (p=0.0078). No difference in relapse rate was observed between the high and low risk groups based on NK cell cytotoxicity model. The study concluded that the KIR receptor-ligand model was a better predictor of relapse than the KIR ligand incompatibility model as described by the Perugia group. However, it should be noted that the KIR ligand-receptor model was not tested statistically for superiority to the KIR ligand incompatibility model and the small number of transplants would not have allowed significance between the models to be achieved.

Based on the Leung study, Ruggeri et al (Ruggeri et al. 2007) reanalysed their own data in 112 patients who received haploidentical transplants (83 with KIR

ligand-receptor mismatch, 29 without) to compare the KIR receptor-ligand model with the KIR ligand incompatibility model. No significant difference was observed in event free survival (EFS) for the KIR ligand-receptor model (p=0.28) whereas the KIR ligand incompatibility model showed a clear benefit of potential NK alloreactivity (p=0.001). Event free survival in the KIR ligand-receptor incompatible group was worse than that after transplantation from NK ligand incompatible donors. They concluded that KIR ligand incompatibility in the GVH direction was the only model impacting beneficially on transplantation outcome.

9.2.2 KIR Ligand Incompatibility in Unrelated Donor HSCT

As in the haploidentical donor HSCT study reported in this thesis, in unrelated donor HSCT, I also showed the potential for NK alloreactivity in the GVH direction was also associated with higher incidence of GVHD, rejection and a significant decrease in overall survival (chapter 5). Many retrospective studies on the impact of KIR ligand incompatibility in unrelated donor HSCT have now been published and the results are conflicting. Few studies have demonstrated in unrelated donor HSCT the beneficial effects of NK alloreactivity as described by the Perugia group in their haploidentical transplants. Some of the studies reporting beneficial or deleterious effects are discussed below. However, it should be noted that in the data in chapter 5 and most other publications reviewed below, it is not possible to attribute effects of KIR ligand incompatibility to NK cells as opposed to T cells. The publication by Morishima et al and Farag et al clearly show that there is an effect that can be attributed to NK cells (vide infra).

9.2.2.1 Studies finding a beneficial effect of NK ligand incompatibility.

Giebel et al (Giebel et al. 2003), studied 130 paediatric and adult patients (87 patients with myeloid leukaemia) receiving T cell replete unrelated donor HSCT. The majority of patients (97%) received bone marrow as a source of haematopoietic stem cells and all patients were given prophylaxis for GVHD including the use of ATG. In patients with myeloid leukaemia, the study reported significantly better overall survival (100% vs. 45%, p=0.002), disease free survival (100% vs. 37%, p=0.0005) and less relapse (although not significant, 0% vs. 18%, p=0.06) in patients with KIR ligand incompatibility compared to those without ligand incompatibility. A trend towards less severe aGVHD (grade III-IV aGVHD) was also reported in transplants with KIR ligand incompatibility (15%) compared to those without (0%) (p=0.08). If low donor T-cell numbers are required to obtain benefit from NK alloreactivity, the use of anti-thymocyte globulin (ATG) and bone marrow stem cells, which contain fewer T cells than mobilised peripheral blood stem cells, may have favoured the observed beneficial effect of NK cell alloreactivity on transplant outcome.

The possibility that ATG in the conditioning regimen helps realise the benefits of KIR ligand incompatibility in T cell replete unrelated HSCT is supported by a study by Yabe et al (Yabe et al. 2008). This study reanalysed the transplants from Morishima et al (Morishima et al. 2007) but included 94 patients pre-treated with ATG. The cumulative incidence of aGVHD was analysed separately in the non-ATG treated and ATG treated groups. As previously reported (Morishima et al. 2007), in patients who were not treated with ATG, KIR ligand incompatibility in the GVH direction was associated with a significantly higher incidence of grades III-IV aGVHD (47.7% vs. 29.4%, p=0.0014) and poorer

overall survival (21% vs. 42%, p<0.0001) compared to patients with KIR ligand compatibility. In contrast in those treated with ATG, KIR ligand incompatibility provided protection against GVHD. None of the 11 patients given ATG with KIR ligand incompatibility developed grade III-IV (p=0.042) which was significantly less than in the KIR ligand compatible patients. In patients given ATG, there was no significant difference in overall survival between the KIR ligand incompatible and KIR ligand compatible patients (36.4% vs. 39.5%, p=0.79). These data suggest that pre-administration of ATG may abolish the adverse effect of KIR ligand incompatibility on survival presumably through abolition of T cell mediated effects, and may even result in protection from GVHD in the KIR ligand incompatible group through NK cell mediated effects. Other retrospective studies (Bornhauser et al. 2004,Lowe et al. 2003,Schaffer et al. 2004) however have not confirmed the beneficial effects of the use of ATG on potential NK alloreactivity.

In support of the report of Giebel et al (Giebel et al. 2003), in an analysis of 374 patients with myeloid leukaemia receiving unrelated donor HSCT, Beelen et al (Beelen et al. 2005) reported that KIR ligand incompatibility decreased the risk of relapse. Of the 374 patients with myeloid leukaemia receiving unrelated donor HSCT, 2 of the 48 (4%) patients who were KIR ligand incompatible relapsed compared to 49/326 (15%) who were KIR ligand compatible (p=0.04). The lower relapse rate however did not translate into improved overall or disease free survival. The lower relapse rate compensated for the adverse of an increased risk of aGVHD and graft failure in KIR ligand incompatible transplants also observed in this study.

9.2.2.2 Studies finding a deleterious or no effect of NK ligand incompatibility.

The majority of studies in unrelated donor HSCT have reported similar findings to those presented in this thesis (Chapter 5). Some of the studies finding either deleterious (Bornhauser et al. 2004, Davies et al. 2002, De Santis et al. 2005, Kroger et al. 2006, Lowe et al. 2003, Morishima et al. 2007, Schaffer et al. 2004) or no (Hsu et al. 2006, Sun et al. 2005) effect of KIR ligand incompatibility are discussed below.

Schaffer et al (Schaffer et al. 2004) studied 190 patients receiving T-cell replete unrelated donor HSCT, all of whom received ATG during conditioning. KIR ligand incompatibility was shown to be associated with inferior overall survival (p=0.01). Similar results were obtained when only patients with myeloid leukaemia (p=0.03) were analysed. Those patients who were matched for HLA-A, -B, -DRB1 but mismatched at HLA-C and KIR ligand incompatible had inferior survival (p=0.03). No significant differences were observed in relapse rates, engraftment or aGVHD. The inferior survival in patients receiving KIR ligand incompatible HSCT resulted from the significantly higher transplant related mortality (p=0.02) reflecting the correlation between KIR ligand incompatibility and a higher rate of infection-related mortality, significantly more fungal infections and a trend for more septicaemia. Despite the use of ATG in this study, beneficial effects of NK alloreactivity were not observed.

Most of the studies reviewed so far address the effect of KIR ligand incompatibility on transplantation outcome. Transplants with KIR ligand incompatibility (HLA mismatched donors) are compared with transplants in

which donors are KIR ligand compatible (includes HLA-C allele matched and mismatched transplants). The question as to whether KIR ligand incompatible donors result in poorer outcomes than HLA-C mismatched but KIR ligand compatible donors has only recently been addressed in larger studies. The studies of Morishima et al (Morishima et al. 2007) and Farag et al (Farag et al. 2006) suggest that HLA-C mismatches with a KIR ligand incompatibility have a poorer outcome than other HLA mismatches.

Morishima et al (Morishima et al. 2007) studied 1790 leukaemia patients (557 AML, 596 CML and 617 ALL) receiving T-cell replete marrow from unrelated donors. ATG was not given as GVHD prophylaxis. In AML and CML, KIR ligand incompatibility in the GVH direction was associated with significantly higher risk of grade II-IV aGVHD (HR=1.70, p<0.001) and grade III-IV aGVHD (HR=2.35, p<0.001) compared with KIR ligand compatibility. In ALL, the trend was similar but not significant (p=0.11). KIR ligand incompatibility was also a significant risk for mortality in AML (p<0.005) and CML (p<0.001) patients, but less of a risk in ALL (p=0.09) patients. The large number of patients included in this study enabled the effect of KIR ligand incompatibility in the GVH direction to be compared to KIR ligand incompatibility among HLA-C mismatched transplants. KIR ligand incompatibility among HLA-C mismatched transplants resulted in lower survival in AML (p=0.01), CML (p<0.001) and ALL (p=0.04) patients than for those HLA-C mismatched but KIR ligand compatible transplants. As all transplants were HLA-C mismatched, these data suggest that KIR ligand incompatibility is deleterious compared to KIR ligand compatibility particularly among HLA-C mismatched transplants and are consistent with this effect being NK cell mediated rather than T cell mediated. KIR ligand incompatibility in GVH

direction had no effect on leukaemia relapse in myeloid leukaemias (HR= 1.05, p=0.926) whereas in ALL patients, relapse rates were higher in those with KIR ligand incompatibility (HR=2.55, p=0.017).

Farag et al (Farag et al. 2006), in a study of 1571 patients who received T-cell replete unrelated HSCT for myeloid malignancies compared transplants from KIR ligand incompatible donors with HLA matched donors to determine whether selecting donors with KIR ligand incompatibility would be beneficial in transplantation outcome. Donor–recipient pairs were either, HLA-A, -B, -C, and DRB1 matched (n=1004), KIR ligand incompatible in the GVH direction (n=137), KIR ligand incompatible in the HVG (n=170), or HLA-B and/or –C–mismatched but KIR ligand compatible (n=260). KIR ligand incompatibility resulted in significantly more grade III-IV GVHD (p=0.001), TRM (p<0.001), relapse (p=0.04) and mortality (p<0.001). KIR ligand incompatibility among HLA mismatched transplants resulted in significantly lower survival rates compared to KIR ligand compatible transplants (p<0.001). These data are consistent with those described by Morishima et al (Morishima et al. 2007).

Hsu et al (Hsu et al. 2006) in a study of 1770 transplants receiving T-cell replete unrelated donor HSCT for myeloid and lymphoid leukaemia submitted from 22 participating transplant centres as part of the International Histocompatibility Working Group (IHWG). In 428 HLA-B and/or HLA-C mismatched pairs, there was no statistically significant difference in relapse and survival rates between patients who were KIR ligand incompatible in the GVH direction compared to those who were KIR ligand compatible.

9.2.3 NK alloreactive cells as adoptive immunotherapy of cancer

In addition to the potential role of NK alloreactive cells in HSCT setting, the use of alloreactive NK cells as a form of adoptive cell based immunotherapy in leukaemia has also been considered. Miller et al (Miller et al. 2005) infused haploidentical NK cells into AML patients in a non-transplant setting. In 19 AML patients receiving NK cell infusions, a significantly higher complete remission rate when KIR ligand incompatible compared to ligand compatible donors were used, without the development of aGVHD. Patients received a high dose conditioning regimen consisting of 60mg/kg intravenous cyclophosphamide and 25mg/m^2 intravenous fludarabine for 5 consecutive days 48 hours prior to NK cell infusion. Three of four (75%) patients who were KIR ligand incompatible in the GVH direction achieved a complete remission. In contrast 2 of 15 (13%) patients who were KIR ligand compatible achieved remission (p=0.04). The number of circulating NK cells was significantly higher in patients who achieved complete remission compared to those that did not (51% vs. 8%, p=0.027). Further larger studies are required to confirm the promising preliminary findings of this study if alloreactive NK cells are to be used in adoptive immunotherapy of cancer.

9.2.4 The Influence of Donor KIR genotype on HSCT outcome.

In the haploidentical transplantation study reported in this thesis, I showed high numbers of donor KIR receptors (both inhibitory and activating) were associated with higher incidence and more severe aGVHD (grade III-IV aGVHD) and a trend towards improved survival (chapter 4). In contrast, in unrelated donor HSCT, my findings indicated high numbers of donor KIR receptors was associated with decreased aGVHD and improved survival (chapter 5).

The influence of donor KIR genotype on transplant outcome is still unclear. Some studies have analysed outcomes in terms of the presence or absence of the KIR B haplotype, while others have analysed outcomes in terms of the total number of KIR genes or activating KIR genes. As the KIR B haplotype consists of a high number of activating KIR genes compared to the KIR A haplotype, the presence of the KIR B haplotype is highly correlated with increased total number of KIR genes or activating KIR genes. Therefore the analysis methods used in these studies are comparable. Some studies analysing the effects on clinical outcome of donor KIR genotype in HLA identical sibling and unrelated donor HSCT have found a deleterious or no effect, while others have found a beneficial effect. Some of these studies are discussed below.

9.2.4.1 Studies finding a deleterious effect of high number of donor activating KIR or the KIR haplotype B.

McQueen et al (McQueen et al. 2007) studied 113 patients with myeloid leukaemia who received an allograft from a T-cell replete, HLA identical sibling donor. The combination of the presence of the KIR haplotype B in the donor (Bx donor) and a recipient who lacks the KIR haplotype B (an AA recipient) was a risk factor for relapse and aGVHD grade II-IV which translated into poorer overall survival (p=0.046). Eight out of 59 transplants performed for myeloid leukaemia involved a Bx donor and AA recipient, and four of the eight (50%) relapsed compared to none of five Bx patients grafted from an AA donor relapsed (p=0.02). The incidence of aGVHD in AA recipients receiving a graft from a Bx donor was 46% (5/11) compared to AA recipients receiving a AA donor graft 10% (2/20), p=0.04). The presence of KIR haplotype B or a higher

number of activating KIR genes in the donor was therefore associated with the poorest clinical outcomes.

Clausen et al (Clausen et al. 2007) studied 43 patients receiving T cell replete peripheral blood stem cells (PBSC) from their HLA identical sibling donor for a variety of malignancies including myeloid leukaemia (n=18). A high number of activating KIRs was associated with poorer outcome. If the donor had three or more activating KIRs, the cumulative incidence of non-relapse mortality was significantly higher than transplants from a donor with less than three activating KIRs (40% vs. 0%, p=0.01). The increased risk of non-relapse related mortality translated into poorer overall survival in multivariate analysis (p=0.022).

Kroger et al (Kroger et al. 2006) studied 142 patients receiving T cell depleted unrelated donor HSCT for myeloid leukaemia (n=90) and lymphoid leukaemia (n=52). All patients received ATG. The source of stem cells included both peripheral blood and bone marrow. Patients who received an allograft from a KIR haplotype B donor had poorer disease-free survival (p=0.05) and increased risk of relapse (p=0.03) than those with a KIR haplotype A donor. As the KIR haplotype B consists of a high number of activating KIR genes, the authors also analysed the data based on the number of activating KIR genes in the donor. A high number of donor activating KIR genes (> 3 activating donor KIR genes) was significantly associated with increased risk of relapse (p=0.005) and poorer disease free survival (p=0.04). The detrimental effect of high number of activating KIR genes was mainly found in AML/MDS patients, to a lesser extent in CML patients and but not at all in ALL patients. The number of activating KIR genes had no effect on transplant-related mortality or incidence of GVHD.

Yabe et al (Yabe et al. 2008) observed in patients receiving a graft from a KIR2DS2 positive donor with KIR ligand incompatibility in the GVH direction, a higher incidence of severe aGVHD (70.9% grade III-IV compared with KIR2DS2 negative donor, 33.6%, p=0.012). No association was observed between KIR2DS2 and relapse or overall survival.

9.2.4.2 Studies finding a beneficial effect of high number of donor activating KIR or KIR haplotype B.

Verheyden et al (Verheyden et al. 2005) studied 65 patients, 49 with myeloid and 16 with lymphoid leukaemia, who received HLA identical sibling donor transplants (51% of which were T cell depleted). The combined presence of activating KIR2DS1 and KIR2DS2 in donors was significantly associated with decreased incidence of relapse compared to donors who were positive for only one of these activating KIR or negative for both (17% versus 61.3%, p = 0.018).

Savani et al (Savani et al. 2007) studied 54 patients, 39 with myeloid and 15 with lymphoid leukaemia who received a HSCT using a T cell depleted HLA identical sibling donor. In myeloid leukaemia, a higher total number and a higher number of activating and of inhibitory KIR genes were all associated with reduced relapse (total KIR, p=0.002, activating KIR p=0.006, inhibitory KIR p=0.01).

The largest study to show a beneficial effect of the KIR B haplotype is that of Cooley et al (Cooley et al. 2009) who studied 448 AML patients receiving unrelated donor HSCT. In a multivariate analysis the 3-year relapse-free survival was significantly higher in patients whose donors had at least one KIR

B haplotype (KIR B/x) compared with KIR AA haplotype donors (p=0.002). Transplantations using KIR B/x donors were associated with a trend towards decreased risk of relapse and transplant related mortality. Donor KIR B/x did not affect the incidence of aGVHD but was associated with increased incidence of chronic GVHD.

Triplett et al (Triplett et al. 2009) found in 59 patients transplanted with unrelated donor HSCT, patients who were positive for HLA C2 group and received a donor graft which was negative for KIR2DS1 had a significantly better survival (p=0.02). The improved survival was attributed to lower relapse and transplant-related mortality.

9.2.4.3 Studies finding no effect of high number of donor activating KIR or KIR haplotype B.

Two small underpowered studies failed to find an effect of donor KIR gene number on transplantation outcome. Kim et al (Kim et al. 2007) studied 44 patients receiving unrelated donor HSCT. Most of the donor-recipient pairs were HLA mismatched. This study did not observe a significant association between a particular KIR haplotype and aGVHD. The number of donor activating KIR genes was also not associated with aGVHD. The only significant association found was the occurrence of less aGVHD in patients whose donors had 12 or more KIR genes (activating or inhibitory) compared with donors with 11 or less (p<0.05). These results however were not statistically significant after correction for multiple comparisons. Sun et al (Sun et al. 2005) studied 65 AML who received T-cell replete unrelated donor HSCT. This study reported no

association between high number of activating KIR or KIR haplotype B and aGVHD, relapse and survival.

In a large multi-centre evaluation of 739 HLA matched or mismatched T-cell replete unrelated donor HSCT, Hsu et al (Hsu et al. 2005) found KIR2DS1 or KIR2DS2 was not associated with relapse or improved survival. Likewise, no associated was found between cumulative numbers of donor activating KIR and relapse or survival.

9.2.5 Conclusions from Clinical Studies

The beneficial effects of potential NK alloreactivity in the GVH direction demonstrated by the Perugia study (Ruggeri et al. 1999) were not confirmed in haploidentical donor HSCT (Huang et al. 2007, Leung et al. 2004, Zhao et al. 2007) or unrelated donor HSCT (Bornhauser et al. 2004, Davies et al. 2002, Hsu et al. 2006, Kroger et al. 2006, Lowe et al. 2003, Morishima et al. 2007, Schaffer et al. 2004, Sun et al. 2005) including my own studies. However, it should be noted that none of these studies replicated the transplant protocol used in the Perugia study. The extensive T cell depletion used in Perugia compared to the other studies has been considered as a possible explanation for the differences observed (Bishara et al. 2004, Huang et al. 2007, Yabe et al. 2008). If T cell numbers are critically important, then the haploidentical study reported in this thesis suggests that an average of 10.8 x 10^4 T cells/kg compared to 3 x 10^4 T cells/kg in the Perugia study could nullify the beneficial effects of NK alloreactivity. Huang et al (Huang et al. 2007) demonstrated a significant increase in the incidence of aGVHD in patients with KIR ligand incompatibility who received high T cell numbers (more than 1.48 x 10^8/kg) but

little effect in the ‘low’-T cell group (p=0.039). Yabe et al (Yabe et al. 2008) demonstrated that KIR ligand incompatibility in the GVH direction resulted in higher aGVHD incidence and lower overall survival in patients who were not administered ATG, and that ATG administration in the conditioning regimen prevented these adverse effects. These data support the notion that the presence of T cells may prevent the benefits of NK alloreactivity from being realised. However, other differences in transplant protocols, such as conditioning regimen or stem cell source, or heterogeneous cohorts with differences in the proportions of disease type could also be responsible for the differences observed between the studies above.

As is the case with KIR ligand incompatibility, both beneficial and deleterious effects have been reported to be associated with the KIR genotype of the donor. It is unclear why there are conflicting results. It may be that the influence of donor KIR genotype on transplantation outcome may also be transplant protocol dependent. Larger studies taking into account transplant protocol, disease and disease status are necessary to further clarify the role of donor KIR genotype on transplantation outcome.

9.3 CD107A ASSAY FOR THE DETECTION OF NK ALLOREACTIVE CELLS

If NK cell alloreactivity is to be exploited in stem cell transplants, it will be important to be able to reliably select donors who are alloreactive towards the patient. In chapter 6 I described a rapid flow cytometry based cytotoxicity assay of NK alloreactivity utilising CD107a expression on 12-day polyclonal expanded NK cells, when incubated in the presence of allogeneic target cells. NK

alloreactivity mediated by inhibitory or activating KIR (KIR2DS1) could be detected by measuring CD107a expression following incubation with BLCL targets lacking or expressing the appropriate KIR ligand. The CD107a cytotoxicity assay was shown to be sufficiently sensitive to detect not only NK alloreactive cells dependent on inhibitory KIR but also the activating receptor KIR2DS1. However, further studies are required before the CD107a assay could be considered a reliable routine assay with a clear cut off for positivity for the detection of KIR2DS1-dependent NK cells. The average frequency of C1-, C2-, and Bw4-dependent alloreactive NK cells found in my study using the CD107a assay correlated well to the frequencies reported by NK cloning and chromium release assay (Ruggeri et al. 2005). However, it was clear that the polyclonal expansion phase of the assay slightly favoured the outgrowth of alloreactive clones. Therefore the frequencies detected by the assay will provide a slight overestimate of precursor frequencies.

Alloreactive NK cells of the appropriate specificity as predicted by HLA and KIR genetics were identified in the majority of the NK donors, however there were a few exceptions that could not be explained by the current knowledge of the rules governing NK alloreactivity. Such exceptions may reveal atypical biology of the KIR receptor alleles or HLA ligands and highlight the value of the CD107a assay I developed.

9.3.1 Activating KIR confer potential NK alloreactivity

In chapter 7 of this thesis, we provided evidence that activating receptors, specifically KIR2DS1, can confer NK alloreactivity but only in individuals who did not have C2, the ligand for KIR2DS1. C1 homozygous (i.e. C2 negative),

KIR2DS1-positive individuals possessed alloreactive NK clones that were activated via interaction of KIR2DS1 with HLA-C alleles with the C2 epitope.

In a subsequent study supporting my findings, Morvan et al (Morvan et al. 2008) reported that $KIR2DS1^{+}$ NK cells recognise and are activated by C2 but only in individuals who do not express the C2 epitope. In functional assays (CD107a expression and IFN production), these authors demonstrated that $KIR2DS1^{+}/KIR2DL1/2/3/2DS2/NKG2A^{-}$ NK cells expressed CD107a (65.5%) and produced IFN (29.1%) in the presence of a $C2^{+}$ cell line but only in $C2^{-}$, $KIR2DL1^{+}$ individuals. In $C2^{-}$, $KIR2DL1^{-}$ and $C2^{+}$, $KIR2DL1^{+}$ individuals, the percentage of these cells expressing CD107a and IFN following stimulation with a $C2^{+}$ cell line was low. In the $C2^{-}$, $KIR2DL1^{-}$ individual the percentage of $KIR2DS1^{+}/KIR2DL1/2/3/2DS2/NKG2A^{-}$ NK cells expressing CD107a and producing IFN was 9.3% and 8.4%, respectively whereas in the $C2^{+}$, $KIR2DL1^{+}$ individual the percentage of these NK cells expressing CD107a and producing IFN was 3% and 0.2%, respectively.

Fauriat et al (Fauriat et al. 2010) investigated whether the expression of KIR2DS1 in the presence or absence of its HLA ligand could affect NK cell education. CD107a-based assays were used to determine the NK cell responsiveness of fresh or overnight cultured NK cells expressing KIR2DS1 in the absence of KIR2DL1/2/3, KIR3DL1 and NKG2A, following stimulation with either K562 or HLA-expressing targets. Consistent with my findings, they demonstrated that $KIR2DS1^{+}/KIR2DL1/2/3^{-}/KIR3DL1^{-}/NKG2A^{-}$ (KIR2DS1-dependent) NK cells from C2 homozygous individuals did not lyse $C2^{+}$ target cells whereas such cells from C1 homozygous individuals clearly responded to

C2^{+} target cells. They also showed that KIR2DS1-dependent NK cells from C2 homozygous individuals (freshly isolated) stimulated with K562 (HLA bare) target cells were not responsive and comparable to that of KIR^{-}NKG2A^{-} NK cells. The percentage of KIR2DS1-dependent and KIR^{-}NKG2A^{-} NK cells expressing CD107a was 3.4% and 3.9%, respectively. The responsiveness to K562 target cells following overnight stimulation of KIR2DS1-dependent NK cells with IFN and IL-15 was clearly lower than in KIR^{-}NKG2A^{-} NK cells. These results indicate that KIR2DS1 in C2 homozygous individuals is incapable of inducing NK alloreactivity.

The question remains why functional activating receptors specific for HLA ligands, as is the case for KIR2DS1 in C1 homozygous individuals, exist if these NK cells may never encounter the ligand. The function of such NK cells may be to recognise allogeneic cells in pregnancy where the foetus will express a paternal HLA-C antigen. Maternal NK cell recognition of foetal HLA-C antigens is known to play an important role in placentation (Hiby et al. 2004). Alternatively they may recognise pathogen-encoded, -derived or –altered self ligands. The data from all three studies (Foley et al. 2008, Morvan et al. 2008, Fauriat et al. 2010) suggest that the education of NK cells via activating receptors secures tolerance that complements education via inhibitory KIRs.

The alloreactivity of KIR receptors both activating and inhibitory is dose-dependent. In our study, we have shown a dose-dependent effect of activating KIR receptors in that a KIR2DS1-dependent NK clone (clone C9) was capable of NK alloreactivity through the C2 epitope and in a dose-dependent manner, that is cytotoxicity was stronger against C2/C2 homozygous targets than C1/C2

heterozygous targets. Similarly, Fauriat et al (Fauriat et al. 2010) observed a dose-dependent NK response following the interaction of inhibitory KIR receptors to their corresponding HLA ligands (Fauriat et al. 2010). They showed that KIR2DL1^{+} and KIR2DL3^{+}-mediated NK cell cytotoxicity to K562 target cells was weaker in C1/C2 heterozygous individuals compared to either C2 or C1 homozygous individuals. The dose effect observed in both studies seems to be generalised to inhibitory receptors as well as activating receptors.

The ligands for most activating KIR receptors are unknown. Recently, Graef et al (Graef et al. 2009) have reported on the HLA class I specificity of KIR2DS4. They showed that soluble KIR2DS4-Fc fusion protein (made from the common KIR2DS4*001 allele) bound to three HLA-C alleles with the C1 epitope (Cw*1601, Cw*0102 and Cw*1402), three with the C2 epitope (Cw*0501, Cw*0202 and Cw*0401) and HLA-A*1101 and -A*1102. Of the KIR2DS4 alleles they studied, only KIR2DS4*001 was shown to interact in functional assays with HLA class I alleles. The other seven KIR2DS4 alleles share a deletion of 22bp in exon 5 that changes the reading frame and causes a premature termination resulting in a protein that is soluble. In functional experiments, they showed that the interaction between KIR2DS4*001 on G4-NKL cells and HLA-A*1102 on 721.221 target cells activated NK cell-mediated lysis. The lysis of 721.221 cells transfected with HLA-A*1102 was decreased when the G4-NKL cells also expressed the inhibitory receptor KIR3DL2, the inhibitory receptor that recognises A*1102 (Hansasuta et al. 2004, Pende et al. 1996). In contrast, a functional effect of the interaction of KIR2DS4*001 with HLA-A*1101, or HLA-Cw alleles could not be detected. This result is in contrast to the previous findings of Katz et al (Katz et al. 2001) who showed HLA-Cw*04 to be a

functional ligand for KIR2DS4*001. It is not yet clear if the activating KIR2DS4 receptor has the potential to mediate clinically relevant NK alloreactivity. Further studies to clarify its role are required.

9.3.2 KIR3DL1 and Bw4+ HLA-B alleles

As described in chapter 8 of this thesis, we demonstrated that most Bw4$^+$ HLA-B alleles tested, but not B*1301 and B*1302, are ligands for KIR3DL1 and that donors who expressed HLA-B*1301 or -1302 in the absence of any other Bw4$^+$ allele could not generate KIR3DL1-dependent NK clones.

Subsequently, Sanjanwala et al (Sanjanwala et al. 2008) published a study of the amino acid residues that constitute the Bw4 epitope recognised by NK cells. The Bw4 motif of HLA-B and -A alleles is comprised of three variable (77, 80 and 81) and two conserved residues (82 and 83). In mutagenesis experiments, in which Bw4 residues were replaced with their Bw6 counterparts, Sanjanwala et al (Sanjanwala et al. 2008) demonstrated that none of the variable residues of the Bw4 motif were essential for binding to KIR3DL1 and in the production of IFN or NK cell mediated cytotoxicity in ^{51}chromium release assays. However, both conserved residues were essential for the binding of Bw4$^+$-B*5101 to KIR3DL1 and only residue 83 was essential for the binding of Bw4$^+$-B*1513 to KIR3DL1. This study also showed that peptide-binding residues of MHC class I molecules affect the interaction with KIR3DL1, supporting previous data demonstrating that the peptides bound by a class I molecule can affect the binding to KIR3DL1 (Thananchai et al. 2007).

9.3.3 KIR3DL1 and $Bw4^+$ HLA-A alleles

The interaction between $Bw4^+$ HLA-A alleles and KIR3DL1 has been controversial. In an attempt to clarify this interaction we systematically evaluated the ability of the $Bw4^+$ HLA-A alleles (HLA-A*23, -A*24, -A*25 and –A*32) to inhibit KIR3DL1-mediated NK cell cytotoxicity. In chapter 8 we demonstrated that HLA-A*2402 and HLA-A*3201 are effective ligands for KIR3DL1. HLA-A*2501 was shown to bind weakly to KIR3DL1. Preliminary evidence was provided that the binding of HLA-A*2301 to KIR3DL1 was influenced by the specific allele of KIR3DL1. Previous reports on the interaction of the $Bw4^+$ HLA-A alleles with KIR3DL1 have provided contradictory results (Cella et al. 1994,Gumperz et al. 1995). Such differences may be due to the high degree of KIR3DL1 polymorphism and the differences in binding affinity of the different alleles of KIR3DL1 that is now known (Carr et al. 2005,Pando et al. 2003,Yawata et al. 2006) but which was not known at the time of the first reports (Cella et al. 1994,Gumperz et al. 1995). Some of the recent studies demonstrating the effect of KIR3DL1 polymorphism on binding of HLA ligands are discussed below.

Our findings support those of a study performed in 2007 by Thananchai et al (Thananchai et al. 2007) who showed that HLA-A*2402 tetramers bound to KIR3DL1. HLA-A*2402 tetramers bound to both KIR3DL1*001 and KIR3DL1*005 and the amount bound correlated with KIR3DL1 expression as determined by staining with the anti-KIR3DL1 antibody, DX9. NK cell clones expressing either KIR3DL1*001 or KIR3DL1*005 were also inhibited by 721.221 target cells expressing HLA-A*2402 in cytotoxicity assays, and inhibition was blocked by the addition of DX9 antibody. However, HLA-A*2402 tetramers

loaded with different peptides bound differentially to different KIR3DL1 alleles. Of the six tetramers tested, four bound 3DL1*005, three bound 3DL1*001, and only one bound 3DL1*007 and 3DL1*1502. Whether these peptide specific effects are likely to influence the development of NK alloreactivity in individuals with different KIR3DL1 alleles is not clear.

Our study has been confirmed by Stern et al (Stern et al. 2008) who also demonstrated, in functional studies, that A*2402 and A*3201 but not HLA-A*2501 are ligands of KIR3DL1. However, in contrast to our findings, Stern et al (Stern et al. 2008) also demonstrated that HLA-A*2301 is a ligand of KIR3DL1. NK clones positive for KIR3DL1 and negative for all other KIR and NKG2A were obtained by limiting dilution culture of NK cells depleted for KIR2DL/S1/2/3 and NKG2A and then positively selected for KIR3DL1 using monoclonal antibodies Z27. NK clones expressing KIR3DL1 were inhibited by target cells expressing HLA-A*2301, -A*2402 or -A*3201, and inhibition was reversed by the addition of antibodies to KIR3DL1 or to class I HLA. In contrast, KIR3DL1$^+$ NK clones lysed target cells expressing HLA-A*2501 and lysis was not increased by the addition of anti-KIR3DL1 or anti-HLA antibodies, suggesting that this allele did not mediate even partial inhibition of cytotoxicity. This finding was confirmed by functional analysis of NK clones of donor origin derived from an acute myeloid leukaemia patient possessing HLA-A*2501 who had received a HSCT from a haploidentical brother possessing HLA-A*3201. Of the 43 NK clones of donor origin tested, 3 lysed patient cryopreserved cells. All three NK clones expressed KIR3DL1 in the absence of other inhibitory KIR receptors.

The interaction between HLA-A*2301 and KIR3DL1 in our study provided evidence that KIR3DL1 allelic variation influences HLA-A specificity. NK cells expressing KIR3DL1*005 but not KIR3DL1*01502 were inhibited by target cells expressing HLA-A*2301. Stern et al (Stern et al. 2008), in chromium release assays, demonstrated that HLA-A*2301 was an effective ligand for KIR3DL1. Both KIR3DL1 donors used in their study possessed two KIR3DL1 alleles (KIR3DL1*001/*002 and KIR3DL1*001/*005). One of the donors possessed the KIR3DL1*005 allele which was shown to interact with A*2301 and inhibit lysis of target cells in our study. The interaction between HLA-A*2301 and KIR3DL1 therefore may depend on the allele of KIR3DL1.

Further evidence that HLA-A alleles interact differently with different KIR3DL1 alleles has been provided by Norman et al (Norman et al. 2009). In cytotoxicity assays, NKL cells lines transduced with different KIR3DL1 alleles were tested against HLA-A*3201 and they showed that KIR3DL1*059 inhibited the 721.221 cell line transfected with A*3201 to a degree that was comparable to that to KIR3DL1*015 allele but less than KIR3DL1*001. Taken together the data described above suggest that the effectiveness of Bw4^{+} HLA-A alleles as ligands for KIR3DL1 may be dependent on the allele of KIR3DL1.

Most recently, supporting the data described above, Sharma et al (Sharma et al. 2009) reported that the D0 and D1+D2 domains of KIR3DL1 were essential for its functional interactions with Bw4^{+} HLA class I ligands. KIR3DL1 alleles differ on the basis of two dimorphic motifs; one constituted by positions 2, 47 and 54 in the D0 domain, and the other at positions 182 in D1 and 283 in D2. KIR3DL1*015 and KIR3DL1*005 differ at both these motifs, whereas

KIR3DL1*001 is a recombinant that combines the D0 motif of KIR3DL1*005 and the D1+D2 motif of KIR3DL1*015. A tetramer comprising an HIV nef peptide bound to A*2402 (A24nef), binds with higher avidity to KIR3DL1*001 than either KIR3DL1*005 or KIR3DL1*015. A mutant form of KIR3DL1 that combined the D0 of KIR3DL1*015 and the D1+D2 of KIR3DL1*005 does not bind A24nef at all, even though it is expressed at the cell surface and binds anti-KIR3DL1 antibody. These data demonstrate the critical importance of both D0 and D1+D2 in the binding of HLA class I ligands to KIR3DL1. Different combinations of the D0 and D1+D2 motifs can give binding sites with different avidity for the A24nef ligand.

9.3.4 Conclusions from Functional Studies

The work in this thesis clarifies some of the interactions between $Bw4^+$ HLA-A and HLA-B alleles and KIR3DL1 receptors and has important implications in the selection of NK alloreactive donors (Miller 2008). It also underlines the value of a relatively rapid functional assay to confirm alloreactivity where allelic variants of KIR3DL1 may influence alloreactivity.

The thesis also described the identification of NK alloreactive cell clones dependent on the activating KIR2DS1 receptor in individuals who were C1 homozygous. The role of NK alloreactivity mediated by KIR2DS1 and the other activating receptors (KIR2DS2, KIR2D3, KIR2DS4, KIR2DS5 and KIR3DS1) in transplantation remains to be determined. The CD107a assay may prove to be useful in identifying potential NK alloreactivity mediated by activating KIR.

9.4 FINAL CONCLUSIONS

It is clear from the many retrospective studies of potential NK alloreactivity and donor KIR genotype in HSCT that opposing effects on transplantation outcome have been observed. Although the presence of T cells in the allograft seems to be an important factor which prevents the beneficial effects of NK alloreactivity, other differences, such as the use of ATG, stem cell dose, disease and disease status, preparative regimen and post-transplant immune suppression could also be responsible. The factors that enable the beneficial effects of NK alloreactivity to be realised need to be identified before donor selection based on KIR ligand incompatibility in the GVH direction is used in the HSCT setting.

If NK alloreactivity is to be exploited to improve the transplantation outcome of myeloid leukaemic patients it will be important to clearly define the exceptions to the rules governing NK alloreactivity. The findings in this thesis which have utilised the rapid functional *in vitro* assay I developed, have clarified some of the interactions between HLA class I alleles and KIR receptors and has provided new important information relevant to donor selection in HSCT.

CHAPTER 10: REFERENCES

The references listed below relate to Chapters 1, 2 and 9. References for other chapters can be found at the end of each chapter.

Abbas AK, Lichtman AH, Pober JS (1997) Cellular and Molecular Immunology.

Alter G, Malenfant JM, Altfeld M (2004) CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods 294:15-22

Arnon TI, Markel G, Mandelboim O (2006) Tumor and viral recognition by natural killer cells receptors. Semin. Cancer Biol 16:348-58

Aversa F, Tabilio A, Velardi A, Cunningham I, Terenzi A, Falzetti F, Ruggeri L, Barbabietola G, Aristei C, Latini P, Reisner Y, Martelli MF (1998) Treatment of high-risk acute leukaemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. N. Engl. J. Med 339:1186-93

Barnes DW and Loutit JF (1957). Treatment of murine leukaemia with x-rays and homologous bone marrow. II. Br J Haematol. Jul;3(3):241-52.

Barten R, Torkar M, Haude A, Trowsdale J, Wilson MJ (2001) Divergent and convergent evolution of NK-cell receptors. Trends Immunol 22:52-7

Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285:727-9

Beelen DW, Ottinger HD, Ferencik S, Elmaagacli AH, Peceny R, Trenschel R, Grosse-Wilde H (2005) Genotypic inhibitory killer immunoglobulin-like receptor ligand incompatibility enhances the long-term antileukaemic effect of unmodified allogeneic hematopoietic stem cell transplantation in patients with myeloid leukaemias. Blood 105:2594-600

Bennett M (1987) Biology and genetics of hybrid resistance. Adv Immunol 41:333-445

Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, Koup RA (2003) Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods 281:65-78

Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M, Conte R, Poggi A, Moretta A, Moretta L (1996) The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. J Exp Med 183:645-50

Biassoni R, Pessino A, Malaspina A, Cantoni C, Bottino C, Sivori S, Moretta L, Moretta A (1997) Role of amino acid position 70 in the binding affinity of p50.1 and p58.1 receptors for HLA-Cw4 molecules. Eur. J Immunol 27:3095-9

Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP (1999) Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu. Rev Immunol 17:189-220

Bishara A, De Santis D, Witt CC, Brautbar C, Christiansen FT, Or R, Nagler A, Slavin S (2004) The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. Tissue Antigens 63:204-11

Blery M, Olcese L, Vivier E (2000) Early signaling via inhibitory and activating NK receptors. Hum. Immunol 61:51-64

Bornhauser M, Schwerdtfeger R, Martin H, Frank KH, Theuser C, Ehninger G, Locatelli F, Velardi A, Giebel S (2004) Role of KIR ligand incompatibility in hematopoietic stem cell transplantation using unrelated donors. Blood 103:2860-1

Borrego F, Kabat J, Kim DK, Lieto L, Maasho K, Pena J, Solana R, Coligan JE (2002) Structure and function of major histocompatibility complex (MHC) class I specific receptors expressed on human natural killer (NK) cells. Mol Immunol 38:637-60

Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG (1998) Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. J Exp Med 187:813-8

Brooks AG, Boyington JC, Sun PD (2000) Natural killer cell recognition of HLA class I molecules. Rev Immunogenet. 2:433-48

Bruhns P, Marchetti P, Fridman WH, Vivier E, Daeron M (1999) Differential roles of N- and C-terminal immunoreceptor tyrosine-based inhibition motifs during inhibition of cell activation by killer cell inhibitory receptors. J. Immunol. 162:3168-75

Burshtyn DN, Lam AS, Weston M, Gupta N, Warmerdam PA, Long EO (1999) Conserved residues amino-terminal of cytoplasmic tyrosines contribute to the SHP-1-mediated inhibitory function of killer cell Ig-like receptors. J. Immunol. 162:897-902

Burshtyn DN, Yang W, Yi T, Long EO (1997) A novel phosphotyrosine motif with a critical amino acid at position -2 for the SH2 domain-mediated activation of the tyrosine phosphatase SHP-1. J. Biol. Chem. 272:13066-72

Carr WH, Pando MJ, Parham P (2005) KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. J Immunol 175:5222-9

Cella M, Longo A, Ferrara GB, Strominger JL, Colonna M (1994) NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. J Exp Med 180:1235-42

Ciccone E, Pende D, Viale O, Di Donato C, Orengo AM, Biassoni R, Verdiani S, Amoroso A, Moretta A, Moretta L (1992) Involvement of HLA Class I alleles in natural killer (NK) cell-specific functions: expression of HLA-Cw3 confers selective protection from lysis by alloreactive NK clones displaying a defined specificity (specificity 2). J Exp Med 176:963-71

Clausen J, Wolf D, Petzer AL, Gunsilius E, Schumacher P, Kircher B, Gastl G, Nachbaur D (2007) Impact of natural killer cell dose and donor killer-cell immunoglobulin-like receptor (KIR) genotype on outcome following human leucocyte antigen-identical haematopoietic stem cell transplantation. Clin Exp Immunol 148:520-8

Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL (1993a) HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. Proc. Natl. Acad. Sci U. S. A 90:12000-4

Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL (1993b) Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. Science 260:1121-4

Colonna M, Samaridis J (1995) Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. Science 268:405-8

Colonna M, Spies T, Strominger JL, Ciccone E, Moretta A, Moretta L, Pende D, Viale O (1992) Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. Proc Natl Acad Sci USA 89:7983-5

Cooley S, Trachtenberg E, Bergemann TL, Saeteurn K, Klein J, Le CT, Marsh SG, Guethlein LA, Parham P, Miller JS, Weisdorf DJ (2009) Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukaemia. Blood 113:726-32

Cooper MA, Fehniger TA, Caligiuri MA (2001) The biology of human natural killer-cell subsets. Trends Immunol. 22:633-40

Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ (2001) ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity. 14:123-33

Cudkowicz G, Stimpfling JH (1964) Deficient growth of c57bl marrow cells transplanted in F1 hybrid mice. Association with the histocompatibility-2 locus. Immunology 7:291-306

Davies SM, Ruggieri L, DeFor T, Wagner JE, Weisdorf DJ, Miller JS, Velardi A, Blazar BR (2002) Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. Blood 100:3825-7

De Santis D, Bishara A, Witt CS, Nagler A, Brautbar C, Slavin S, Christiansen FT (2005) HLA natural killer cell HLA-C epitopes and killer cell immunoglobulin-like receptors both influence outcome of mismatched unrelated donor bone marrow transplants. Tissue Antigens 65:519-28

Djeu JY, Jiang K, Wei S (2002) A view to a kill: signals triggering cytotoxicity. Clin Cancer Res 8:636-40

Dohring C, Scheidegger D, Samaridis J, Cella M, Colonna M (1996) A human killer inhibitory receptor specific for HLA-A. J Immunol 156:3098-101

Fan QR, Mosyak L, Winter CC, Wagtmann N, Long EO, Wiley DC (1997) Structure of the inhibitory receptor for human natural killer cells resembles haematopoietic receptors. Nature 389:96-100

Farag SS, Bacigalupo A, Eapen M, Hurley C, Dupont B, Caligiuri MA, Boudreau C, Nelson G, Oudshoorn M, van Rood J, Velardi A, Maiers M, Setterholm M, Confer D, Posch PE, Anasetti C, Kamani N, Miller JS, Weisdorf D, Davies SM (2006) The effect of KIR ligand incompatibility on the outcome of unrelated donor transplantation: a report from the center for international blood and marrow transplant research, the European blood and marrow transplant registry, and the Dutch registry. Biol Blood Marrow Transplant. 12:876-84

Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA (2002) Natural killer cell receptors: new biology and insights into the graft-versus-leukaemia effect. Blood 100:1935-47

Fassas ABT, Morris C, Badros A, Van Rhee F, Tricot G (2002) Separating Graft-versus-tumour from Graft-versus-host reactions. Leukemia & Lymphoma 43:725-733

Fauriat C, Ivarsson MA, Ljunggren HG, Malmberg KJ, Michaelsson J (2010) Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. Blood 115:1166-74

Foley BA, De Santis D, Van Beelen E, Lathbury LJ, Christiansen FT, Witt CS (2008) The reactivity of Bw4+ HLA-B and HLA-A alleles with KIR3DL1: implications for patient and donor suitability for haploidentical stem cell transplantations. Blood 112:435-43

Fukuda M (1991) Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. J Biol Chem 266:21327-30

Gagne K, Brizard G, Gueglio B, Milpied N, Herry P, Bonneville F, Cheneau ML, Schleinitz N, Cesbron A, Follea G, Harrousseau JL, Bignon JD (2002) Relevance of KIR gene polymorphisms in bone marrow transplantation outcome. Hum Immunol. 63:271-80

Gardiner CM, Guethlein LA, Shilling HG, Pando M, Carr WH, Rajalingam R, Vilches C, Parham P (2001) Different NK cell surface phenotypes defined by the DX9 antibody are due to KIR3DL1 gene polymorphism. J Immunol 166:2992-3001

Giebel S, Locatelli F, Lamparelli T, Velardi A, Davies S, Frumento G, Maccario R, Bonetti F, Wojnar J, Martinetti M, Frassoni F, Giorgiani G, Bacigalupo A, Holowiecki J (2003) Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. Blood 102:814-9

Gomez-Lozano N, Vilches C (2002) Genotyping of human killer-cell immunoglobulin-like receptor genes by polymerase chain reaction with sequence-specific primers: an update. Tissue Antigens 59:184-93

Goulmy E, Schipper R, Pool J, Blokland E, Falkenburg JH, Vossen J, Gratwohl A, Vogelsang GB, van Houwelingen HC, van Rood JJ (1996) Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. N. Engl. J Med 334:281-5

Graef T, Moesta AK, Norman PJ, Abi-Rached L, Vago L, Older Aguilar AM, Gleimer M, Hammond JA, Guethlein LA, Bushnell DA, Robinson PJ, Parham P (2009) KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A*11 while diminishing avidity for HLA-C. J Exp Med 206:2557-72

Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P (1995) The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. J Exp Med 181:1133-44

Hansasuta P, Dong T, Thananchai H, Weekes M, Willberg C, Aldemir H, Rowland-Jones S, Braud VM (2004) Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. Eur. J. Immunol. 34:1673-9

Hiby, S. E., J. J. Walker, K. M. O'shaughnessy, C. W. Redman, M. Carrington, J. Trowsdale, and A. Moffett. 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J.Exp.Med* 200:957-965.

Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, Rimm AA, Ringden O, Rozman C, Speck B, . (1990) Graft-versus-leukaemia reactions after bone marrow transplantation. Blood 75:555-62

Hsu KC, Gooley T, Malkki M, Pinto-Agnello C, Dupont B, Bignon JD, Bornhauser M, Christiansen F, Gratwohl A, Morishima Y, Oudshoorn M, Ringden O, van Rood JJ, Petersdorf E (2006) KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. Biol Blood Marrow Transplant. 12:828-36

Hsu KC, Keever-Taylor CA, Wilton A, Pinto C, Heller G, Arkun K, O'Reilly RJ, Horowitz MM, Dupont B (2005) Improved outcome in HLA-identical sibling hematopoietic stem cell transplantation for acute myelogenous leukaemia (AML) predicted by KIR and HLA genotypes. Blood 105:4878-84

Hsu KC, Liu XR, Selvakumar A, Mickelson E, O'Reilly RJ, Dupont B (2002) Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. J. Immunol. 169:5118-29

Huang XJ, Zhao XY, Liu DH, Liu KY, Xu LP (2007) Deleterious effects of KIR ligand incompatibility on clinical outcomes in haploidentical hematopoietic stem cell transplantation without in vitro T-cell depletion. leukemi 21:848-51

Janeway Jr CA, Travers P, Walport MJ, Capra JD (1997) Immuno Biology The Immune System in Health and Disease.

Karre K, Ljunggren HG, Piontek G, Kiessling R (1986) Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature 319:675-8

Katz G, Markel G, Mizrahi S, Arnon TI, Mandelboim O (2001) Recognition of HLA-Cw4 but not HLA-Cw6 by the NK cell receptor killer cell Ig-like receptor two-domain short tail number 4. J Immunol 166:7260-7

Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, French AR, Sunwoo JB, Lemieux S, Hansen TH, Yokoyama WM (2005) Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature 436:709-13

Kim SY, Choi HB, Yoon HY, Choi EJ, Cho B, Kim HK, Kim YJ, Kim HJ, Min CK, Kim DW, Lee JW, Min WS, Kim CC, Kim TG (2007) Influence of killer cell immunoglobulin-like receptor genotypes on acute graft-vs-host disease after unrelated hematopoietic stem cell transplantation in Koreans. Tissue Antigens 69 Suppl 1:114-7

Kroger N, Binder T, Zabelina T, Wolschke C, Schieder H, Renges H, Ayuk F, Dahlke J, Eiermann T, Zander A (2006) Low number of donor activating killer immunoglobulin-like receptors (KIR) genes but not KIR-ligand mismatch prevents relapse and improves disease-free survival in leukaemia patients after in vivo T-cell depleted unrelated stem cell transplantation. Transplantation 82:1024-30

Lanier LL (1998) NK cell receptors. Annu. Rev. Immunol. 16:359-93

Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH (1998) Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. Nature 391:703-7

Lee N, Llano M, Carretero M, Ishitani A, Navarro F, Lopez-Botet M, Geraghty DE (1998) HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. Proc. Natl. Acad. Sci U. S. A 95:5199-204

Leung W, Iyengar R, Turner V, Lang P, Bader P, Conn P, Niethammer D, Handgretinger R (2004) Determinants of antileukaemia effects of allogeneic NK cells. J Immunol. 172:644-50

Little MT, Storb R (2002) History of haematopoietic stem-cell transplantation. Nat. Rev Cancer 2:231-8

Litwin V, Gumperz JE, Parham P, Phillips JH, Lanier LL (1994) NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. J Exp Med 180:537-43

Ljunggren HG, Karre K (1990) In search of the "missing self": MHC molecules and NK recognition. Immunol Today 11:237-44

Lowe EJ, Turner V, Handgretinger R, Horwitz EM, Benaim E, Hale GA, Woodard P, Leung W (2003) T-cell alloreactivity dominates natural killer cell alloreactivity in minimally T-cell-depleted HLA-non-identical paediatric bone marrow transplantation. Br. J Haematol. 123:323-6

Martin AM, Freitas EM, Witt CS, Christiansen FT (2000) The genomic organization and evolution of the natural killer immunoglobulin-like receptor (KIR) gene cluster. Immunogenetics 51:268-80

Martin AM, Kulski JK, Gaudieri S, Witt CS, Freitas EM, Trowsdale J, Christiansen FT (2004) Comparative genomic analysis, diversity and evolution of two KIR haplotypes A and B. Gene 335:121-31

Martin PJ, Schoch G, Fisher L, Byers V, Anasetti C, Appelbaum FR, Beatty PG, Doney K, McDonald GB, Sanders JE, . (1990) A retrospective analysis of therapy for acute graft-versus-host disease: initial treatment. Blood 76:1464-72

Mathe G, Amiel Jl, Schwarzenberg L, Cattan A, Schneider M, Devries MJ, Tubiana M, Lalanne C, Binet Jl, Papiernik M, Seman G, Matsukura M, Mery Am, Schwarzmann V, Flaisler A (1965) Successful allogenic bone marrow transplantation in man: Chimerism, induced specific tolerance and possible anti-leukaemic effects. Blood 25:179-96

McQueen KL, Dorighi KM, Guethlein LA, Wong R, Sanjanwala B, Parham P (2007) Donor-recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation. Hum. Immunol 68:309-23

Miller JS (2008) How killers kill. Blood 112:213

Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, McKenna D, Le C, Defor TE, Burns LJ, Orchard PJ, Blazar BR, Wagner JE, Slungaard A, Weisdorf DJ, Okazaki IJ, McGlave PB (2005) Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood 105:3051-7

Moretta A, Biassoni R, Bottino C, Pende D, Vitale M, Poggi A, Mingari MC, Moretta L (1997) Major histocompatibility complex class I-sepcific receptors on human natural killer and T lymphocytes. Immunol Rev 155:105-17

Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, Biassoni R, Moretta L (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu. Rev Immunol 19:197-223

Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R, Bottino C, Moretta L (1995) Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. J Exp Med 182:875-84

Moretta A, Vitale M, Bottino C, Orengo AM, Morelli L, Augugliaro R, Barbaresi M, Ciccone E, Moretta L (1993) P58 molecules as putative receptors for major

histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. J Exp Med 178:597-604

Morishima Y, Yabe T, Matsuo K, Kashiwase K, Inoko H, Saji H, Yamamoto K, Maruya E, Akatsuka Y, Onizuka M, Sakamaki H, Sao H, Ogawa S, Kato S, Juji T, Sasazuki T, Kodera Y (2007) Effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukaemia patients undergoing transplantation with T-cell-replete marrow from an unrelated donor. Biol Blood Marrow Transplant. 13:315-28

Morvan M, David G, Sebille V, Perrin A, Gagne K, Willem C, Kerdudou N, Denis L, Clemenceau B, Follea G, Bignon JD, Retiere C (2008) Autologous and allogeneic HLA KIR ligand environments and activating KIR control KIR NK-cell functions. Eur. J Immunol 38:3474-86

Norman PJ, Abi-Rached L, Gendzekhadze K, Hammond JA, Moesta AK, Sharma D, Graef T, McQueen KL, Guethlein LA, Carrington CV, Chandanayingyong D, Chang YH, Crespi C, Saruhan-Direskeneli G, Hameed K, Kamkamidze G, Koram KA, Layrisse Z, Matamoros N, Mila J, Park MH, Pitchappan RM, Ramdath DD, Shiau MY, Stephens HA, Struik S, Tyan D, Verity DH, Vaughan RW, Davis RW, Fraser PA, Riley EM, Ronaghi M, Parham P (2009) Meiotic recombination generates rich diversity in NK cell receptor genes, alleles, and haplotypes. Genome Res 19:757-69

Norman PJ, Stephens HA, Verity DH, Chandanayingyong D, Vaughan RW (2001) Distribution of natural killer cell immunoglobulin-like receptor sequences in three ethnic groups. Immunogenetics 52:195-205

Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P (2003) The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. J Immunol 171:6640-9

Pende D (1996) The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors which is characterised by three Ig-like domains and is expressed as a 140 kD disulphide-linked dimer. J Exp Med 184:505-18

Pende D, Cantoni C, Rivera P, Vitale M, Castriconi R, Marcenaro S, Nanni M, Biassoni R, Bottino C, Moretta A, Moretta L (2001) Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. Eur. J Immunol 31:1076-86

Raulet DH, Vance RE, McMahon CW (2001) Regulation of the natural killer cell receptor repertoire. Annu. Rev Immunol 19:291-330

Remington RD, Schork MA (1985). Statistics with Applications to the Biological and Health Sciences. 2nd ed.

Ruggeri L, Capanni M, Casucci M, Volpi I, Tosti A, Perruccio K, Urbani E, Negrin RS, Martelli MF, Velardi A (1999) Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. Blood 94:333-9

Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F, Martelli MF, Velardi A (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 295:2097-100

Ruggeri L, Mancusi A, Burchielli E, Perruccio K, Aversa F, Martelli MF, Velardi A (2006) Natural killer cell recognition of missing self and haploidentical hematopoietic transplantation. Semin. Cancer Biol 16:404-11

Ruggeri L, Mancusi A, Capanni M, Martelli MF, Velardi A (2005) Exploitation of alloreactive NK cells in adoptive immunotherapy of cancer. Curr Opin Immunol 17:211-7

Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, Stern M, Pende D, Perruccio K, Burchielli E, Topini F, Bianchi E, Aversa F, Martelli MF, Velardi A (2007) Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukaemia: challenging its predictive value. Blood 110:433-40

Sanjanwala B, Draghi M, Norman PJ, Guethlein LA, Parham P (2008) Polymorphic sites away from the Bw4 epitope that affect interaction of Bw4+ HLA-B with KIR3DL1. J Immunol 181:6293-300

Savani BN, Mielke S, Adams S, Uribe M, Rezvani K, Yong AS, Zeilah J, Kurlander R, Srinivasan R, Childs R, Hensel N, Barrett AJ (2007) Rapid natural killer cell recovery determines outcome after T-cell-depleted HLA-identical stem cell transplantation in patients with myeloid leukaemias but not with acute lymphoblastic leukaemia. leukemi 21:2145-52

Schaffer M, Malmberg KJ, Ringden O, Ljunggren HG, Remberger M (2004) Increased infection-related mortality in KIR-ligand-mismatched unrelated allogeneic hematopoietic stem-cell transplantation. Transplantation 78:1081-5

Sharma D, Bastard K, Guethlein LA, Norman PJ, Yawata N, Yawata M, Pando M, Thananchai H, Dong T, Rowland-Jones S, Brodsky FM, Parham P (2009) Dimorphic motifs in D0 and D1+D2 domains of killer cell Ig-like receptor 3DL1 combine to form receptors with high, moderate, and no avidity for the complex of a peptide derived from HIV and HLA-A*2402. J Immunol 183:4569-82

Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, Shlomchik MJ, Emerson SG (1999) Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science 285:412-5

Spits H, Lanier LL, Phillips JH (1995) Development of human T and natural killer cells. Blood 85:2654-70

Stern M, Ruggeri L, Capanni M, Mancusi A, Velardi A (2008) Human leukocyte antigens A23, A24, and A32 but not A25 are ligands for KIR3DL1. Blood 112:708-10

Storb R, Deeg J, Whitehead J, Applebaum F, Beatty P, Besinger W, Buckner D, Clift R, Doney K, Farewell V, Hansen J, Hill R, Lum L, Martin P, McGuffin R, Sanders J, Stewart P, Sullivan K, Witherspoon R, Yee G, Thomas ED (1986) Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukaemia. N Engl J Med 314:730-5

Storb R, Thomas ED (1985) Graft-versus-host disease in dog and man: the Seattle experience. Immunol Rev 88:215-38

Sullivan KM, Mori M, Sanders J, Siadak M, Witherspoon RP, Anasetti C, Appelbaum FR, Bensinger W, Bowden R, Buckner CD, . (1992) Late complications of allogeneic and autologous marrow transplantation. Bone Marrow Transplant. 10 Suppl 1:127-34

Sun JY, Gaidulis L, Dagis A, Palmer J, Rodriguez R, Miller MM, Forman SJ, Senitzer D (2005) Killer Ig-like receptor (KIR) compatibility plays a role in the prevalence of acute GVHD in unrelated hematopoietic cell transplants for AML. Bone Marrow Transplant. 36:525-30

Sun JY, Gaidulis L, Miller MM, Goto RM, Rodriguez R, Forman SJ, Senitzer D (2004) Development of a multiplex PCR-SSP method for Killer-cell immunoglobulin-like receptor genotyping. Tissue Antigens 64:462-8

Thananchai H, Gillespie G, Martin MP, Bashirova A, Yawata N, Yawata M, Easterbrook P, McVicar DW, Maenaka K, Parham P, Carrington M, Dong T, Rowland-Jones S (2007) Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. J Immunol 178:33-7

Triplett BM, Horwitz EM, Iyengar R, Turner V, Holladay MS, Gan K, Behm FG, Leung W (2009) Effects of activating NK cell receptor expression and NK cell reconstitution on the outcomes of unrelated donor hematopoietic cell transplantation for hematologic malignancies. leukemi 23:1278-87

Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, Tyan D, Lanier LL, Parham P (1997) Human diversity in killer cell inhibitory receptor genes. Immunity 7:753-63

Vago L, Forno B, Sormani MP, Crocchiolo R, Zino E, Di Terlizzi S, Lupo Stanghellini MT, Mazzi B, Perna SK, Bondanza A, Middleton D, Palini A, Bernardi M, Bacchetta R, Peccatori J, Rossini S, Roncarolo MG, Bordignon C, Bonini C, Ciceri F, Fleischhauer K (2008) Temporal, quantitative, and functional characteristics of single-KIR-positive alloreactive natural killer cell recovery account for impaired graft-versus-leukaemia activity after haploidentical hematopoietic stem cell transplantation. Blood 112:3488-99

Vales-Gomez M, Reyburn H, Strominger J (2000) Molecular analyses of the interactions between human NK receptors and their HLA ligands. Hum. Immunol 61:28-38

Vales-Gomez M, Reyburn HT, Erskine RA, Strominger J (1998) Differential binding to HLA-C of p50-activating and p58-inhibitory natural killer cell receptors. Proc. Natl. Acad Sci U. S. A 95:14326-31

Valiante NM, Uhrberg M, Shilling HG, Lienert-Weidenbach K, Arnett KL, D'Andrea A, Phillips JH, Lanier LL, Parham P (1998) Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. Immunity 7:739-51

Verheyden S, Schots R, Duquet W, Demanet C (2005) A defined donor activating natural killer cell receptor genotype protects against leukaemic relapse after related HLA-identical hematopoietic stem cell transplantation. leukemi 19:1446-51

Vilches C, Pando MJ, Parham P (2000a) Genes encoding human killer-cell Ig-like receptors with D1 and D2 extracellular domains all contain untranslated pseudoexons encoding a third Ig-like domain. Immunogenetics 51:639-46

Vilches C, Rajalingam R, Uhrberg M, Gardiner CM, Young NT, Parham P (2000b) KIR2DL5, a novel killer-cell receptor with a D0-D2 configuration of Ig-like domains. J Immunol 164:5797-804

Wagtmann N, Biassoni R, Cantoni C, Verdiani S, Mainatl MS, Vitale M, Bottino C, Moretta L, Moretta A, Long EO (1995) Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. Immunity 2:439-49

Whiteside TL, Herberman RB (1994) Human natural killer cells in health and disease: biology and therapeutic potential. Clin. Immunother 1:56-66

Wilson MJ, Torkar M, Haude A, Milne S, Jones T, Sheer D, Beck S, Trowsdale J (2000) Plasticity in the organization and sequences of human KIR/ILT gene families. Proc Natl. Acad. Sci U. S. A 97:4778-83

Winter CC, Gumperz JE, Parham P, Long EO, Wagtmann N (1998) Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. J Immunol 161:571-7

Witt CS, Dewing C, Sayer DC, Uhrberg M, Parham P, Christiansen FT (1999) Population frequencies and putative haplotypes of the killer cell immunoglobulin-like receptor sequences and evidence for recombination. Transplantation 68:1784-9

Yabe T, Matsuo K, Hirayasu K, Kashiwase K, Kawamura-Ishii S, Tanaka H, Ogawa A, Takanashi M, Satake M, Nakajima K, Tokunaga K, Inoko H, Saji H, Ogawa S, Juji T, Sasazuki T, Kodera Y, Morishima Y (2008) Donor killer immunoglobulin-like receptor (KIR) genotype-patient cognate KIR ligand combination and antithymocyte globulin preadministration are critical factors in outcome of HLA-C-KIR ligand-mismatched T cell-replete unrelated bone marrow transplantation. Biol Blood Marrow Transplant. 14:75-87

Yawata M, Yawata N, Draghi M, Little AM, Partheniou F, Parham P (2006) Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. J Exp Med 203:633-45

Yu YYL, Kumar V, Bennett M (1992) Murine natural killer cells and marrow graft rejection. Annu Rev Immunol 10:189-213

Zhao XY, Huang XJ, Liu KY, Xu LP, Liu DH (2007) Prognosis after unmanipulated HLA-haploidentical blood and marrow transplantation is correlated to the numbers of KIR ligands in recipients. Eur. J Haematol. 78:338-46

APPENDICES

APPENDIX I:

PRIMER SEQUENCES

APPENDIX II:

SOLUTIONS AND BUFFERS

APPENDIX III:

ANTIBODIES

APPENDIX IV:

SUPPLEMENTARY DATA FOR CHAPTER 6: RAPID, FLOW CYTOMETRIC ASSAY FOR NK ALLOREACTIVITY REVEALS EXCEPTIONS TO RULES GOVERNING ALLOREACTIVITY.

APPENDIX I: PRIMER SEQUENCES

Sense primers including M13F sequence (bold):

KIR gene	Primer name	Primer sequence
KIR3DL1	Ft624	5'-**TGTAAAACGACGGCCAGT**CCATYGGTCCCATGATGCT
KIR2DL3	KIR2DL3(D)F	5'-**TGTAAAACGACGGCCAGT**CCTTCATCGCTGGTGCTG
KIR2DL5	Fcon173	5'-**TGTAAAACGACGGCCAGT** GTCTGCCTGGCCCAGCG
KIR3DS1	KIR3DS1(D)F	5'-**TGTAAAACGACGGCCAGT** GGCAGAATATTCCAGGAGG
KIR2DS4	2DS4HsuFor	5'-**TGTAAAACGACGGCCAGT** ATCCTGCAATGTTGGTCG
KIR2DS4*004	2DS4HsuFor	5'-**TGTAAAACGACGGCCAGT** ATCCTGCAATGTTGGTCG
KIR2DS1	KIR2DS1-F	5'-**TGTAAAACGACGGCCAGT** TCTCCATCAGTCGCATGAR
KIR2DS2	KIR2DS2-F	5'-**TGTAAAACGACGGCCAGT** TGCACAGAGAGGGGAAGTA
KIR2DL2	Fa538	5'-**TGTAAAACGACGGCCAGT** ACTTCCTTCTGCACAGAGAA
KIR2DS3	KIR2DS3-F	5'-**TGTAAAACGACGGCCAGT** TCACTCCCCCTATCAGTTT
KIR2DL1	KIR2DL1(D)F	5'-**TGTAAAACGACGGCCAGT** ACTCACTCCCCCTATCAGG
KIR2DL1v	KIR2DL1(D)F	5'-**TGTAAAACGACGGCCAGT** ACTCACTCCCCCTATCAGG
KIR2DS5	KIR2DS5-F	5'-**TGTAAAACGACGGCCAGT** AGAGAGGGGACGTTTAACC

Anti-sense primers including M13R sequence (bold):

KIR gene	Primer name	Primer sequence
KIR3DL1	KIR3DL1(D)R	5'- **CAGGAAACAGCTATGACC**AGAGAGAAGGTTTCTCATATG
KIR2DL3	KIR2DL3(D)R	5'-**CAGGAAACAGCTATGACC** CAGGAGACAACTTTGGATCA
KIR2DL5	Rc939	5'-**CAGGAAACAGCTATGACC** GAGAGAGCCGAAGCATG
KIR3DS1	KIR3DS1(D)R	5'-**CAGGAAACAGCTATGACC** AGGGGTCCTTAGAGATCCA
KIR2DS4	2DS4HsuRev	5'-**CAGGAAACAGCTATGACC** CTGGATAGATGGTACATGTC
KIR2DS4*004	KIR1DHsuRev	5'-**CAGGAAACAGCTATGACC** CTGGATAGATGGAGCTGCAG
KIR2DS1	KIR2DS1-R	5'-**CAGGAAACAGCTATGACC** AGGGCCCAGAGGAAAGTT
KIR2DS2	KIR2DS2-R	5'-**CAGGAAACAGCTATGACC** CACGCTCTCTCCTGCCAA
KIR2DL2	KIR2DL2(D)R	5'-**CAGGAAACAGCTATGACC** GCCCTGCAGAGAACCTACA
KIR2DS3	KIR2DS3-R	5'-**CAGGAAACAGCTATGACC** GCATCTGTAGGTTCCTCCT
KIR2DL1	KIR2DL1(D)R	5'-**CAGGAAACAGCTATGACC** AGGGCCCAGAGGAAAGTCA
KIR2DL1v	Rat780	5'-**CAGGAAACAGCTATGACC** CTGCAGGACAAGGTCACAT
KIR2DS5	KIR2DS5-R	5'-**CAGGAAACAGCTATGACC** TCCGTGGGTGGCAGGGT

Internal SSP-PCR primers

HGHI CAGTGCCTTCCCAACCATTCCCTTA

HGHII ATCCACTCACGGATTTCTGTTGTGTTTC

Stock KIR typing primers were diluted to 20ρmol/μL using TE buffer, pH 8.0.

APPENDIX II: SOLUTIONS AND BUFFERS

PCR REAGENTS

1 M $MgCl_2$	$MgCl_2$	20.3g
	Sterile deionised water	100mL
1 M Tris-HCL pH 7.5, 8.3	Trizma Base	60.6g
	Sterile deionised water	500mL
1 M KCL	KCL	37.3g
	Sterile deionised water	500mL
20mg/mL Gelatin	Gelatin powder	2g
	Sterile deionised water	100mL

40mM deoxynucleotide triphosphates (dNTPs) (Invitrogen, CA USA)

100mM dATP	200µL
100mM dCTP	200µL
100mM dGTP	200µL
100mM dTTP	200µL
Sterile deionised water	1200µL
	2000µL

10 x PCR Buffer (final concentration 2mM $MgCl_2$)

1M $MgCl_2$	0.5mL
1M Tris-HCL pH 8.3	2.5mL
1M KCL	12.5mL
20mg/mL gelatin	0.125mL
Sterile deionised water	9.38mL
Total	25mL

10 x PCR Buffer (final concentration 2.5mM $MgCl_2$)

1M $MgCl_2$	0.625mL
1M Tris-HCL pH 8.3	2.5mL
1M KCL	12.5mL
20mg/mL gelatin	0.125mL
Sterile deionised water	9.25mL
Total	25mL

0.5 M EDTA, pH 8.0

EDTA	93.06g
Sterile deionised water	500mL

Adjust to pH 8.0 with concentrated HCL

Tris-EDTA (TE) Buffer, pH 8.0

Tris-HCL, pH 7.5	2mL
0.5 M EDTA, pH 8.0	0.4mL

Make up to 200mL with sterile deionised water, adjust pH with HCL

10 x TBE Electrophoresis Buffer

Trizma Base	215.6g
Boric Acid	110g
EDTA	16.4g

2% Agarose gel

ME Agarose	9g
0.5 x TBE	450mL
Ethidium bromide(100mg/ul)	17ul

Type IV loading buffer

Bromophenol Blue	0.05g
Sucrose	8g
Milli-Q water	20mL

1 kb Plus DNA Lambda ladder

1 kb Plus DNA ladder	1mL
Type IV loading buffer	2mL
Sterile deionised water	7mL

CELL LINES AND TISSUE CULTURE

Heat inactivated fetal calf serum (HIFCS)

Fetal calf serum (Thermo Trace, Melbourne AUS) was incubated at 56ºC with shaking for 30 minutes, aliquotted and stored at -20ºC.

RPMI

RPMI 1640 (Invitrogen, Carlsbad USA) supplemented with 500IU/mL penicillin, 500µg/mL streptomycin, 2mM L-glutamine and 10% sterile FCS.

2-Mercaptoethanol (2-ME)

2-ME (ICN Biochemicals, OH USA) was diluted 1:250 in RPMI and stored at –20°C. A 1:1000 dilution of this stock was then added to NK cell culture medium.

NK Culture Medium

RPMI 1640 supplemented with Penicillin-Streptomycin (500IU/mL,500µg/mL), 2mM L-glutamine, 0.1mM non-essential amino acids, 1mM pyruvate, 2-ME and 10% sterile HIFCS.

1 x PBS

17g NaCl, 2.68g $Na_2HPO_42H_2O$ (disodium hydrogen orthophosphate) and 0.78g $NaH_2PO_42H_2O$ (sodium dihydrogen orthophosphate) were dissolved in 2L Milli-Q water and the pH adjusted to 7.2.

Freezing Medium

Freezing medium contained RPMI 1640, 20% HIFCS and 10% DMSO (BDH, Poole UK). Freezing medium was always made fresh and added to the cells drop-wise on ice.

Trypan Blue

0.5g Trypan Blue (ICN Biochemicals, Ohio USA) was dissolved in 100mL 1 x PBS. The solution was sterilised using a 0.22μM filter and stored at room temperature.

IMMUNOPHENOTYPING

Flow Buffer

2% HIFCS and 0.1% sodium azide was added to 1 x PBS and stored at 4ºC.

2% Paraformaldehyde

2g paraformaldehyde was dissolved in 100mL PBS and solution heated to 56ºC with gently shaking. Solution was cooled and pH adjusted to 7.4. Solution was stored at 4°C wrapped in foil.

APPENDIX III: ANTIBODIES

Antibody	Fluorochrome	Isotype	Clone	Dilution	Supplier	Method
CD56	PC5	IgG1	N901(NKH1)	1/20	Beckman Coulter	Phenotyping
CD56	PeCy7	IgG1	B159	1/40	BD Biosciences	CD107a assay
CD158a	PE	IgG1	EB6B	1/10	Beckman Coulter	Phenotyping
CD158b	PE	IgG1	GL183	1/10	Beckman Coulter	Phenotyping
CD158a	APC	IgG1	EB6B	1/25	Beckman Coulter	CD107a assay
CD158b	APC	IgG1	GL183	1/25	Beckman Coulter	Phenotyping/CD107a Assay
CD158a	PE	IgM	HP-3E4	1/10	BD Biosciences	Phenotyping/CD107a Assay
CD158b	PE	IgG2b	CH-L	1/10	BD Biosciences	Phenotyping/CD107a Assay
CD158e	PE	IgG1	z27.3.7	1/10	Beckman Coulter	Phenotyping
NKB1	PE	IgG1	DX9	1/10	BD Biosciences	Phenotyping/CD107a Assay
NKG2A	PE	IgG2b	z199	1/20	Beckman Coulter	Phenotyping
CD107a	FITC	IgG1	H4A3	1/20	BD Biosciences	CD107a assay
IgG	isotype	IgG1	679.1Mc7	1/20	Beckman Coulter	Isotype control
IgM	isotype	IgM	GC323	1/100	Beckman Coulter	Isotype control

APPENDIX IV:

SUPPLEMENTARY DATA FOR CHAPTER 6: RAPID, FLOW CYTOMETRIC ASSAY FOR NK ALLOREACTIVITY REVEALS EXCEPTIONS TO RULES GOVERNING ALLOREACTIVITY.

Donor Number	Donor ID	HLA-A	HLA-Bw4	HLA-Bw6	HLA-C Group 1	HLA-C Group 2	C1/C2* Assay	Bw4† Assay
1	P06 9900734E	02	0702	4402	07	05	+	-
2	P06 7421654C	11	1301	15	0304	0401	+	+
3	P06 9900837M	03, 3201	4402	55	03	05	+	-
4	S06 0275326S	02, 11		15,35	0303	04	+	+
5	P06 9901345W	01, 11	1301,5701		03	06	+	+
6	S06 0275327Z	01, 03		14,35	08	04	+	-
7	S07 0303098B	24, 29	4403,5701		1601	0602	+	-
8	S07 0303100F	24		41,45	1601	17	+	+
9	S07 0303126E		44	4001	0304	05	+	-
10	S07 0303319H	01, 02	4402	08	07	05	+	+
11	P06 9900864U	02, 03		0702,3502	07	04	+	-
12	P06 9901389R	01, 02	5701	15	03	0602	+	-
13	P06 7421624A	11	1301	15	03	04	+	+
14	P06 9900619P	0206, 11		15,56	07	04	+	-
15	P06 0268080U	01, 02		08,18	07	05	+	-
16	S07 0303099H	2902, 6601	4403	4102	1601	17	+	-
17	S07 0303320R	01, 02	4403	07	07	04	-	+
18	S07 0303442C	02, 23	44	08	07	05	-	+
19	S07 0303443J	01, 32	5701	1401	0802	0602	-	+
20	P06 9901346C	01		08	07		+	-
21	P06 9900806D	01, 03		08	07		+	+
22	P06 9900883Y	11	4403	5601	0102,1601		+	-

Donor Number	Donor ID	HLA-A	HLA-Bw4	HLA-Bw6	HLA-C Group 1	HLA-C Group 2	C1/C2* Assay	Bw4† Assay
23	P06 9900547Q	02, 2902	44,57		07,1601		+	+
24	S07 0303127L	11, 2902	3801,4403		1203,1601		+	+
25	P06 9900597C	01, 11		08,18	07,1203		+	-
26	P06 9901403B	24, 34		5601,5602	0102		+	-
27	P06 0277334P-35	03, 33		07,14	07,08		+	-
28	S07 0305312B	03, 2501		0702,18	07,1203		+	+
29	S07 0305313H	02, 03		0702,15	0304,0702		+	+
30	P06 9900018E	01, 24	51	08	0102,07		+	+
31	P06 9900830R	02, 33	5801	4601	0102,0302		+	+
32	S07 0303128S	02, 03		07,35	0702		+	-
33	P06-9900868W	02, 26	3801	0702	07,1203		-	+
34	P06 9900820Z	02, 25	57	40		0202,0602	+	-
35	P06 9900548X	11, 24	4403,51			04,15	+	-
36	S07 0305993A	03, 31	1302	35		0401,0602	+	+
37	R98 0902569E	03, 19	1302	35		04,0602	+	-
38	R99 0901306J	3,29	44	35		04,0602	+	-
39	R02 0901472Y	03, 24	13,51			04,0602	+	-
40	R98 0903855U	01,02	4405	3503		0202	+	-
41	R99 0901482K	02		15		04	+	-
42	S07 0302099N	23, 31	4403	3501		04	-	+

Table I Supplementary data. HLA types of NK cell donors used in the CD107a assay.
Where a four-character HLA allele could not be assigned by sequencing of exons 2 and 3, the 2-character allele name is shown.
*Donors used in the CD107a assay for the detection of C1- and C2-dependent NK cells.
†Donors used in the CD107a assay for the detection of Bw4-dependent NK cells.

Donor Number	Donor ID	2DL1	2DL2	2DL3	3DL1	2DL5	2DS1	2DS2	2DS3	2DS4	2DS4 *003	2DS5	3DS1
1	P06 9900734E												
2	P06 7421654C												
3	P06 9900837M												
4	S06 0275326S												
5	P06 9901345W												
6	S06 0275327Z												
7	S07 0303098B												
8	S07 0303100F												
9	S07 0303126E												
10	S07 0303319H												
11	P06 9900864U												
12	P06 9901389R												
13	P06 07421624A												
14	P06 9900619P												
15	P06 0268080U												
16	S07 0303099H												
17	S07 0303320R												
18	S07 0303442C												
19	S07 0303443J												
20	P06 9901346C												
21	P06 9900806D												
22	P06 9900883Y												
23	P06 9900547Q												
24	S07 0303127L												
25	P06 9900597C												

Donor Number	Donor ID	2DL1	2DL2	2DL3	3DL1	2DL5	2DS1	2DS2	2DS3	2DS4	2DS4 *003	2DS5	3DS1
26	P06 9901403B	■	■	■	■	■		■	■		■		
27	P06 0277334P	■	■	■	■	■	■	■		■		■	■
28	S07 0305312B	■	■	■	■			■			■		
29	S07 0305313H	■		■	■	■	■			■		■	■
30	P06 9900018E	■		■	■					■	■		
31	P06 9900830R	■	■	■	■					■	■		■
32	S07 0303128S	■	■	■	■			■			■		
33	P06-9900868W	■		■	■					■	■		
34	P06 9900820Z	■	■	■	■			■		■	■		
35	P06 9900548X	■		■	■	■	■			■		■	■
36	S07 0305993A		■		■			■		■	■		
37	R98 0902569E		■		■			■		■	■		
38	R99 0901306J	■		■	■	■	■			■		■	■
39	R02 0901472Y	■	■	■	■			■	■				■
40	R98 0903855U	■		■	■						■		
41	R99 0901482K	■	■	■	■	■	■	■	■		■	■	■
42	S07 0302099N	■		■	■	■	■				■	■	■

Table II Supplementary data. KIR genotype of donors used in CD107a assay. A shaded box indicates presence of the KIR gene