Human natural killer cell responses to tumour-priming

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Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
For Goggi
Abstract

As one of the central components of host anti-tumour immunity, natural killer (NK) cells exert cellular cytotoxicity against tumour cells and secrete a milieu of immunoregulatory cytokines to inhibit tumour progression. NK cell-mediated cytotoxicity requires successful progression through discrete activation events that begin with NK cell adhesion to a target cell and culminate in the polarized release of cytotoxic granules into the immunological synapse. These activation events are tightly regulated by a complex array of signalling molecules, the engagement of which by ligands on target cells can determine susceptibility to NK cell-mediated killing. Resistance to NK cell cytotoxicity can be attributable to a deficiency in any of the signalling requirements for the events leading to granule exocytosis. Tumour resistance to NK cell lysis may be overcome by priming of NK cells with cytokines or by binding of NK cell activating receptors to ligands expressed on target cells. Here, the activation profiles of normal, freshly isolated human peripheral blood NK cells upon tumour-priming with the NK-resistant leukemic cell line, CTV-1 are defined, and candidate NK cell receptors involved in the delivery of the tumour-priming signal are identified. Results from this study demonstrate that NK cell responses to tumour-priming are distinct from those induced by the gold standard in immunotherapy, cytokine-priming. Tumour-priming of NK cells resulted in a significant downregulation of various activating NK cell receptors including CD16, NKp46, NK group 2, member D (NKG2D), and intracellular adhesion molecule (ICAM)-1. Tumour-primed NK cells (TpNKs) also exhibited a strong pro-inflammatory profile marked by ample secretion of macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal T cell expressed and secreted (RANTES), tumour necrosis factor (TNF)-α and interferon (IFN) -γ after short incubations with CTV-1. Their secretory profiles were distinct from the profiles of NK cells stimulated with exogenous cytokines or the NK-sensitive target cell line, K562. Collectively, data from this study demonstrates that NK cell responses can differ according to the type of stimulus, as well as the ligand combination presented by the target cell. The co-engagement of NK cell receptors LFA-1 and CD2 by their respective ligands on CTV-1 cells, ICAM-1 and CD15, seems to play an important role in the delivery of the tumour-priming signal.
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Table of Contents

Abstract ........................................................................................................................................... 4

Acknowledgements .......................................................................................................................... 5

List of figures .................................................................................................................................... 12

List of tables ..................................................................................................................................... 14

Abbreviations ................................................................................................................................... 15

Chapter 1 General Introduction .................................................................................................... 20

1.1 The human immune system ..................................................................................................... 20

1.1.1 Innate and adaptive immunity ............................................................................................. 20

1.1.2 Immune cells, tissues and organs ......................................................................................... 21

1.1.2.1 Lymphocytes .................................................................................................................... 22

1.1.2.1.1 B cells .......................................................................................................................... 22

1.1.2.1.2 T cells .......................................................................................................................... 23

1.1.2.1.3 NK cells ....................................................................................................................... 24

1.2 NK cell defence: innate or adaptive? ......................................................................................... 25

1.2.1 Priming .................................................................................................................................... 25

1.2.2 Antigen specificity and clonal expansion .............................................................................. 26

1.2.3 Immunologic memory ........................................................................................................... 27

1.3 NK cell subsets and development ............................................................................................ 28

1.3.1 CD56$^{\text{bright}}$ NK cells ....................................................................................................... 28

1.3.2 CD56$^{\text{dim}}$ NK cells ............................................................................................................ 29

1.3.3 NK cell development model ................................................................................................. 29

1.4 NK cell recognition and regulation ........................................................................................ 33

1.4.1 Inhibitory NK cell receptors ............................................................................................... 34

1.4.2 NK cell education ................................................................................................................. 34
1.4.3 Activating NK cell receptors ................................................................. 38
1.4.4 NK cell activation ..................................................................................... 39
1.5 NK cells and cancer .................................................................................... 41
1.5.1 Cancer immunosurveillance by NK cells .................................................... 41
1.5.1.1 Target cell elimination ........................................................................ 41
1.5.1.2 Cytokine secretion .............................................................................. 42
1.5.2 Cancer evasion of NK cells ...................................................................... 43
1.5.3 NK cell modulation for cancer therapy ...................................................... 46
1.6 Thesis Aims .................................................................................................. 47

Chapter 2 Materials and Methods .................................................................... 48
2.1 Blood Donors ............................................................................................... 48
2.2 Cell Lines ...................................................................................................... 48
2.3 Cell Culture Medium .................................................................................... 49
2.4 Cell Counting and Viability Assessment ....................................................... 49
2.5 Cryopreservation of Cells .......................................................................... 50
2.6 Thawing of Frozen Cells ............................................................................ 50
2.7 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) ......................... 50
2.8 Isolation of Human NK cells ...................................................................... 51
2.9 In vitro stimulation of NK cells ................................................................... 52
2.10 Immunophenotyping of NK Cells ............................................................... 52
    2.10.1 Monoclonal Antibodies ..................................................................... 53
2.11 Analysis of NK cell secretory profiles ......................................................... 58
2.12 PKH-26 labelling of Target Cells ............................................................... 61
2.13 Cytotoxicity Assays .................................................................................. 61
2.14 CD107a Degranulation Assay ................................................................... 62
2.15 Antibody blocking experiments ................................................................. 63
2.16 Bead Stimulation ........................................................................................................ 64
2.16.1 Dynabeads Sheep anti-mouse IgG ........................................................................ 64
2.16.2 Dynabeads Protein G .......................................................................................... 65
2.17 Statistical Analyses .................................................................................................. 65

Chapter 3  Phenotypic profiles of Tumour-primed NK cells ........................................ 66
3.1 Introduction ................................................................................................................ 66
3.2 Experimental Aims .................................................................................................... 67
3.3 Methods ..................................................................................................................... 68
3.4 Results ....................................................................................................................... 69
  3.4.1 Differentiation markers .......................................................................................... 69
    3.4.1.1 CD56 expression .......................................................................................... 69
    3.4.1.1 CD16 expression ......................................................................................... 71
    3.4.1.2 CD62L expression ..................................................................................... 73
    3.4.1.3 CD57 expression ......................................................................................... 75
    3.4.1.4 Compound expression of NK cell differentiation receptors ........................... 77
  3.4.2 Activating receptors .............................................................................................. 79
    3.4.2.1 Natural cytotoxicity receptors ...................................................................... 79
    3.4.2.2 Adhesion receptors ..................................................................................... 81
    3.4.2.3 Co-activating receptors .............................................................................. 83
  3.4.3 Homing receptors .................................................................................................. 85
  3.4.4 CD107a expression and different NK cell subsets .............................................. 87
    3.4.4.1 Correlation between CD107a expression and cytotoxicity ........................... 87
    3.4.4.2 CD107a expression in resting and primed NK cells .................................... 89
    3.4.4.3 CD107a expression in different NK cell subsets ......................................... 91
  3.5 Discussion .................................................................................................................. 94

Chapter 4  Secretory profiles of tumour-primed NK cells ............................................. 99
  4.1 Introduction ............................................................................................................... 99
4.2 Experimental Aims ........................................................................................................ 101
4.3 Methods ....................................................................................................................... 101
4.4 Results ......................................................................................................................... 102
  4.4.1 Inflammatory cytokines .......................................................................................... 102
    4.4.1.1 IL-1β ............................................................................................................... 102
    4.4.1.2 IL-1RA ........................................................................................................... 103
    4.4.1.3 IL-6 ................................................................................................................ 103
    4.4.1.4 IL-8 ................................................................................................................ 104
    4.4.1.5 GM-CSF ......................................................................................................... 105
    4.4.1.6 TNF-α ............................................................................................................ 105
  4.4.2 Th1/Th2 cytokines .................................................................................................... 110
    4.4.2.1 IFN-γ ............................................................................................................. 110
    4.4.2.2 IL-2R ............................................................................................................. 110
    4.4.2.3 IL-2, IL-4, IL-5 and IL-10 ............................................................................ 111
  Cytokines II ...................................................................................................................... 116
    4.4.2.4 IFN-α ............................................................................................................. 116
    4.4.2.5 IL-7 ................................................................................................................ 116
    4.4.2.6 IL-12p40/p70 ................................................................................................. 117
    4.4.2.7 IL-13 and IL-17 ............................................................................................. 117
    4.4.2.8 IL-15 .............................................................................................................. 118
  4.4.3 Chemokines ............................................................................................................. 123
    4.4.3.1 Eotaxin and MIG .......................................................................................... 123
    4.4.3.2 IP-10 ............................................................................................................. 123
    4.4.3.3 MCP-1 .......................................................................................................... 124
    4.4.3.4 MIP-1α ........................................................................................................ 124
    4.4.3.5 MIP-1β ........................................................................................................ 125
    4.4.3.6 RANTES ....................................................................................................... 125
  4.5 Discussion .................................................................................................................. 131
Chapter 5  Dissecting the tumour-priming signal ................................................................. 138

5.1  Introduction .................................................................................................................. 138

5.2  Experimental Aims ....................................................................................................... 139

5.3  Methods ........................................................................................................................ 140

  5.3.1  Blockade studies ...................................................................................................... 140

  5.3.2  Bead Stimulation ..................................................................................................... 140

    5.3.2.1  Dynabeads Sheep anti-mouse IgG ................................................................. 140
    5.3.2.2  Dynabeads Protein G ................................................................................... 141

5.4  Results .......................................................................................................................... 142

  5.4.1  Critical ligands provided by CTV-1 for tumour-priming ...................................... 142

    5.4.1.1  LFA-1-mediated tumour priming of NK cells .............................................. 142
    5.4.1.2  CD2-mediated tumour priming of NK cells ................................................. 145

  5.4.2  Microbead-mediated priming of NK cells .............................................................. 148

    5.4.2.1  NK cell priming with antibody-coated microbeads .................................... 148
    5.4.2.2  NK cell priming with CTV-1 ligand-coated microbeads .............................. 151

5.5  Discussion ...................................................................................................................... 154

Chapter 6  General Discussion ............................................................................................. 157

6.1  NK Cells and tumours: a few burning questions ...................................................... 157

6.2  Tumour-associated NK Cells: impaired or tumour-primed? .................................... 158

6.3  What are the activating signals involved in tumour-priming? ............................... 162

  6.3.1  Contact/Adhesion ................................................................................................. 162

  6.3.2  Conjugate/Synapse formation .............................................................................. 163

  6.3.3  Granule Polarization ............................................................................................ 164

  6.3.4  Granule maturation and exocytosis .................................................................... 164

  6.3.5  Tumour-priming model for NK cell activation .................................................. 167

6.4  Is it time to replace the missing-self hypothesis? .................................................... 169
6.5 Is NK cell priming different from NK cell memory? ......................................................... 171
6.6 Conclusion: The remaining questions ............................................................................. 173

Chapter 7 References ........................................................................................................... 176

Appendix 1: Publications .................................................................................................. 206

Appendix 2: Abstracts ......................................................................................................... 208

Index I: Standard curves for cytokine concentration ......................................................... 209
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Modified NK cell development model</td>
<td>32</td>
</tr>
<tr>
<td>1.2</td>
<td>NK cell activation events</td>
<td>40</td>
</tr>
<tr>
<td>1.3</td>
<td>Tumour Evasion Strategies</td>
<td>45</td>
</tr>
<tr>
<td>2.1</td>
<td>Basic gating strategy for flow cytometric analysis</td>
<td>57</td>
</tr>
<tr>
<td>2.2</td>
<td>Standard curves for analysis of cytokine secretion</td>
<td>60</td>
</tr>
<tr>
<td>2.3</td>
<td>Basic gating strategy for flow cytometric analysis</td>
<td>57</td>
</tr>
<tr>
<td>3.1</td>
<td>CD56 expression in resting and primed NK cells</td>
<td>70</td>
</tr>
<tr>
<td>3.2</td>
<td>CD16 expression in resting and primed NK cells</td>
<td>72</td>
</tr>
<tr>
<td>3.3</td>
<td>CD62L expression in resting and primed NK cells</td>
<td>74</td>
</tr>
<tr>
<td>3.4</td>
<td>CD57 expression in resting and primed NK cells</td>
<td>76</td>
</tr>
<tr>
<td>3.5</td>
<td>Tumour-priming completes NK cell maturation</td>
<td>78</td>
</tr>
<tr>
<td>3.6</td>
<td>NKp46 expression by resting and primed NK cells</td>
<td>80</td>
</tr>
<tr>
<td>3.7</td>
<td>ICAM-1 expression by resting and primed NK cells</td>
<td>82</td>
</tr>
<tr>
<td>3.8</td>
<td>NKG2D expression by resting and primed NK cells</td>
<td>84</td>
</tr>
<tr>
<td>3.9</td>
<td>CXCR-1, -4 and -7 expression by resting and primed NK cells</td>
<td>86</td>
</tr>
<tr>
<td>3.10</td>
<td>Correlation between NK cell cytotoxicity and CD107a expression</td>
<td>88</td>
</tr>
<tr>
<td>3.11</td>
<td>CD107a expression in resting and primed NK cells</td>
<td>90</td>
</tr>
<tr>
<td>3.12</td>
<td>CD107a expression in different NK cell subsets</td>
<td>92</td>
</tr>
<tr>
<td>3.13</td>
<td>CD107a expression in different subsets of tumour-primed NK cells</td>
<td>93</td>
</tr>
<tr>
<td>4.1</td>
<td>Secretion of inflammatory cytokines by resting and target cell-stimulated NK cells</td>
<td>106</td>
</tr>
<tr>
<td>4.2</td>
<td>Secretion of inflammatory cytokines by cytokine-stimulated NK cells</td>
<td>107</td>
</tr>
<tr>
<td>4.3</td>
<td>Secretion of inflammatory cytokines by tumour cells</td>
<td>108</td>
</tr>
<tr>
<td>4.4</td>
<td>Kinetics of NK cell secretion of inflammatory cytokines upon stimulation with CTV-1, K562 or IL-2.</td>
<td>109</td>
</tr>
<tr>
<td>4.5</td>
<td>Secretion of Th1/Th2 cytokines by resting and target cell-stimulated NK cells</td>
<td>112</td>
</tr>
<tr>
<td>4.6</td>
<td>Secretion of Th1/Th2 cytokines by cytokine-stimulated NK cells</td>
<td>113</td>
</tr>
</tbody>
</table>
Figure 4.7 Secretion of Th1/Th2 cytokines by target cells. ................................................................. 114
Figure 4.8 Secretion of cytokines II by target cell-stimulated NK cells. ........................................ 119
Figure 4.9 Secretion of cytokines II by cytokine-stimulated NK cells........................................... 120
Figure 4.10 Secretion of cytokines II by target cells. ........................................................................ 121
Figure 4.11 Kinetics of NK cell secretion of cytokines II upon stimulation with CTV-1, K562 or IL-2. .................................................................................................................................................. 122
Figure 4.12 Secretion of chemokines by resting and target cell-stimulated NK cells. ................. 127
Figure 4.13 Secretion of chemokines by cytokine-stimulated NK cells........................................ 128
Figure 4.14 Secretion of cytokines by target cells. ........................................................................... 129
Figure 4.15 Kinetics of NK cell secretion of chemokines upon stimulation with CTV-1, K562 or IL-2. .................................................................................................................................................. 130
Figure 5.1 Effect of ICAM-1 blockade on CTV-1-mediated priming of NK cells........................ 143
Figure 5.2 Effect of ICAM-1 blockade on lytic activity of CTV-1-primed NK cells...................... 144
Figure 5.3 Effect of CD15 or CD2 blockade on CTV-1-mediated priming of NK cells............... 146
Figure 5.4 Effect of CD15 or CD2 blockade on lytic activity of CTV-1-primed NK cells........... 147
Figure 5.5 Effect of antibody-coated microbeads on NK cell priming........................................ 149
Figure 5.6 Effect of antibody-coated microbeads on the lytic activity of NK cells..................... 150
Figure 5.7 Effect of CTV-1 ligand-coated microbeads on NK cell priming................................. 152
Figure 5.8 Effect of CTV-1 ligand-coated microbeads on lytic activity of NK cells................... 153
Figure 6.1 NK cell activation events and receptor requirements for natural cytotoxicity........ 166
Figure 6.2 NK cell activation stages and events. .............................................................................. 168
List of Tables

Table 1-1 Human NK cell receptors .................................................................36
Table 2-1 Monoclonal antibodies used in this Thesis .......................................54
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent Cell-mediated Cytotoxicity</td>
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<td>AICL</td>
<td>Activation-induced C-type Lectin</td>
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<td>ALL</td>
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<td>AML</td>
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<td>APC</td>
<td>Antigen-Presenting Cell</td>
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<td>Chemokine C-C Motif Ligand 20</td>
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<td>Cluster of differentiation</td>
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<tr>
<td>CM</td>
<td>Complete Media</td>
</tr>
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<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
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<td>CRACC</td>
<td>CD2-like Receptor for Activating Cytotoxic Cells</td>
</tr>
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<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>DAP-10/12</td>
<td>DNAX-activation protein-10/12</td>
</tr>
<tr>
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</tr>
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</tr>
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</tr>
<tr>
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<td>-----------</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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</tr>
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<td>Goat Anti-Mouse</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>Good Manufacturing Practice</td>
</tr>
<tr>
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<td>Graft-versus-Host Disease</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
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<td>Human Leukocyte Antigen</td>
</tr>
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</tr>
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<td>Hematopoietic Stem Cell</td>
</tr>
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<td>HSCT</td>
<td>Hematopoietic Stem Cell Transplantation</td>
</tr>
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</tr>
<tr>
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<td>Interleukin</td>
</tr>
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<td>IP₃</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>IP-10</td>
<td>IFN-gamma-inducible Protein 10</td>
</tr>
<tr>
<td>IS</td>
<td>Immunological Synapse</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immune Tyrosine-based Activation Motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immune Tyrosine-based Inhibitory Motif</td>
</tr>
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<td>Killer Immunoglobulin-like Receptor</td>
</tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>Lysosomai-associated Membrane Protein 1</td>
</tr>
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<td>Leukocyte Ig-like Receptor 1</td>
</tr>
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</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
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<td>Macrophage-1 Antigen</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic Cell Sorting</td>
</tr>
<tr>
<td>MCMV</td>
<td>Murine Cytomegalovirus</td>
</tr>
<tr>
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<td>Monocyte Chemoattractant Protein-1</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Median Fluorescence Intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MICA/B</td>
<td>MHC class I Chain-related Molecules A and B</td>
</tr>
<tr>
<td>MIG</td>
<td>Monokine Induced by IFN-gamma</td>
</tr>
<tr>
<td>MIP-1, -3 α/β</td>
<td>Macrophage Inflammatory Protein-1 or -3 alpha/beta</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>NKG2D</td>
<td>NK group 2 member D</td>
</tr>
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<td>NK Receptor- Protein 1A</td>
</tr>
<tr>
<td>NTB-A</td>
<td>NK, T and B Cell Antigen</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral Blood</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
</tr>
<tr>
<td>PVR</td>
<td>Poliovirus Receptor</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination-Activating Gene</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RE</td>
<td>Recycling Endosome</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SECTM1</td>
<td>Secreted and Transmembrane 1</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2</td>
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<tr>
<td>SHIP-1</td>
<td>SH2 Domain-containing Inositol Phosphatase-1</td>
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<tr>
<td>SHP1/2</td>
<td>SH2 Domain-containing Phosphatase 1/2</td>
</tr>
<tr>
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<td>Secondary Lymphoid Tissue</td>
</tr>
<tr>
<td>TACTILE</td>
<td>T Cell-activated Increased Late Expression</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-beta</td>
</tr>
<tr>
<td>TNF-α/β</td>
<td>Tumour Necrosis Factor- alpha/beta</td>
</tr>
<tr>
<td>TpNK</td>
<td>Tumour-primed NK</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related Apoptosis-inducing Ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>T Regulatory Cell</td>
</tr>
<tr>
<td>UBLP</td>
<td>UL16-binding Protein</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule-1</td>
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<tr>
<td>VLA</td>
<td>Very Late Antigen</td>
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<tr>
<td>VSV</td>
<td>Vesicular Stomatitis Virus</td>
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<tr>
<td>ZAP70</td>
<td>Zeta-chain-associated Protein Kinase 70</td>
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Chapter 1 General Introduction

1.1 The human immune system

The immune system is a collection of cells, tissues and molecules that act together in a dynamic network to protect us against the effects of pathogens, toxins and cancer cells (Kindt et al. 2007). Protection by the immune system involves two parts: recognition and response. Immune recognition is remarkable for its capacity to recognize molecular patterns that characterize groups of common pathogens and detect subtle differences that distinguish one pathogen from another. It can also discriminate between foreign and self-cells as well as recognize host cells that have undergone alterations that may lead to cancer. In a normal immunocompetent setting, immune recognition leads to an effector response whereby the threat is neutralized or eliminated. The type of threat faced by the immune system dictates how the initial recognition event is converted into a variety of effector responses by the multiple components of the immune system.

1.1.1 Innate and adaptive immunity

Traditionally, the immune system is divided into innate and adaptive arms, which collaborate for an effective immune response (Kindt et al. 2007). The innate immune system is always active and ready to mobilize, providing the initial line of defense against any threat to the immune system. The main components of the innate immune system are physical epithelial barriers, phagocytic leukocytes, dendritic cells (DCs), natural killer (NK) cells, and circulating plasma proteins. The molecular and cellular mechanisms involved in innate defense are developed before the encounter with pathogen or stressed cell and most infections are prevented or eliminated within hours of initial encounter. The adaptive immune system is called into action against pathogens that are able to evade innate responses or persist in spite of them. It adapts to recognize, eliminate and then remember the invading pathogen, such that if the same, or a closely related, pathogen infects the body, memory cells provide the
means for the adaptive cells to mount a rapid and more potent secondary response. There are two types of adaptive immune responses: humoral immunity, mediated by antibodies produced by B lymphocytes, and cell-mediated immunity, mediated by T lymphocytes.

1.1.2 Immune cells, tissues and organs

Hematopoiesis, the formation and development of red and white blood cells, begins in the embryonic yolk sac during the first few weeks of development. Subsequently, the BM becomes the major site of hematopoiesis; where hematopoietic stem cells (HSCs) differentiate along two separate pathways into two main types of progenitor cells, myeloid and lymphoid. Myeloid progenitors give rise to neutrophils, eosinophils, basophils, monocytes, mast cells, and DCs (Edvardsson et al. 2006), whereas lymphoid progenitors develop into B, T and NK cells (Rolink et al. 2007). Some of these immune cells complete their maturation in the BM, whereas others migrate through the circulatory system to other tissues where they achieve full maturation and acquire further physiological and functional characteristics. The primary lymphoid organs (BM and thymus) provide the appropriate microenvironment required for the development and maturation of lymphocytes. The secondary lymphoid organs and tissues (lymph nodes and spleen) are sites where mature lymphocytes can optimally interact with antigen presenting cells (APCs) and antigen captured from nearby tissues. Blood vessels and lymphatic systems connect the various organs and tissues into a whole that functions to maintain homeostatic conditions. Abnormality or dysfunction of the immune system may lead to disease; for example, immune responses can be mounted against self-antigens, resulting in autoimmune diseases that include Crohn’s disease etc.
1.1.2.1 Lymphocytes

Lymphocytes comprise 20–40% of white blood cells and are the only cells in the body capable of recognizing specific antigens through an array of highly specific cell surface receptors (Kindt et al. 2007). The three major lymphocytic subpopulations, B cells, T cells and NK cells, each bear their own distinctive family of cell surface receptors. Before antigen exposure, B and T cells are referred to as naïve or unprimed, characterized by a small morphology and a short life span of up to a few weeks. The interaction of naïve lymphocytes with antigen induces their enlargement into lymphoblasts, which eventually differentiate into effector and memory cells. Memory cells from B or T cell progeny persist to provide lifelong immunity against many pathogens.

1.1.2.1.1 B cells

B cells mature in the BM, where they come to express a unique antigen-binding receptor on their membrane, known as an antibody. Antibodies are glycoproteins that consist of two identical polypeptides called the heavy chains and two shorter, identical polypeptides called the light chains. Each heavy chain is joined to a light chain by disulfide bonds and the heavy/light chains are joined to each other by additional disulfide bonds. The amino-terminal ends of the pairs of heavy and light chains form the receptor site to which the antigen binds. The antigen specificity of each B cell is determined by the antibody expressed by the cell. As a B cell matures in the bone marrow, its specificity is created by random rearrangements of a series of gene segments that encode the antibody molecule. At maturity, each B cell possesses a single functional gene encoding the antibody heavy chain and a single functional gene encoding the antibody light chain. All antibody molecules on a given B lymphocyte have identical specificity, giving each B lymphocyte and the clone of daughter cells to which it gives rise, a distinct specificity for a single antigen. When a naïve B cell encounters antigen that matches its specific antibody, it rapidly divides into effector cells known as plasma cells and memory B cells. Plasma cells are highly specialized for antibody secretion and have little or no membrane-bound antibody,
whereas memory B cells express the same membrane-bound antibody as their parent B cell.

1.1.2.1.2 T cells

T cells arise in the BM but migrate to the thymus gland in order to complete their maturation. Maturing T cells express a unique antigen-binding molecule on their membranes called the T-cell receptor (TCR). In contrast to membrane-bound antibodies on B cells, which recognize free antigen, most T cell receptors can only recognize antigen that is bound to major histocompatibility complex (MHC) molecules. MHC molecules are polymorphic glycoproteins found on cell membranes, and fall into two groups: class I MHC molecules and class II MHC molecules. Class I MHC molecules are expressed by nearly all nucleated cells of vertebrate species, whereas class II MHC molecules are expressed only by APCs. Similar to B cell maturation, the process of T cell maturation also includes random rearrangements of a series of gene segments that encode the cell’s antigen-binding receptor, to generate an enormous number of unique antigenic specificities. Once a naïve T cell recognizes antigen combined with MHC, it proliferates and differentiates into effector and memory T cells. T cell effector cells include cytokine-secreting CD4+ T helper cells (T_{H1}), as well as cytotoxic CD8+ T lymphocytes (CTLs). T_{H1} cells can further differentiate into several subtypes upon activation, including T_{H1}, T_{H2}, and T_{H17}, all with their distinct cytokine secretion profiles. CTLs degranulate to release cytotoxic granules that kill infected or stressed cells. Memory T cells confer lifelong immunity against a specific antigen, such that any subsequent encounters with the same antigen generates a faster and more potent immune response. A small subset of T cells known as gamma delta T cells (γδ T cells) possess a distinct TCR on their surface and don’t appear to be MHC-restricted in their recognition of target cells (Morita et al 2000).
1.1.2.1.3 NK cells

NK cells comprise 5–15% of circulating lymphocytes and are characterized by a large, granular morphology. They are not exclusively found in peripheral blood (PB) but populate different tissues and organs including the gut, liver, lungs and uterus (Carrega and Ferlazzo 2012). With a wide array of cell surface receptors designed for robust immune surveillance, NK cells are strongly equipped to act as initiators of immune responses upon recognition of infected or malignant cells. They secrete a milieu of cytokines including tumour necrosis factor (TNF)-α and interferon (IFN)-γ, and exert cellular cytotoxicity against tumour targets and infected cells (Vivier et al. 2008). Unlike B and T cells, they perform these effector functions without the requirement for prior exposure to antigen or somatic rearrangement of their surface receptors during maturation. The importance of NK cells in host protection is illustrated by studies showing that the selective absence of these cells or impairment in their function results in increased episodes of viral infections (Biron et al. 1989; Eidenschenk et al. 2006; Etzioni et al. 2005) and a reduced ability to control the development of some tumours (Ballas et al. 1990; Waldhauer and Steinle 2008a; Yang et al. 2002). Recent studies have also shown their protective role against bacteria (Small et al. 2008), fungi (Schmidt et al. 2011) and parasites (Korbel et al. 2004). Although many questions regarding their distribution, development, activation and response specificity remain unanswered, the last two decades have brought major advances in our understanding of NK cell biology; these advances have challenged the classical view of NK cells as simple effector cells that are classified within the innate immune system.
1.2 NK cell defence: innate or adaptive?

NK cells were discovered in 1975 based on their ability to spontaneously or ‘naturally’ lyse tumour cells without prior sensitization (Herberman et al. 1975a; Herberman et al. 1975b; Kiessling et al. 1975a; Kiessling et al. 1975b). They were initially believed to have a short life span and were characterized by their rapid response, and invariant, germ-line encoded receptors. Now a growing body of evidence suggests that under certain experimental conditions NK cells share some of the attributes of adaptive immunity, including priming, antigen specificity, clonal expansion and immunologic memory (Paust et al. 2010b).

1.2.1 Priming

For many years, NK cell effector functions were believed to be triggered completely independently of prior stimulation. However, recent findings in mice (Ganal et al. 2012; Lucas et al. 2007) and humans (Bryceson et al. 2006b; North et al. 2007; Sabry et al. 2011; Sabry and Lowdell 2013) have shown that NK cell priming is necessary to generate full NK cell effector responses. In vivo, NK cells can be primed by DCs, which produce and trans-present IL-15 to resting NK cells to stimulate cytokine production and cytotoxicity (Lucas et al. 2007). Mononuclear phagocytes have also been shown to be another important source for in vivo priming of NK cells during viral infections (Ganal et al. 2012). Bryceson et al showed that pre-treatment with IL-2 is a requirement for resting NK cells to exhibit natural cytotoxicity against target cells expressing a single ligand for an NK cell activating receptor (Bryceson et al. 2006). Similar studies have also reported that pre-activation of NK cells by IL-12 or IL-15 enhances cytokine production and cytotoxicity against tumour target cells (Hart et al. 2005; Strowig et al. 2010). Our group has shown that tumour cells can also prime resting NK cells to kill previously resistant targets (North et al. 2007). Thus, NK cell priming appears to induce more potent NK cell effector functions by increasing their cytotoxic armoury and lowering the threshold for NK cell activation.
1.2.2 Antigen specificity and clonal expansion

Antigen specificity and diversity allow adaptive lymphocytes to distinguish subtle differences between pathogens and respond by clonal expansion and rapid proliferation of antigen-specific cells. This ability is in contrast to the pattern recognition molecules of innate immune cells which can only distinguish broad families of pathogens. NK cells have a wide array of activating cell surface receptors that bind a variety of ligands on target cells stressed with a pathogen infection or transformation. Many of these NK cell receptors are shared with adaptive T lymphocytes and activate a common killing mechanism that relies on cytotoxic molecules stored within secretory lysosomes (Narni-Mancinelli et al. 2011). Diversity in adaptive recognition is achieved by employing recombination-activating gene (RAG) proteins to generate random rearrangements of V(D)J gene segments for B and T cell receptors. Transient expression of RAG proteins and incomplete V(D)J recombination were recently reported to be observed in low frequency during NK cell development (Borghesi et al. 2004;Igarashi et al. 2002;Pilbeam et al. 2008;Yokota et al. 2003). In addition, NK cell behaviour resembling clonal expansion by adaptive lymphocytes has also been shown to occur. For example, in mice infected with murine cytomegalovirus (MCMV), an NK cell subset expands in an antigen-dependent manner to exhibit long-lasting functional changes (Dokun et al. 2001). Specifically, NK cells expressing the surface receptor Ly49H, which binds the viral protein m157, were shown to proliferate following receptor engagement, and persist up to several months after clearance of infection. These Ly49H⁺ NK cells demonstrated higher functional competence relative to naïve NK cells that had not been exposed to the virus, and were 10 times more efficient at mediating protection against MCMV in adoptive transfer experiments. Several viral infections in humans have also been reported to result in the clonal expansion of a specific NK cell subset, with more potent effector functions than its naïve counterparts (Rolle et al. 2013). For instance, the expansion of NKG2C⁺ NK cells was observed in human cytomegalovirus (HCMV) (Foley et al. 2012;Lopez-Verges et al. 2011), acute
hantavirus (Bjorkstrom et al. 2011), chikungunya virus (Petitdemange et al. 2011), hepatitis B virus (HBV) (Beziat et al. 2012) and human immunodeficiency virus (HIV)-1 (Brunetta et al. 2010). The selective proliferation of this NK cell subset, which could be detected over a month later and exhibited enhanced cytolytic and cytokine-producing effector functions upon restimulation with the virus, is reminiscent of memory T cells.

1.2.3 Immunologic memory

Although the innate immune system is commonly considered to lack the capacity for immunologic memory, recent studies have shown that specificity and memory might exist in invertebrates, which rely solely on innate defence (Kurtz 2005). The half-life of mature NK cells has been estimated to be 17 days in steady-state conditions (Jamieson et al. 2004), but some mature NK cells can be long-lived to mount a robust recall response upon restimulation. The first evidence of NK cell-mediated, antigen-specific adaptive recall responses was observed in a model of hapten-induced contact hypersensitivity mice devoid of T and B cells (O'Leary et al. 2006). Haptens, which are compounds that chemically modify proteins, were used to provoke a hypersensitivity reaction that was transferable to naïve animals by adoptive NK cell transfer. This NK cell-mediated response persisted for at least 4 weeks, and was triggered only by haptens to which mice were previously sensitized. Further studies extended these observations to NK cell recall responses against viruses including MCMV, influenza, vesicular stomatitis virus (VSV), and HIV-1 (Paust et al. 2010a; Sun et al. 2009). In some cases such as MCMV, memory NK cells isolated from the first host can be adoptively transferred to a second recipient and undergo subsequent rounds of proliferation in response to the virus (Sun et al. 2009). These memory NK cells were detected in essentially all tissues including spleen, lymph nodes, liver, lung and kidney after 2 months from the initial infection with MCMV virus. In addition to the specific antigens that drive the expansion of NK subsets and the formation of NK cell memory, the involvement of cytokines for the generation of long-lived NK cell populations with superior effector function has been reported.
Recent studies in both mouse and human NK cells indicate that a short \textit{in vitro} exposure to a combination of IL-12, IL-15, and IL-18 yields memory-like NK cells that display superior effector function and longevity \textit{in vitro} and \textit{in vivo}, and those properties are also inheritable to daughter cells (Cooper et al. 2009; Ni et al. 2012; Romee et al. 2012; Sun et al. 2009). Thus, NK cells appear to remember their past, a trait initially considered possible only for the adaptive immune system.

1.3 NK cell subsets and development

Human NK cells are phenotypically defined by their surface expression of the neural cell adhesion molecule CD56 and lack of the T cell receptor CD3. Two main NK cell subsets can be identified according to the cell surface density of CD56 (Cooper et al. 2001). CD56\textsuperscript{dim} NK cells constitute the majority of PB NK cells and express high levels of CD56 and the low affinity IgG receptor CD16. In contrast, CD56\textsuperscript{bright} NK cells express high levels of CD56, but display little or no CD16 expression. CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells have unique phenotypic and functional attributes. Mouse NK cells resemble their human counterparts in many regards, but the murine homolog for CD56 has not been identified, which has made it difficult to translate our knowledge from mouse to human NK cell biology.

1.3.1 CD56\textsuperscript{bright} NK cells

CD56\textsuperscript{bright} NK cells comprise approximately 10\% of PB NK cells, and are classically viewed as the predominant source of NK cell immunoregulatory cytokines (Cooper et al. 2001; Jacobs et al. 2001). The CD56\textsuperscript{bright} subset lacks perforin and displays weak cytotoxic activity against target cells. However, recent evidence suggests that this may be dependent on the type of stimulation. Following cytokine stimulation, CD56\textsuperscript{bright} NK cells produce significantly greater levels of cytokines such as IFN-\textgreek{g}, and TNF-\textgreek{b}, than their CD56\textsuperscript{dim} counterparts (Cooper et al. 2001). CD56\textsuperscript{bright} NK cells primed with IL-2 also demonstrate enhanced cytotoxicity compared to their resting counterparts. By contrast, CD56\textsuperscript{bright} NK cells exhibit weak cytokine production (Fauriat et al 2010) and cytotoxicity (Penack et al. 2005) after target cell stimulation. Thus, CD56\textsuperscript{bright} NK cells may be the cytokine-responsive NK cell subset.
1.3.2 CD56\textsuperscript{dim} NK cells

CD56\textsuperscript{dim} NK cells are the most abundant subset in circulating NK cells and are considered to be the main cytolytic subset (Campbell et al. 2001; Cooper et al. 2001; Jacobs et al. 2001). Most CD56\textsuperscript{dim} NK cells co-express CD16 and have high levels of perforin and granzymes (Jacobs et al. 2001). They also exhibit a strong capacity to form conjugates, which enables them to mediate potent cytotoxicity. Recent studies have shown that following target cell recognition or crosslinking of NK cell activating receptors; CD56\textsuperscript{dim} NK cells secrete substantial levels of inflammatory cytokines (De et al. 2011; Fauriat et al. 2010; Juelke et al. 2010). This suggests that in addition to mediating cytotoxicity CD56\textsuperscript{dim} NK cells can be an important source of cytokines and chemokines following target cell stimulation. This suggests that CD56\textsuperscript{dim} NK cells may be the target-responsive subset (Zhang and Yu 2010).

1.3.3 NK cell development model

In contrast to other lymphocytes, many of the details of human NK cell development and differentiation remain unclear. An important role for the bone marrow was identified early on by selective bone marrow ablation studies in mice (Kumar et al. 1979; Seaman et al. 1978). These studies provided the first evidence that NK cells are bone marrow-derived, and for more than three decades it was believed that NK cell development occurs solely in the bone marrow (Farag and Caligiuri 2006). Although it was clear that CD34\textsuperscript{+} hematopoietic progenitor cells (HPCs) within the bone marrow gave rise to NK cells, many details of the process, including the in vivo stages of human NK differentiation and the maturation sites remained uncharacterized (Galy et al. 1995). The discovery of CD56\textsuperscript{bright} NK cells in human lymph nodes and tonsils, revealed a potential role for the secondary lymphoid tissue (SLT) in human NK cell development (Freud et al. 2005). Although CD56\textsuperscript{dim} NK cells dominate the bone marrow, blood and spleen, CD56\textsuperscript{bright} NK cells are more abundant in the SLT, where there is a selective enrichment of CD34\textsuperscript{+} and CD45RA\textsuperscript{+} pre-NK cells (Freud et al. 2005). Moreover, the SLT contains an abundance of DCs and other APCs that express membrane-bound IL-15, a critical requirement for NK cell maturation (Mrozek et al. 1996). All of this evidence suggested that the SLT
may be an important site for human NK cell development.

Distinct stages in NK cell development are characterized through expression analysis of CD34, CD117, CD94 and CD56 cell surface antigens. Stage 1 of NK cell development begins with multi-potent CD34+CD117−CD56−CD94− cells, which is followed by the gain of CD117 (stage 2; CD34+CD117+CD56−CD94−). Cells from stage 1 and 2 can give rise to other cell lineages including T cells and DCs. In the first part of stage 3, CD34 expression is lost (stage 3a; CD34−CD117+CD56−CD94−) (Eissens et al 2012). The second part of stage 3 is defined by loss of multi-potency and acquirement of NK cell lineage commitment through CD56 acquisition (stage 3b; CD34−CD117+CD56+CD94−). Subsequently, cells gain CD94 expression and develop into immature CD56bright NK cells (stage 4; CD34−CD117+CD56−CD94−). CD56bright NK cells are believed to be precursors of CD56dim NK cells (Takahashi et al. 2007), as evidenced by observations that CD56bright NK cells have longer telomeres than CD56dim NK cells (Romagnani et al. 2007), are more abundant in blood at an early stage following hematopoietic stem cell transplantation (HSCT) (Shilling et al. 2003) and differentiate into CD56dim NK cells in humanized mice engrafted with human HSCs (Huntington et al. 2009). CD56dim cells continue to develop first through loss of CD117 (stage 5a; CD34−CD117−CD56+CD94−), followed by loss of CD94 expression (stage 5b; CD34−CD117−CD56−CD94−). After the linear progression from CD56bright to CD56dim NK cells, the mature CD56dim NK cells were thought to retain the same phenotypic and functional properties until recent studies demonstrated that CD56dim NK cells continue to differentiate. The differentiation process is accompanied by loss of NKG2A and CD62L expression, acquisition of killer cell inhibitory immunoglobulin-like receptors (KIRs) and the T cell terminal differentiation marker CD57, as well as a gradual decline in proliferative capacity (Bjorkstrom et al. 2010). Collectively, studies involving ex vivo analysis of NK cell phenotype, function, telomere length, and age-dependent frequencies suggest that CD56dimCD62L−CD57+ NK may be the terminally differentiated NK cell subset (Lopez-Verges et al. 2010). Figure 1.1 illustrates an NK cell development model modified from the models proposed by Freud et al. and
Eissens et al. to include studies discussed above (Freud et al. 2006; Freud and Caligiuri 2006; Eissens et al. 2012).
Figure 1.1 NK cell development model

NK cells exhibit different maturational stages with distinct NK cell populations from CD34^+CD45RA^+ HPCs to CD3CD56^{bright} NK cells. After linear progression from CD56^{bright} to CD56^{dim} NK cells, CD56^{dim}CD62L^{+} NK cells appear as an intermediary stage, before CD62L expression is lost and expression of the terminally differentiation marker CD57 is acquired.

HSC indicates hematopoietic stem cell; iNK, immature NK; PB, peripheral blood; SLT, secondary lymphoid tissue.
1.4 NK cell recognition and regulation

Prior to the discovery of NK cell receptors, it was unclear how NK cells could distinguish target cells from normal cells for lysis. The ‘missing-self’ hypothesis was proposed based on the observation that NK cells kill targets with reduced or absent self MHC class-I molecules, a characteristic common to virally-infected and transformed cells (Karre et al. 1986; Ljunggren and Karre 1990). The subsequent characterization of NK cell inhibitory receptors supported this hypothesis by explaining the molecular mechanisms by which NK cells sensed the downregulation of MHC class I expression (Brooks et al. 1997; Carretero et al. 1997; Colonna and Samaridis 1995; D’Andrea et al. 1995; Karlhofer et al. 1992; Lazetic et al. 1996; Moretta et al. 1994; Phillips et al. 1996; Sivori et al. 1996; Wagtmann et al. 1995). The missing-self could also explain the hybrid resistance phenomenon, in which (AxB) F1 mice rejected BM transplants from either parent, despite the fact that the transplant did not express foreign MHC molecules (Murphy et al. 1987). Further confirmation came from experiments demonstrating selective rejection of an MHC class I-deficient version of the tumour cell line RMA in mouse models, in which the results were reversed after treating the mice with an NK-depleting agent (Karre et al. 1986). However, when studies began to show that the absence of MHC class I molecules on tumour cells was insufficient to trigger NK cell lysis, it became clear that our understanding of NK cell target recognition was incomplete (Costello et al. 2002; Ruggeri 2002). As a wide array of activating receptors started to be uncovered, the ‘dynamic equilibrium’ hypothesis was formulated, postulating that the integration of opposing signals from activating and inhibitory receptors determines the functional outcome of NK cell activity (Brumbaugh et al. 1998).
1.4.1 Inhibitory NK cell receptors

Human NK cell inhibitory receptors fall into two groups: the killer immunoglobulin-like receptors (KIRs), and the lectin-like receptor NKG2A, which forms a complex with CD94. KIRs have a two- or three-domain extracellular structure and bind to human leukocyte antigen (HLA)-A, -B, or -C, whereas the NKG2/CD94 complexes ligate HLA-E. Human KIRs contain either two (KIR2D) or three (KIR3D) immunoglobulin (Ig)-like domains in their extracellular domain. KIR2D receptors recognize HLA-C alleles, whereas KIR3D receptors recognize HLA-A or HLA-B alleles. The cytoplasmic domains of KIRs can be either short (S) or long (L), corresponding to their function as either activating or inhibitory, respectively (Biassoni et al. 2001). Another KIR-related inhibitory receptor, LILRB1 (also known as LIR-1, CD85j, or ILT2), is also expressed on human NK cells and recognizes a shared epitope present in all humans MHC class I proteins (Chapman et al. 1999). The common pathway generated by ligation of inhibitory receptors is characterized by tyrosine phosphorylation of immune tyrosine-based inhibitory motifs (ITIM) that recruit tyrosine phosphatases such as the Src homology 2 domain-containing phosphatase (SHP)-1 and SHP-2, which are responsible for the inhibition of various NK cell effector functions (Lanier 2005). Recent studies suggest that the intrinsic responsiveness of NK cells to activation stimuli may be determined by interaction of inhibitory receptors with MHC-1 in a way that is dependent on the ITIM (Kim et al. 2005a).

1.4.2 NK cell education

NK cell education refers to the mechanisms through which inhibitory input by MHC class I during development translates into functional responsiveness in mature NK cells (Anfossi et al. 2006). Unlike the educational processes in T- or B-cell development, NK cell education remains a topic of intense debate, with several models proposed to explain how NK cell responsiveness relates to inhibitory signaling. NK cells that lack ITIM-bearing inhibitory receptors for self-MHC-I and NK cells from hosts that lack MHC-1 ligands for ITIM-bearing inhibitory receptors have a reduced responsiveness to activation signals, such as stimulation by sensitive target cells or cross-linking of NK cell activating receptors (Anfossi et al. 2006; Fernandez et al. 2005; Johansson et al. 2005; Kim et al. 2005b). These results have led to the
two main models in NK cell education. The first ‘disarming’ model proposes that in the absence of inhibition, continuous stimulation of NK cells leads to a state of hyporesponsiveness (Raulet and Vance 2006). The second model proposes that inhibitory receptors provide an ITIM-dependent signal to the NK cells that renders them responsive (Yokoyama and Kim 2006). This model is referred to as ‘arming’ (Raulet and Vance 2006) or ‘licensing’ (Kim et al. 2005), although the latter term is now understood to include any process by which NK cells that receive signals through inhibitory receptors for self-MHC-I gain responsiveness (Elliott and Yokoyama 2011). Studies reporting that NK cell responsiveness is calibrated according to the strength of inhibitory signals received (Brodin et al. 2009; Johansson et al. 2005; Joncker and Raulet 2008), have led to a third ‘rheostat’ model that aimed to reconcile the two opposing models, and account for the quantitative tuning of NK cell responsiveness (Hoglund and Brodin 2010; Joncker et al. 2009; Joncker and Raulet 2008). The rheostat model postulates that NK cell responsiveness is dynamically calibrated based on the strength of inhibitory signals received. More recent data demonstrating that NK cell ‘tuning’ or ‘licensing’ may be set by transient signals and can be reversible have led to an updated model known as the ‘revocable license’ (Long et al. 2013). The revocable license model argues that NK cells can keep their license as long as they are tightly regulated by inhibitory signals, but once this inhibitory input is lost, their license is revoked. Many questions regarding the molecular basis of licensing and the effect of subsequent activation signals on licensed vs. unlicensed cells remain unanswered. In many cases, the original concept of ‘missing-self’ and the self-tolerance of NK cells in an MHC-1-devoid environment cannot be explained without the involvement of NK cell activating receptors. Table 1-1 summarizes the human NK cell receptors and their cognate ligands (Bryceson et al. 2006a; Lanier 2003; Lanier 2005; Long et al. 2013; Sutlu and Aliche 2009).
Table 1-1 Human NK cell receptors

<table>
<thead>
<tr>
<th>Receptor Name</th>
<th>Type of Signal</th>
<th>Specificity</th>
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<tr>
<td>CD16 (FcγRIIIA)</td>
<td>Activating</td>
<td>IgG</td>
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<tr>
<td>NKG2D (CD314)</td>
<td>Activating</td>
<td>ULBP-1-4, MICA/B</td>
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<tr>
<td>NKp46 (CD335)</td>
<td>Activating</td>
<td>Viral haemagglutinin</td>
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<td>NKp44 (CD336)</td>
<td>Activating</td>
<td>Viral haemagglutinin</td>
</tr>
<tr>
<td>NKp30 (CD337)</td>
<td>Activating</td>
<td>BAT3, B7-H6</td>
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<td>AICL</td>
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<tr>
<td>CCR6</td>
<td>Inhibitory</td>
<td>MIP-3α</td>
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AICL indicates activation-induced C-type lectin; BAT-3, HLA-B-associated transcript-3; CCL20, chemokine C-C motif ligand 20; CD, cluster of differentiation; CRACC, CD2-like receptor for activating cytotoxic cells; DNAM-1, DNAX accessory molecule-1; HCMV, human cytomegalovirus; HLA, human leukocyte antigen; ICAM-1,-2 and-3, intracellular adhesion molecule-1, -2, and 3; KIR, killer immunoglobulin-like receptor; KLRG-1, killer cell lectin-like receptor subfamily G member 1; Lag3, lymphocyte activation gene-3; LAIR-1, leukocyte-associated Ig-like receptor; LFA-1,-2 and -3, lymphocyte function-associated antigen-1, -2 and -3; LIR-1, leukocyte Ig-like receptor 1; LLT-1, lectin-like transcript 1; MAC-1, macrophage-1 antigen; MICA/B, MHC class I chain-related molecules A and B; MIP-3α, macrophage inflammatory protein-α; NKG2D, NK group 2 member D; NKR-P1A, NK Receptor- Protein 1A; NTB-A, NK, T and B cell antigen; PVR, poliovirus receptor; SECTM1, secreted and transmembrane 1; TACTILE, T cell-activated increased late expression; UBLP, UL16-binding protein; VCAM-1, vascular cell adhesion molecule-1; VLA-4 and 5, very late antigen-4 and 5.
1.4.3 Activating NK cell receptors

Activating NK cell receptors can be grouped into three categories: those that associate with immunoreceptor tyrosine-based activation motif (ITAM)-containing subunits, the DAP10-associated NK group 2 member D (NKG2D) receptor and a number of other receptors including DNAX accessory molecule-1 (DNAM-1), CD2 and 2B4. Receptors that associate with the ITAM-containing adapter proteins transmit signals through the recruitment of tyrosine kinases Syk or ZAP70, and include CD16, which mediates antibody-dependent cellular cytotoxicity (Moretta et al. 2002), and the natural cytotoxicity receptors (NCRs) NKp30, NKp44, NKp46 and NKp80, which are known to play an important role in NK-mediated cytotoxicity against tumour cells. NKp30 and NKp46 are constitutively expressed on all peripheral blood NK cells, whereas NKp44 is expressed only on activated NK cells. NKp30 binds the nuclear factor HLA-B-associated transcript (BAT)-3, NKp46 binds to influenza haemagglutinin (Mandelboim et al. 2001) and the cellular ligand for NKp80 is the activation-induced C-type lectin (AICL). NKG2D associates with the DAP10 adaptor protein and signals through a phosphoinositol 3-kinase (PI3K)-binding motif. It binds several ligands associated with stress, infection or transformation including MHC class I chain-related protein A (MICA) and MICB and the UL16-binding proteins 1-4 (UBLP1-4) (Mistry and O'Callaghan 2007).
1.4.4 NK cell activation

NK cells require the co-engagement of multiple activating receptors in order to exhibit natural cytotoxicity against tumour target cells (Bryceson et al. 2006). Work by our group further defined this co-stimulation into two discrete stages: priming and triggering (North et al. 2007). The priming signal can be delivered by an activating cytokine in the tumour microenvironment or a target cell expressing the appropriate intensity and combination of ligands for NK cell activating receptors. The second stage, ‘triggering’, requires the co-engagement of at least one additional NK cell activating receptor, specific to stressed cells, in order to avoid autoreactivity. Upon encounter with potential target cells, an immunological synapse forms at the point of contact between the NK cell and the target cell, where NK cell receptors can interact with their respective ligands. Given sufficient activation signals, NK cell cytoskeletal rearrangements are initiated, which result in the polarization of NK cell lytic granules toward the immunological synapse, where they eventually fuse and release their cytotoxic contents on to the target cell (Orange 2008) (Figure 1.2). In contrast to CTLs, NK cells have their cytotoxic granules preformed before target cell recognition, and so their release is initially constrained until sufficient signaling is achieved (Orange 2008). NK cells have also been shown to establish cytoskeletal polarity more slowly than CTLs, and to have a unique sensitivity to minor interference with cytoskeletal dynamics (Wulfing et al. 2003). This stepwise progression in activation events with specific requirements for synergistic signaling may provide a mechanistic explanation of how the spontaneous cytotoxic capacity of NK cells is regulated.
Figure 1.2 NK cell activation events.

NK cell encounter with a tumour cell target generates an immunological synapse at the point of contact. If the ligand combination on the tumour target engages NK cell activating receptors sufficiently, cytoskeletal rearrangements take place resulting in granule polarization and the eventual release of cytotoxic granules on to the target cell.
1.5 NK cells and cancer

1.5.1 Cancer immunosurveillance by NK cells

The theory of cancer immunosurveillance, as proposed by Burnet and Thomas in 1957 (Burnet 1957), postulates that immune cells continuously monitor the body such that any threat to the immune system is detected and eliminated. Although the theory was abandoned shortly after for lack of sufficient experimental evidence (Rygaard and Povlsen 1974a; Rygaard and Povlsen 1974b; Stutman 1974; Stutman 1979), the subsequent discovery of NK cells led to considerable enthusiasm over the possibility that they function as one of the main effector cells of immunosurveillance (Herberman and Holden 1978). Recent data from both mouse and human studies clearly show the existence of cancer immunosurveillance and support the concept that NK cells play a critical role in tumour control and eradication (Waldhauer and Steinle 2008). The two main anti-tumoural effector functions observed by NK cells are target cell elimination and cytokine secretion (Vivier et al. 2008). Until recently, these two effector functions were thought to follow similar mechanisms of activation, but now it is recognized that cytokine secretion by NK cells is distinct from cytotoxicity (Reefman et al. 2010).

1.5.1.1 Target cell elimination

NK cells can directly eliminate malignant cells through granule exocytosis or death receptor ligation. Following target cell recognition and formation of an activating immunological synapse (1.4.4), NK cells release the membrane disrupting protein perforin and a family of serine proteases termed granzymes, which are the critical effector molecules contained in their granules (Smyth et al. 1999). After its release from secretory lysosomes, perforin results in the disruption of endosomal trafficking and binds in a calcium-dependent manner to phospholipid components of the lipid bilayer to facilitate entry of granzymes into the target cell cytosol (Lavrik et al. 2005). Once granzymes enter the target cell, they induce apoptosis, or programmed cell death. In addition to granule exocytosis, NK cells can directly eliminate target cells through the engagement of cell surface death receptors. NK cells express Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), which are both members
of the TNF family and are shown to induce target cell apoptosis when they bind their receptors on target cells (Lavrik et al. 2005).

1.5.1.2 Cytokine secretion

Resting NK cells secrete a plethora of cytokines that help eliminate target cells and amplify activation signals for a more efficient immune response. Cytokine or target cell stimulation of NK cells can result in enhanced secretion of immunoregulatory cytokines, which in turn influence the activity of resting NK cells and other immune cells. Pro-inflammatory cytokines secreted by NK cells, which include IL-1, IL-6, IL-8 and IL-12, can enhance the activation and proliferation of T cells, DCs and macrophages (Elenkov and Chrousos 2002). By contrast, anti-inflammatory cytokines such as IL-4 and IL-10 suppress T cell and macrophage function, but activate humoral responses (Elenkov and Chrousos 2002). Chemokines, which are chemotactic cytokines, play an important role in directing various immune cells to target sites, such that more potent responses are achieved. Chemokines released by NK cells include the macrophage inflammatory protein (MIP)-1α and MIP-1β; chemokine (C-C motif) ligand 5 (CCL5), also known as regulated on activation normal T cell expressed and secreted (RANTES); monocytes chemoattractant protein (MCP)-1; and eotaxin (Cuturi et al. 1989; Roda et al. 2006). Similar to cytotoxicity, a hierarchy is observed for cytokine release by NK cells, whereby chemokine secretion precedes other cytokines and requires less stimulation through the engagement of NK cell activating receptors (Bryceson et al. 2009; Fauriat et al. 2010). The signaling requirements for cytokine/chemokine secretion are different from degranulation and NK cell cytotoxicity (Bryceson et al. 2009). Thus, target cells that are unsusceptible to NK cell-mediated killing can still trigger cytokine release by NK cells. The signaling pathways and mechanisms required for cytokine secretion also appear to be distinct from secretion of cytotoxic granules (Reefman et al. 2010). The localization and trafficking of IFN-γ and TNF-α were shown to take place in compartments and vesicles that do not overlap with perforin or other late endosome granule markers. Recycling endosomes (REs) are not needed for release of perforin, but are required for cytokine secretion in NK cells. Although perforin granules are released in a polarized fashion
at lytic synapses, distinct carriers transport both IFN-γ and TNF-α to points all over the cell surface, including within the synapse, for non-polarized release.

1.5.2 Cancer evasion of NK cells

Although the development of any malignancy is under surveillance by immune cells, malignant cells obtain means to escape from the immune system and proliferate. The recent addition of immune evasion as an emerging ‘hallmark’ of cancer, supports the revival of support for the theory of immunosurveillance (Hanahan and Weinberg 2011). It is now believed that tumours acquire a set of biological capabilities during their development that allow them to overcome challenges posed by the immune system, one of which is likely to be NK cell-mediated anti-tumour immunity. These capabilities are acquired with the help of recruited inflammatory cells and soluble factors in the tumour microenvironment, which play an active role in the process of tumourigenesis. Early on in the study of NK cell interactions with tumours, Kiessling et al. (Kiessling et al. 1999) proposed that cancer evasion of NK cells involves two stages: the early stages of tumour formation and growth are associated with antigen-specific tolerance, whereas the later stages elicit a more generalized state of immunodeficiency. The concept of cancer immunoediting, as introduced by Dunn et al. argues that the immune system plays a role during tumour formation by selecting less immunogenic variants for survival in an immunologically intact environment (Dunn et al. 2002). Tumours are thus ‘imprinted’ by the immunologic environment in which they form, and only those that have acquired capabilities to evade or suppress immune attack remain.

Based on evidence for a two-stage hypothesis for NK cell-mediated killing of tumours, we propose that tumours evade NK cell attack directly by lacking either the priming or triggering ligands such that the activation threshold for NK cell granule exocytosis is not met. Once successful evasion of NK cell attack is achieved, the tumour begins to create the microenvironment necessary for its continued growth. Direct evasion of NK cells by tumour targets can be accompanied by various other escape mechanisms. For example, tumours have been shown to minimally express or shed ligands for important NK cell receptors, such as NKG2D ligands UL16-binding protein 2, major histocompatibility complex (MHC) class I chain-related molecules A and B molecules (MICA/MICB). They have also been reported to upregulate MHC class I, soluble MIC and FasL expression in order to increase inhibitory
signaling (Bennett et al. 1998; Costello et al. 2002; Maki et al. 1998; Yamauchi et al. 1996). The release of immunosuppressive factors such as IL-10, TGF-β and indoleamine 2,3-dioxygenase (IDO) by tumour targets has also been reported, which can suppress the adaptive anti-tumour immune response or skew the immune response toward a Th2 response with significantly less anti-tumour capacity (Morse et al. 2002; Orleans-Lindsay et al. 2001; Shields et al. 2010; Yang et al. 2010; Zagury and Gallo 2004) (Figure 1.3). Indirect mechanisms for NK cell evasion by tumours can involve numerous cell types from the immune system. Recruitment of inflammatory cells that are actively immunosuppressive has been demonstrated, including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and phagocytes secreting reactive oxygen species (ROS) (Romero et al. 2006). Some tumours alter their expressions of IL-6, IL-10, vascular epithelial growth factor or GM-CSF, impairing DC function and maturation, and, thereby, NK cell priming. Tumour growth has also been shown to decrease NK cell count by reducing the numbers of its lymphoid progenitor (Richards et al. 2008).
Tumour cells can evade NK cell attack via direct or indirect mechanisms. Direct mechanisms include:
(a) shedding soluble ligands for NK cell activating receptors;
(b) upregulation of HLA molecules; and (c) release of inhibitory cytokines. Indirect mechanisms include:
(d) activation of inhibitory regulatory T cells; (e) dendritic cell killing; and (f) phagocyte-derived inhibitory cytokines. Tumour cells have also been shown to decrease the number of NK progenitor cells (g), thereby lowering NK cell counts.

HLA indicates human leukocyte antigen; iDC, immature dendritic cell; IDO, indoleamine 2,3-dioxygenase; IL-10, interleukin-10; MICA/B, MHC-class I chain-related molecules A and B; ROS, reactive oxygen species; TGF-β, transforming growth factor-beta; Treg, regulatory T cell
1.5.3 NK cell modulation for cancer therapy

The ability of NK cells to kill tumour cells has made them very attractive in immunotherapy. NK cell impairments associated with tumour development and progression have been frequently reported in cancer patients, including weakened effector functions and an altered phenotype with downregulation of activating NK cell receptors (Sutlu and Alici 2009). Different strategies have been employed to repair, replace or enhance the biological functions of autologous or allogeneic NK cells in vivo and ex vivo. In a clinical setting, the key factors to be considered are the number, purity, proliferative capacity and activation state of NK cells (Sutlu and Alici 2009). The most limiting of these factors is obtaining a sufficient number of NK cells, hence the extensive development of ex vivo expansion methods for NK cell adoptive immunotherapy applications. Genetic modification of NK cells is another approach for the induction of better proliferation, survival and targeting of malignant cells. The genetically modified NK-92 cell line expresses high levels of activating receptors and low levels of inhibitory ones (Gong et al. 1994), is easily grown and expanded under good manufacturing practice (GMP) conditions, and can be readily available for adoptive NK-cell therapy. Preclinical and clinical studies in melanoma, leukemia, lymphoma, refractory renal cell cancer and breast cancer suggest that it is well-tolerated and can be beneficial. The delivery of IL-2, IL-12 and IL-15 genes to NK cell lines has also been shown to enhance proliferative and cytotoxic capabilities. These cytokines are known to play important roles in the enhancement of survival and activation of many immune cells including T cells, B cells and NK cells. Strategies to enhance endogenous NK cell function in vivo through cytokines were pioneered by Rosenberg et al. who demonstrated great initial potential for IL-2 administration in advanced cancer patients (Rosenberg et al. 1987). In vitro stimulation of NK cells by activating cytokines such as IL-2 is known as the lymphokine-activated killer (LAK) phenomenon (Grimm et al. 1982). In early experiments, NK cells were activated ex vivo and adoptively transferred to patients with advanced metastatic renal cancer and melanoma along with IL-2 infusions (Rosenberg et al. 2009). However, overall data from clinical trials since then have failed to provide a convincing proof of efficacy (Sutlu and Alici 2009). LAK therapy requires continual IL-2 administration which carries a strong dose-limiting toxicity. Our group has previously reported an alternative tumour-priming approach, in which human NK cells are activated by co-incubation with the CTV-1 acute lymphoblastic...
leukemia cell line in the absence of IL-2. The resulting tumour-primed NK cells (TpNKs) are cytotoxic to a range of NK-resistant tumour cells in vitro (North et al. 2007). Moreover, their clinical potential has been explored in acute myeloid leukemia and multiple myeloma with promising results in autologous and allogeneic settings (Katodritou et al. 2011). The TpNK primed state is maintained in the absence of IL-2 and is even retained after cryopreservation, which suggests that this approach can yield many clinical benefits in the future.

1.6 Thesis Aims

This central objective of this thesis was to provide novel mechanistic insights into the efficacy of cancer immunotherapy involving TpNKs, by analyzing resting NK cell responses to tumour-priming and dissecting the tumour-induced changes responsible for NK cell activation. The experimental aims of the thesis were as follows:

1. To compare the activation profiles of TpNKs with cytokine-primed NK cells by investigating: a) antigen expression (Chapter 3) and b) cytokine/chemokine secretion (Chapter 4).

2. To identify the specific NK cell receptors involved in the delivery of the tumour-priming signal (Chapter 5).

3. To replicate this stimulatory signal in an artificial priming system in the absence of tumour cells or cytokines using antibody-coated beads (Chapter 5).
Chapter 2  Materials and Methods

2.1 Blood Donors

Fresh blood samples were obtained from normal healthy donors and used within 3 hours after collection unless stated otherwise. Informed consent was obtained in accordance with the declaration of Helsinki and the study was approved by the Royal Free NHS Trust Research and Development review board. Blood samples were taken directly using needle and syringe by phlebotomist staff in the Academic Haematology Department at the Royal Free Hospital, London and were dispensed into 50 ml tubes (Nalge Nunc, Rochester, NY, USA).

2.2 Cell Lines

All cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) or from the Laboratory of the Government Chemist (London, UK) and cultured as recommended by the repository. The main cell lines used in this thesis are described below:

**CTV-1**: Human T cell leukemia cell line that was originally reported to be of myeloid origin, but was recently re-classified as a T cell acute lymphoblastic leukemia (T-ALL). CTV-1 cells have been shown to express cyCD3, CD6, CD7 and CD15, but lack expression of CD2, smCD3, CD4, CD5, CD8, CD13, CD19, CD33, CD34, HLA-DR, TCRalpha/beta and TCRgamma/delta. These cells are round or multiformed and are single cells or clustered in suspension.

**RAJI**: Human Burkitt lymphoma cell line that is one of the prototypical NK-resistant lines. RAJI cells express CD10, CD19, CD20, CD37, CD38, cyCD79a, CD80, HLA-DR and cyIgM, but do not express the markers CD3, CD13, CD34, CD138, sm/cyIgG, smIgM, sm/cy kappa or sm/ycy lamda. They appear as round single cells in suspension, but can form clusters or clumps.
**K562:** Human erythroleukemia cell line that is a prototypical NK-sensitive cell line, which lacks expression of HLA molecules. K562 cells also stain negatively for CD3, CD14 and CD19, but express CD15, CD33, CD71 and CD235a. These cells are non-adherent and rounded.

### 2.3 Cell Culture Medium

Cell cultures were performed in complete medium (CM), which consisted of Roswell Park Memorial Institute medium with Glutamax (RPMI 1640; Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS), penicillin (100 IU), and streptomycin (100 IU) (Invitrogen, Carlsbad, CA, USA). All cell lines were maintained in continuous suspension culture and harvested in exponential growth phase to use as stimulator or target cells. For culture involving short term activation of NK cells, CM was supplemented with cytokines, as outlined in Section 2.9.

### 2.4 Cell Counting and Viability Assessment

Viable cell number was estimated using the Trypan Blue exclusion method; a 10 μl aliquot of cell suspension was mixed with 90 μl of 0.4% Trypan Blue solution (Sigma-Aldrich), which is extruded by viable cells but taken up by non-viable cells. After mixing thoroughly, 10ul of the cell mixture was loaded onto a bright-line haemocytometer counting chamber (Hawksley, Sussex, UK) under a glass cover slip. Cells were counted using a phase contrast microscope (Nikon) excluding non-viable cells that appeared in blue. The number of cells in the central 25 squares was counted and the cell concentration (x10^6/ml) was calculated by multiplying the total number of cells by 10^4. Unless otherwise specified, cells were resuspended and used at concentrations of 10^6 viable cells/ml in CM.
2.5 Cryopreservation of Cells

Cells were brought to a minimum concentration of $1 \times 10^7$/ml in CM and left on ice to reach a temperature of 4º C. Freezing medium was made up with 80% of 4.5% human serum albumin (HSA, Bio Products Laboratory) and 20% dimethylsulfoxide (DMSO, Sigma-Aldrich). The cryopreserving solution was mixed drop by drop under mild agitation to an equal volume of cells, up to a maximum total volume of 1.5 ml in cryopreservation vials (Nalge Nunc, Rochester, NY, USA) and placed in isopropanol freezing containers (Mr Frosty, Nalge) at −80º C for 24 hours. The vials were then transferred for storage in liquid nitrogen at −196º C or vapour phase nitrogen at -152 ºC.

2.6 Thawing of Frozen Cells

Frozen cells were transferred from nitrogen storage straight into a 37 ºC water bath. Cryopreservation vials were partially immersed and gently agitated until partially thawed. The cells were then pipetted into a 15ml Falcon tube containing 10ml of complete RPMI 1640 medium and centrifuged at 200xg for 10 minutes at room temperature. Cells were washed once more in 15ml RPMI 1640 medium to remove any DMSO and the cell pellet was resuspended for future use. The cell viability of frozen cells was usually between 80–90%. Cells were allowed to recover for 2 hours prior to magnetic separation or immunofluorescence labelling.

2.7 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

All blood samples from human donors for research into innate immunity to leukemia were obtained with informed consent, and the research was approved by the institutional ethical review board. Fresh peripheral blood samples obtained from normal healthy donors were added to 100 µl of preservative-free heparin (Monaparin) and diluted with Hanks Balanced Salt Solution (HBSS, Life Technologies) at a 1:1 ratio. An equal volume of whole blood was layered over an equal volume of Lymphoprep (Axis Shield Diagnostics). Tubes were centrifuged at 400xg for 20 minutes at room temperature with the brake off. PBMCs were collected.
from the interface layer using Pasteur pipettes into 50ml Falcon tubes and the cells were washed twice by centrifugation at 200xg for 10 minutes at room temperature with HBSS. The resulting cell pellet was resuspended in CM.

2.8 Isolation of Human NK cells

Human NK cells were isolated from PBMCs using magnetic-activated cell sorting (MACS, Miltenyi Biotec), a cell separation system that comprises three components: MACS MicroBeads, MACS Columns and MACS Separators (Miltenyi Biotec). Cells were separated by labeling with magnetic microbeads conjugated to an antibody of choice, and running through a column placed in the magnetic field of a separator. The labeled cells were magnetically retained in the column and the unlabeled cells pass through. Thus, cells could be positively or negatively selected with respect to the particular antigen.

CD56+ cells were positively selected by labeling PBMCs with CD56 MicroBeads (Miltenyi Biotec). An LS-25 column (Miltenyi Biotec) was prepared by inserting into an MACS separator, with a collection tube placed under the column. The column was rinsed by applying 3 ml of MACS buffer on top of the column and letting the buffer run through. The effluent was discarded and the collection tube changed. PBMCs were resuspended at 10⁸ cells in 500 µl of buffer. The cell suspension was then applied onto the prepared column, where the unlabeled cells passed through to be collected into a negative fraction tube. The column was washed three times with 3ml buffer, adding buffer each time the column reservoir was empty. The column was removed from the separator and placed onto a new collection tube. After pipetting 5 ml of buffer onto the column, the positive fraction was immediately flushed out by firmly applying the plunger supplied with the column.

CD56+CD16+ NK cells were isolated from human PBMCs using the CD56+CD16+ NK Cell Isolation Kit (Miltenyi Biotec). First, non-NK cells were labeled with a cocktail of biotin-conjugated antibodies and anti-biotin microbeads and subsequently
depleted by separation over an LS column, which was placed in the magnetic field of a MACS separator. In the second step, cells were labeled with CD16 microbeads and isolated from the pre-enriched cell fraction by positive selection over a second MS column (Miltenyi Biotec). MACS buffer was prepared using phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA) and 2mM EDTA by diluting MACS BSA stock solution 1:20 with autoMACS rinsing solution. Buffer and CD56 microbeads were kept cold during handling, and air bubbles were avoided to prevent clogging of the column and to ensure high quality of separation. All selected cells were confirmed as ≥ 97% positive, and electronic gating on CD56⁺CD3⁻ identified the pure NK cell population using flow cytometry.

2.9 In vitro stimulation of NK cells

Human PB NK cells were isolated from the PBMCs of normal healthy donors by magnetic sorting for CD56⁺ cells. In vitro stimulation of NK cells was achieved by incubating freshly isolated NK cells (1x10⁶ cells/ml) with CTV-1 or K562 cells (2x10⁶ cells/ml) or the exogenous cytokines IL-2 (200IU/ml) IL-7, IL-15, IL-21 (10 ng/ml), or IL-12 (20 ng/ml) (R&D Systems, Minneapolis, Minnesota, USA) for 20 hours at 37°C, 5 % CO₂ in CM, unless stated otherwise. Activation marker expression (CD69, CD25 and median fluorescence intensity [MFI] expression) and the capacity to kill RAJI cells in a 4 hour cytotoxicity assay were used as measures of NK cell activation.

2.10 Immunophenotyping of NK Cells

Cell surface staining was carried out in 12 x 75 mm polystyrene tubes (Sarstedt, city country). A total of 10⁵ cells were routinely used in a final volume of 100 µl HBSS. Between 5–20 µl of fluorochrome conjugated antibodies were added to purified CD56⁺ PBMCs (titration experiments confirmed adequate cell labeling) and incubated in the dark at room temperature for 15 minutes. Cells were then diluted in 2 ml of HBSS and centrifuged at 200g for 5 minutes at room temperature, and the cell pellet was resuspended in approximately 300 µl FACS Flow (Becton Dickenson,
Oxford, UK). Samples were kept at 4 °C and acquired within 8 hours of labelling on a FACS Aria (Becton Dickenson, Oxford, UK) using FACS Diva software or on the MACS Quant (Miltenyi Biotec, Surrey, UK) using MACS Quantify software for up to 8 parameters. A minimum of 20,000 CD56^+CD3^- events were acquired after gating of viable cells using forward scatter (FSC) and side scatter (SSC) signals and data analysed using MACS Quantify and FlowJo v7.6 software (TreeStar, Olten, Switzerland). Appropriate controls matched for isotype, fluorochrome and manufacturer, as well as single labelled and non-labelled samples were used to set voltages and compensation.

2.10.1 Monoclonal Antibodies

Monoclonal antibodies (mAbs) used were conjugated to the following fluorochromes:

B1: Fluorescein isothiocyanate (FITC)

B2: Phycoerythrin (PE)

B3: Peridinin Chlorophyll Protein Cyanine-5.5 (PerCPCy5.5), Phycoerythrin Cyanine-5 (PE-Cy5)

B4: Phycoerythrin Cyanine-7

R1: Allophycocyanin (APC)

R2: Allophycocyanin Cyanine-7 (APC Cy7)

V1: Violet Blue, Pacific Blue, eFluor450

V2: Violet Green
A summary of all mAbs used in this thesis is shown in Table 2.1. A forward-scattered light (FSC) versus side-scattered light (SSC) dot plot was created for each sample and a gate drawn around the area of live lymphocytes (Figure 2.1). The live lymphocyte gate was projected onto a second dot plot of CD56 versus FSC and a second gate drawn to identify the CD56⁺ lymphocytes. For the assessment of antigen expression on CD56⁺CD3⁻ NK cells, a new dot plot was constructed.

Table 2-1 Monoclonal antibodies used in this Thesis.

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Figure 2.1 Basic gating strategy for flow cytometric analysis.

A FSC versus SSC plot was created for each sample and a gate drawn around the area of live lymphocytes. The live lymphocyte gate was projected onto a second dot plot of CD56 versus CD3 and a second gate drawn to identify CD56⁺CD3⁻ NK cells.
2.11 Analysis of NK cell secretory profiles

An aliquot (2 x 10^5) of resting NK cells were washed twice and mixed with 4x10^5 CTV-1 or K562 cells (4 x 10^5), and IL-2 (200IU/ml) IL-7, IL-15, IL-21 (10 ng/ml), or IL-12 (20 ng/ml) Cells were incubated for 6 hours or overnight at 37 ºC in 5% CO₂. Thereafter, supernatants were collected and stored at −20 ºC pending measurement. The concentrations of cytokines were quantified by a multiplex immunoassay (Luminex 100 IS; Invitrogen) using a kit that simultaneously quantifies 25 different cytokines, listed below in four multiplex groupings for analysis.

**Inflammatory Panel:** IL-1β, IL-1RA, IL-6, IL-8 and TNF-α

**Th1/Th2 Panel:** IFN-γ, IL-2, IL-2R, IL-4, IL-5 and IL-10

**Cytokine II Panel:** IFN-α, IL-7, IL-12p40/p70, IL-13, IL-15 and IL-17

**Chemokine Panel:** Eotaxin, IP-10, MCP-1, MIG, MIP-1α, MIP-1β and RANTES

This multiplex assay relies on polystyrene beads (5.6μm), which are internally dyed with red and infrared fluorophores of differing intensities. Each bead has a unique number or bead region that allows differentiation of one bead from another. Beads of defined spectral properties are conjugated to protein-specific capture antibodies and added along with samples (including standards of known protein concentration, control samples and test samples), into the wells of a filter-bottom microplate where proteins bind to the capture antibodies over the course of a 2 hour incubation. After the beads were washed, protein-specific biotinylated detector antibodies were added and incubated with the beads for 1 hour. During this incubation, the protein-specific biotinylated detector antibodies bind to the appropriate immobilized proteins. After removal of excess biotinylated detector antibodies, streptavidin conjugated to the fluorescent protein R-Phycoerythrin (Streptavidin-RPE) was added and allowed to incubate for 30 minutes. The Streptavidin-RPE binds to the biotinylated detector...
antibodies associated with the immune complexes on the beads, forming a four-
member solid phase sandwich. After washing to remove unbound Streptavidin-RPE,
the beads were analyzed with the Luminex detection system. By monitoring the
spectral properties of the beads and the amount of associated RPE fluorescence, the
protein concentrations in test samples were determined from standard curves
constructed from samples of known protein concentration, using Graphpad Prism
v6.0 (Graphpad Software, California, USA) (Figure 2.2). The mean fluorescence
values of blank samples were subtracted from each test sample for the equivalent
protein. In the case of target cell-stimulated NK cells, the mean baseline value
subtracted from test samples also included the corresponding values from target cells
incubated alone.
Figure 2.2 Standard curves for analysis of cytokine secretion.

Typical standard curves collected from analytes assayed with the Human Cytokine 25-Plex Panel using GraphPad Prism v 6.
2.12 PKH-26 labelling of Target Cells

Target cell were washed by adding HBSS and centrifuging at 200xg for 10 minutes with brake on. The cell pellet was resuspended in 1 ml of Diluent C and pipetted gently to insure complete dispersion. In a separate polypropylene tube, a dye solution was prepared by adding 2 µl of the PKH67 to 1 mL of Diluent C. The dye solution was mixed well to disperse and rapidly added to the sample and immediately mixed by pipetting. The staining was stopped by adding an equal volume of serum (neat FCS) or other suitable protein solution (e.g., 1% BSA) and leaving to incubate for 1 minute in order to allow binding of excess dye. After the 1 minute incubation, the sample was centrifuged at 200x g for 10 minutes at 20-25 °C and the supernatant was carefully removed. The pellet was resuspended in 10 mL CM, transferred to a fresh sterile conical poly-propylene tube, and centrifuged again at 200x g for 10 minutes with brake on, to ensure removal of unbound dye. After the wash was complete, the cell pellet was resuspended in complete medium at 1x10^6 cells/ml.

2.13 Cytotoxicity Assays

Target cells were recovered from suspension culture and washed in HBSS before resuspension in 1 ml PKH-26-labeling diluent at a concentration of 4x10^6/ml. A 4 µl aliquot of PKH-26 was added to 1.0 ml labeling diluent and then added to the target cell suspension for 2 min at room temperature. The labeling reaction was stopped by the addition of 1 ml neat FCS for 1 min. Finally, the labeled cells were washed twice in CM and resuspended in CM at 10^6/ml. A total of 1x10^5 PKH-26-labeled target cells in 100 µl RPMI 1640 (10% FCS) were added to 5x10^5 NK cells in 400 µl (effector:target [E:T] cell ratio of 5:1), pelleted at 200 x g for 1 min at room temperature, and then incubated for 4 h at 37°C and 5% CO₂. After the incubation period, the cells were resuspended in a solution of TO-PRO-3 iodide (Life Technologies, Paisley, UK) in PBS (1 µM) and analyzed by flow cytometry. At least 10^4 target cells were acquired with 1024-channel resolution after electronic gating on PKH-26 positivity, and the mean proportion of TO-PRO iodide-positive cells from the triplicate samples was determined. Background target cell death was determined.
from the spontaneous lysis of cells incubated in the absence of effector cells. Cell-mediated cytotoxicity was calculated as the mean (percentage cell death minus percentage background cell death). Less than 5% spontaneous lysis of target cells was observed in these experiments.

2.14 CD107a Degranulation Assay

PBMCs were isolated by density gradient centrifugation on a discontinuous density gradient (Lymphoprep), and CD56+ cells were positively selected using CD56 microbeads (Miltenyi Biotec). To detect spontaneous degranulation, a control sample without target cells was included in every experiment. An E:T ratio of 1:1 (2x10^5 target cells in a volume of 200 µl) was used in all experiments. In each tube containing 200 µl E/T cell suspensions, 15 µl of PE-Cy5 conjugated anti-CD107a mAb was added prior to incubation. NK cells and target cells were co-incubated at 37°C for 3 h in total; after the first hour, 5 µl of the secretion inhibitor monensin (Sigma; 2mM in 100% ethanol) was added. Cells were then washed and stained with mAbs (CD56 and CD3) for flow cytometric analysis.
2.15 Antibody blocking experiments

To investigate the involvement of NK cell receptor-ligand interactions in CTV-1-mediated priming, purified CD56⁺ NK cells from normal healthy donors were pre-incubated with Anti-CD2 (RPA 2.10), CD15 (MEM158), and/or NKG2D (149810) mAbs, and CTV-1 cells were pre-treated with anti-Intercellular Adhesion Molecule-1 (ICAM-1) mAb. After a 16 h incubation, activation marker expression (CD69, CD25 and MedCF expression) and the capacity to kill RAJI cells in a 4 h cytotoxicity assay were used as measures for NK cell priming.

To investigate the involvement of LFA-1, CD2 and NKG2D in CTV-1-mediated NK cell priming, purified CD56⁺ NK cells from normal donors were pre-incubated for 30 mins at room temperature in the presence or absence of saturating concentrations of antibodies. After washing with HBSS, and overnight co-incubation of NK cells (1x10⁶ cells) with CTV-1 (2x10⁶ cells) immunophenotyping was carried out for the analysis of antigen expression (MACS Quant Analyzer) with isotype controls used for every experiment. NK cell activation was also determined by comparing NK cell activity against NK-resistant RAJI cells in a 4h cytotoxicity assay. Statistical comparisons between treatments were performed using the paired t-test. Results are presented as mean ± SD. Corrected P values < 0.05 were considered statistically significant. Statistical analyses were performed with GraphPad Prism v.6 software.
2.16 Bead Stimulation

2.16.1 Dynabeads Sheep anti-mouse IgG

Dynabeads Sheep anti-Mouse IgG have a diameter of 4.5µm. They are uniform superparamagnetic polystyrene beads coated with polyclonal Sheep anti-Mouse IgG antibodies. Dynabeads were washed before use by resuspending in the vial, transferring 50 µl to a tube and adding 1ml of PBS buffer with 0.1% BSA, pH 7.4. The tube was placed in a magnet for 1 minute and the supernatant was discarded. After removing the tube from the magnet, the Dynabeads were resuspended in the same volume of buffer as the initial volume of Dynabeads used (50 µl). Thereafter, washed beads were transferred to a tube and 1.5 µg of each primary monoclonal antibody (mAb) added: anti-LFA-1 (R7.1), anti-CD2 (RPA 2.11) and anti-NKG2D (149810). After incubation for 1 hour at 4 ºC with rotation, the antibody-beads complex was washed again by placing in a magnet for 1 min and discarding the supernatant. The beads were washed twice using 1ml of buffer. After removing the tube from the magnet, Dynabeads were resuspended in 50 µl PBS buffer with 0.1% BSA, pH 7.4. Beads were then used to stimulate NK cells at a ratio of 8 beads per cell. Dynabeads (4 × 10^6) coated with saturating amounts of mAb, were incubated with resting NK cells (5 × 10^5) in 500 µl RPMI supplemented with 10% human serum for the indicated duration at 37ºC. The cultures were rotated end-over-end during the stimulation. For each primary mAb, coating of Dynabeads was ≥ 98% efficient as determined by flow cytometry.
2.16.2 Dynabeads Protein G

Dynabeads coupled to Protein G were then used to generate an artificial priming signal so that cognate ligands, instead of mAbs, engaged activating NK cell receptors. Dynabeads Protein G have a diameter of 2.5μm. Beads were resuspended by vortexing for >30 seconds and then transferred to a fresh tube. The tube was placed on a magnet to separate the beads from the solution, and the supernatant was removed. The tube was removed from the magnet, and 1.5 μg of recombinant Human ICAM-1 Fc Chimera, recombinant Human MICA Fc Chimera, or anti-CD2 (RPA 2.11) were added to the Dynabeads. CD15 was not used as it is not commercially available. The beads were then incubated for 30 minutes at room temperature with rotation. Thereafter, the tube was placed on a magnet and the supernatant removed. Beads were then used to stimulate NK cells at a ratio of 8 beads per cell. Dynabeads (4 × 10^6) coated with saturating amounts of mAb, were incubated with resting NK cells (5 × 10^5) in 500 μl RPMI supplemented with 10% human serum for the indicated duration at 37°C. The cultures were rotated end-over-end during the stimulation. Coating of Dynabeads was ≥ 98% efficient as determined by flow cytometry.

2.17 Statistical Analyses

Statistical comparisons between pairs of unstimulated-stimulated NK cells were tested for significant differences in their means by the multiple t test, corrected for multiple comparisons with the Holm-Sidak method. Results are presented as mean ± SD. Corrected p values < 0.05 were considered statistically significant. Unless stated otherwise, p values are indicative of significant difference relative to unstimulated NK cells at the same time point. Statistical analyses were performed with GraphPad Prism v.6 software.
Chapter 3  Phenotypic profiles of Tumour-primed NK cells

3.1 Introduction

NK cell activation occurs through engagement of germ-line encoded membrane receptors, which trigger downstream signalling pathways that culminate in the secretion of lytic granules onto a susceptible target cell. In recent years, it has become clear that NK cells represent a heterogeneous population, displaying differences in phenotype, function, tissue localization and activation status. NK cells also exhibit heterogeneity in responses to varying stimuli, be it a target cell or an activating cytokine. Human NK cells are phenotypically defined by their surface expression of CD56 and lack of the T cell receptor CD3. Two main NK cell subsets can be identified according to the cell surface density of CD56 and the low affinity IgG receptor CD16 (Cooper et al. 2001). Approximately 90% of NK cells found in blood are CD56\textsuperscript{dim}CD16\textsuperscript{+}, displaying high cytotoxicity, but little capacity for cytokine production. In contrast, about 10% of circulating NK cells are CD56\textsuperscript{bright}CD16\textsuperscript{dim}/\textsuperscript{-}; these are potent cytokine producers, but with little or no ability to spontaneously kill tumour cell targets. Studies aiming to further dissect the heterogeneity of CD56\textsuperscript{dim} NK cells, have characterized other subsets according to the expression of markers related to differentiation, migration or competence (Juelke et al. 2010; Lopez-Verges et al. 2010; Yu et al. 2010).

Exposure to activating cytokines such as interleukin (IL)-2 or IL-12 is known to enhance NK cell effector function. Work by our group previously showed that tumour-mediated priming of resting NK cells is equivalent to cytokine-mediated priming, as measured by activation marker expression, interferon (IFN)-\(\gamma\) secretion and the ability to kill NK-resistant RAJI cells (North et al. 2007). In this Chapter, the characteristics of tumour-primed PB NK cells were investigated, in term of expression of differentiation markers, activating receptors, and chemokine receptors, in comparison with cytokine-primed NK cells.
3.2 Experimental Aims

The aim of the set of experiments described in this Chapter was to characterize the antigen expression profile of PB NK cells after tumour-priming, and compare it with that observed after priming with a panel of innate cytokines, namely, IL-2, IL-7, IL-12 and IL-15. These cytokines each play distinct physiological roles in NK cell biology in vivo, and trigger different NK cell signalling pathways, thus providing a broad panel of expression profiles against which tumour-mediated priming of NK cells could be compared. Furthermore, to investigate heterogeneity in NK cell responses associated with different phenotypes, the expression of CD107a, a degranulation marker (Alter et al. 2004), was used to analyse different subsets of resting, tumour-primed and cytokine-primed NK cells.
3.3 Methods

Human PB NK cells were isolated from the PBMCs of normal healthy donors by magnetic sorting for CD56+ cells. They were then incubated overnight alone (1x10^6 cells), with CTV-1 cells (2x10^6 cells), IL-2 (200IU/ml), IL-7 (10ng/ml), IL-12 (20ng/ml) or IL-15 (10ng/ml). After washing the cells the next day with HBSS, immunophenotyping was carried out for the analysis of antigen expression (MACS Quant Analyzer, Miltenyi Biotec) and the CD107a degranulation assay was performed. CD56^{dim}CD16+ NK cells were positively selected from PBMCs when stated, to investigate specific responses of this NK cell subset (MACS, Miltenyi Biotec). Statistical comparisons between pairs of unstimulated-stimulated NK cells were tested for significant differences in their means by multiple t tests, corrected for multiple comparisons with the Holm-Sidak method. Results are presented as mean ± SD. Corrected p values < 0.05 were considered statistically significant. Unless stated otherwise, p values are indicative of significant difference relative to unstimulated NK cells at the same time point. Statistical analyses were performed with GraphPad Prism v.6 software. Antigen expression analysis was divided into 3 groups for simplicity:

1) Differentiation receptors (CD56, CD16, CD57 and CD62L)
2) Activating receptors (NKp30, NKp44, NKp46, NKp80, LFA-1, CD2, ICAM-1, NKG2D, DNAM-1 and 2B4)
3) Homing receptors (CD49d, CXCR1, CXCR4, CXCR7 and α4β7)
3.4 Results

3.4.1 Differentiation markers

3.4.1.1 CD56 expression

In the initial set of experiments, I set out to investigate differences in the proportion of cells belonging to the two main NK cell subsets, CD56\textsuperscript{bright} and CD56\textsuperscript{dim}, following stimulation. CD56 expression in resting and primed NK cells is shown in Figure 3.1. Consistent with previous studies (Cooper et al. 2001), the mean percentage of CD56\textsuperscript{bright} NK cells within resting PB NK cells was (2.37 ± 1.94\%, n=10). Tumour-priming with CTV-1 cells did not result in a significant change in the mean percentage of NK cells belonging to the CD56\textsuperscript{bright} NK cell subset (2.13 ± 1.39\%, n=6). By contrast, exogenous cytokine stimulation by IL-12 increased the mean percentage of CD56\textsuperscript{bright} NK cells to 16.35 ± 2.70\% (n=5; p < 0.0001). However, IL-2, IL-7, and IL-15 did not have a significant effect on the proportion of NK cells belonging to the CD56\textsuperscript{bright} NK cell subset (mean, 3.34 ± 2.75, n=5; 2.47 ± 1.95, n=5; 2.77± 2.09, n=5, respectively).
Figure 3.1 CD56 expression in resting and primed NK cells.

(A) Percentage of CD56\textsuperscript{bright} NK cells; (B) representative FACS analysis of CD56\textsuperscript{bright} NK cells from one donor. Freshly isolated resting NK cells (1x10\textsuperscript{6}) were incubated alone or with CTV-1 cells (2x10\textsuperscript{6}), IL-2 (200 IU/ml), IL-7 (10 ng/ml), IL-12 (20 ng/ml) or IL-15 (10 ng/ml) overnight. Thereafter, cells were washed with HBSS and stained with anti-CD56 (clone B159) and anti-CD3 (clone SK7). After electronic gating on the viable lymphocyte population and CD56\textsuperscript{bright}CD3\textsuperscript{−} NK cells, the percentage of cells belonging to the CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cell sub-populations was compared. For each treatment group, the mean percentage expression is indicated by a horizontal line.

FSC indicates forward-scattered light; LAK-2,-7,-12 and -15, lymphokine-activated NK cell using IL-2, -7, -12 or -15; rNK, resting NK cells; TpNK, tumour-primed NK cells with CTV-1; %#, percentage of NK cell population; *P <0.05; **P <0.005; ***P < 0.0005.
3.4.1.2 CD16 expression

CD16 expression in resting and primed NK cells is shown in Figure 3.2. A high percentage of resting NK cells expressed the CD16 marker (mean, 93.14 ± 1.62%, n=10). In accordance with our previously published findings (North et al. 2007; Sabry et al. 2011), co-incubation with CTV-1 led to a significant reduction in the percentage of cells expressing CD16 (mean decrease, 25.60 ± 3.43%, n=10; p < 0.0001). The median fluorescence intensity (MFI) was also significantly lower in tumour-primed NK cells compared with resting NK cells (mean decrease, 29.40 ± 8.10%, n=10; p < 0.0001). By contrast, priming with exogenous cytokines had no effect on CD16 expression by NK cells (LAK-2 mean, 91.78 ± 3.76, n=10; LAK-7 mean 95.23 ± 13.46, n=3; LAK-12 mean 95.33 ± 11.08, n=3; LAK-15 mean 94.22 ± 2.95, n=3).
Figure 3.2 CD16 expression in resting and primed NK cells.

(A) Percentage of CD16+ NK cells; (B) representative FACS analysis of CD16+ NK cells from one donor. Freshly isolated resting NK cells (1x10^6) were incubated alone or with CTV-1 cells (2x10^6), IL-2 (200 IU/ml), IL-7 (10ng/ml), IL-12 (20ng/ml) or IL-15 (10 ng/ml) overnight. Thereafter, cells were washed with HBSS and stained with anti-CD56 (clone B159), anti-CD3 (clone SK7) and anti-CD16 (clone VEP13). After electronic gating on the viable lymphocyte population and CD56+CD3– NK cells, the percentage of cells expressing CD16 was analysed.

FSC indicates forward-scattered light; LAK-2,-7,-12 and-15, lymphokine-activated NK cell using IL-2, -7,-12 or -15; rNK, resting NK cells; TpNK, tumour-primed NK cells with CTV-1; %#, percentage of NK cell population, *P <0.05; **P <0.005; ***P < 0.0005.
3.4.1.3 CD62L expression

CD62L is an NK cell homing receptor, the expression of which was recently shown to define an intermediate stage in NK cell development between CD56\textsuperscript{bright} and CD56\textsuperscript{dim}CD62L\textsuperscript{−} NK cells (Juelke et al. 2010). CD62L expression in resting and primed NK cells is shown in Figure 3.3. The proportion of CD62L\textsuperscript{+} resting NK cells in normal donors ranged between 18–52%. Priming by co-incubation with CTV-1 resulted in a significant decrease in the number of CD62L\textsuperscript{+} cells (mean decrease, 50.10 ± 6.22%, n= 4; p = 0.026) and a more modest decrease in CD62L MFI (mean decrease, 35.4 ± 7.45%, n=4). Exposure to IL-2 or IL-15 had a similar effect on NK cells, significantly reducing the proportion of CD62L\textsuperscript{+} NK cells (LAK-2 mean decrease, 55.00 ± 7.45%, n= 4; p = 0.017), (LAK-15: mean decrease, 54.90 ± 9.80%, n=4; p = 0.017) and decreasing CD62L MFI (LAK-2: mean decrease 50.2 ± 7.45%, n=4; p = 0.021) (LAK-15: mean decrease, 56.9 ± 7.45%, n=4; p = 0.027).
Figure 3.3 CD62L expression in resting and primed NK cells

(A) Percentage of CD62L⁺ NK cells; (B) representative FACS analysis of CD62L⁺ NK cells from one donor. Freshly isolated resting NK cells (1x10⁶) were incubated alone or with CTV-1 cells (2x10⁶), IL-2 (200 IU/ml), IL-7 (10ng/ml), IL-12 (20ng/ml) or IL-15 (10 ng/ml) overnight. Thereafter, cells were washed with HBSS and stained with anti-CD56 (clone B159), anti-CD3 (clone SK7) and anti-CD62L (clone DREG-56). After electronic gating on the viable lymphocyte population and CD56⁺CD3⁻ NK cells, the number of CD62L⁺ was compared. For each treatment group, the mean percentage expression is indicated by a horizontal line.
FSC indicates forward-scattered light; LAK-2,-7,-12 and-15, lymphokine-activated NK cell using IL-2, -7,-12 or -15, rNK, resting NK cells; TpNK, tumour-primed NK cells with CTV-1; %#, percentage of NK cell population; *P <0.05; **P <0.005; ***P < 0.0005.

3.4.1.4 CD57 expression

CD57 is a T cell senescence marker that was recently described to mark the terminal differentiation of NK cells (Lopez-Verges et al. 2010) CD57 expression in resting and primed NK cells is shown in Figure 3.4. The mean percentage of CD57+ resting NK cells was 56.5 ± 19.57%. Tumour-priming led to a significant increase in the proportion of CD57+ NK cells in culture (mean increase, 23.6 ± 9.75%, n= 10; p = 0.024) and a non-significant increase in CD57 expression (mean increase in MFI, 6.14 ± 4.28%, n=10). However, cytokine-mediated priming did not result in any significant changes in CD57 expression (LAK-2 mean 62.44 ± 22.31%, n=3; LAK-7 mean 60.83 ± 23.02%, n=3; LAK-12 mean 52.32 ±27.15%, n=3; LAK-15 mean 59.56 ±21.19%, n=3).
Figure 3.4 CD57 expression in resting and primed NK cells

(A) Percentage of CD57^+ NK cells; (B) CD57 MFI of rNK and TpNK. Freshly isolated resting NK cells (1x10^6) were incubated alone or with CTV-1 cells (2x10^6), IL-2 (200 IU/ml), IL-7 (10 ng/ml), IL-12 (20 ng/ml) or IL-15 (20 ng/ml) overnight. Thereafter, cells were washed with HBSS and stained with anti-CD56 (clone B159), anti-CD3 (clone SK7) and anti-CD57 (clone TB01). After electronic gating on the viable lymphocyte population and CD56^+CD3^- NK cells, percentage CD57 positive and MFI were compared. For each treatment group, the mean percentage expression is indicated by a horizontal line.

LAK-2,-7,-12 and-15 indicate lymphokine-activated NK cell using IL-2, -7,-12 or -15; rNK, resting NK cells; TpNK, tumour-primed NK cells with CTV-1; MFI, median fluorescence intensity; *P <0.05; **P <0.005; ***P < 0.0005.
3.4.1.5 Compound expression of NK cell differentiation receptors

It was hypothesized that tumour-priming completes NK cell maturation toward better effector function. Thus, the compound expression of several NK cell differentiation receptors was analysed. An increase in the proportion of the terminally differentiated CD56\textsuperscript{dim}CD62L\textsuperscript{−}CD57\textsuperscript{+} NK cell subset was observed after tumour-priming of resting NK cells (mean increase, 61.2 ± 5.4\%, n= 4; p = 0.02) (Figure 3.5), which suggests a tumour-priming role in promoting a more mature NK phenotype. However, a heterogeneity in response to tumour-priming was observed between different NK cell subsets, as defined by their expression of differentiation markers. CD56\textsuperscript{dim} NK cells uniquely downregulated CD16 expression following stimulation with CTV-1 cells (mean decrease, 28.18 ± 5.43\%, n=3; p < 0.001). In contrast, CD56\textsuperscript{bright} NK cells responded to tumour-priming by upregulating CD16 expression (mean increase, 19.81 ± 1.12\%, n=3), as did CD56\textsuperscript{bright} NK cells primed by IL-2, IL-12, and IL-15. Validation studies to investigate the nature of the CD56\textsuperscript{dim}CD16\textsuperscript{−} population emerging after tumour-priming are described in 3.4.4.
Figure 3.5 Tumour-priming completes NK cell maturation

Freshly isolated resting NK cells (1x10^6) were incubated alone or with CTV-1 cells (2x10^6) overnight. Thereafter, cells were washed with HBSS and stained with anti-CD56 (clone B159), anti-CD3 (clone SK7) anti-CD62L (clone DREG-56) and anti-CD57 (clone TB01). After electronic gating on the viable lymphocyte population, CD56^+CD3^- and CD56^{dim} NK cells, the proportion of CD56^{dim}CD62L^-CD57^+ NK cells was compared. NK cell priming with CTV-1 resulted in a decrease in the proportion of CD56^{dim}CD62L^-CD57^+ NK cells. One representative donor and percentage of CD56^{dim}CD62L^-CD57^+ NK cells as analysed by flow cytometry shown.

rNK indicates resting NK cells; TpNK, tumour-primed NK cells with CTV-1.
3.4.2 Activating receptors

3.4.2.1 Natural cytotoxicity receptors

NK cell natural cytotoxicity receptors (NCRs) are important mediators of NK cell cytotoxicity, through recognition of various cognate cellular and viral ligands (Lanier 2005). Resting NK cells showed constitutive expression of NKp30 (mean percentage expression, 87.36 % ± 1.8%, n=3), NKp46 (mean percentage expression, 94.18 ± 1.3%, n=5) and NKp80 (mean percentage expression, 95.11% ± 1.22%, n=3), but little or no expression of NKp44 (mean percentage expression, 1.93 ± 0.8%, n=3), which is known to be up-regulated post-cytokine stimulation (Lanier 2005). Figure 3.6 shows that the percentage of cells expressing NKp46 was significantly lower in tumour-primed NK cells compared with resting NK cells (mean decrease, 27.6 ± 15.55%, n= 4; p = 0.002), whereas cytokine-mediated priming did not result in a significant change in NKp46 expression (LAK-2 mean, 92.86 ± 4.15%, n=3; LAK-7 mean, 94.57 ± 3.04 %, n=3; LAK-12 mean, 95.58 ± 1.74%, n=3; LAK-15 mean, 91.02 ± 6.94%, n=3). Tumour-priming also resulted in a non-significant decrease in NKp30 expression (MFI mean decrease, 19.3 ± 2.7%, n= 3) and NKp80 expression (MFI mean decrease 29.4 ± 2.18%, n=3). In contrast, cytokine-mediated priming resulted in similar or in some cases higher NCR expression in NK cells. For example, IL-2 or IL-15 stimulation of resting NK cells resulted in a significant increase in NKp44 expression (LAK-2 mean increase 147.3 ± 2.91%, n=3; p <0.01) (LAK-15 mean increase 184.2 ±1.57%, n=3; p <0.01).
Figure 3.6 NKp46 expression by resting and primed NK cells

(A) Percentage of NKp46⁺ NK cells; (B) representative FACS analysis of CDNKp46⁺ NK cells from one donor. Freshly isolated resting NK cells (1x10⁶) were incubated alone or with CTV-1 cells (2x10⁶) or IL-2 (200IU/ml) overnight. Thereafter, cells were washed with HBSS and stained with anti-CD56 (clone B159), anti-CD3 (clone SK7), and anti-NKp46 (clone 9E2) mAbs. After electronic gating on the viable lymphocyte population and CD56⁺CD3⁻ NK cells, NKp46 expression was analysed (MACS Quant Analyser).

FSC indicates forward-scattered light; LAK-2,-7,-12 and-15, lymphokine-activated NK cell using IL-2, -7,-12 or -15, rNK, resting NK cells; TpNK, tumour-primed NK cells with CTV-1; %#, percentage of NK cell population; *P <0.05; **P <0.005; ***P < 0.0005.
3.4.2.2 Adhesion receptors

NK cell interactions with target cells must be stabilized by cell-cell adhesion forces, and the formation of stable conjugates, prior to cytolysis (Davis 2009). Adhesion signals can be provided by receptor-ligand interactions, some of which can also promote activation signalling such as LFA-1 (Barber et al. 2004) and CD2 (Bryceson et al. 2006). Figure 3.7 shows ICAM-1 expression by resting and primed NK cells. Resting NK cells constitutively expressed high levels of lymphocyte function-associated antigen (LFA)-1 (mean, 98.3 ± 1.3%, n=4) and its cognate ligand intracellular adhesion molecule (ICAM)-1 (mean, 94.5 ± 1.9%, n=7) as well as CD2 (mean, 61.3 ± 2.67%, n=3). Tumour-priming resulted in a loss of ICAM-1 expression (MFI mean decrease 26.4%, SD± 1.43, n=5; p = 0.05) and a decrease in the number of cells expressing ICAM-1 (mean decrease 17.8%, SD± 1.52, n=5; p = 0.05). Cytokine-mediated priming did not affect ICAM-1 expression, (LAK-2 mean, 96.74 ± 1.95%, n=5; LAK-7 mean 90.93 ± 3.71%, n=5; LAK-12 mean 93.16 ± 5.88, n=3; LAK-15 mean 95.08 ± 3.58, n=3). Neither priming mechanisms had any significant changes on LFA-1 (TpNK mean, 93.96 ± 6.23, n= 7; LAK-2 mean, 98.02 ± 1.34%, n=7; LAK-7 mean 98.27 ± 0.58 %, n=3; LAK-12 mean 99.16 ± 2.45%, n=3; LAK-15 mean 97.14 ± 2.23%, n=3) or CD2 expression (TpNK mean, 95.76. ± 3.16%, n= 7; LAK-2 mean, 97.21 ± 1.91%, n=7; LAK-7 mean 98.56 ± 1.79 %, n=3; LAK-12 mean 96.55 ± 2.18, n=3; LAK-15 mean 96.04 ± 2.41, n=3).
Figure 3.7 ICAM-1 expression by resting and primed NK cells

(A) Percentage of ICAM-1⁺ NK cells; (B) representative FACS analysis of ICAM-1⁺ NK cells from one donor. Freshly isolated resting NK cells (1x10⁶) were incubated alone or with CTV-1 cells (2x10⁶) or IL-2 (200IU/ml) overnight. Thereafter, cells were washed with HBSS and stained with anti-CD56 (clone B159), anti-CD3 (clone SK7), and anti-ICAM-1 (clone RR1/1) mAbs. After electronic gating on the viable lymphocyte population and CD56⁺CD3⁻ NK cells ICAM-1 expression was analysed (MACS Quant Analyser).

FSC indicates forward-scattered light; rNK, resting NK cells; TpNK, tumour-primed NK cells with CTV-1; LAK-2,-7,-12 and -15, lymphokine-activated NK cell using IL-2, -7,-12 or -15; %#, percentage of NK cell population; *P <0.05; **P <0.005; ***P < 0.0005.
3.4.2.3 Co-activating receptors

In resting NK cells, co-engagement of several activating receptors is required for the triggering of natural cytotoxicity (Bryceson et al 2006; Bryceson et al. 2009). NKG2D expression by resting and primed NK cells is shown in Figure 3.8. Resting NK cells expressed high levels of the co-activating receptors NKG2D (mean, 92.4 ± 4.52%, n= 5), DNAM-1 (mean, 87.1 ± 5.3%, n=3), but little or no expression of 2B4 (mean, 0.98 ± 0.81%, n=3). Tumour-priming resulted in a significant down-regulation of NKG2D expression (mean decrease, 33.7 ± 2.7%, n= 5; p = 0.001) and non-significant downregulation of DNAM-1 expression (mean decrease, 11.5 ± 3.2%, n=3). Although cytokine-priming did not significantly affect NKG2D or DNAM-1 expression relative to resting NK cells, in most cases expression levels were similar or higher than those expressed by unstimulated NK cells. IL-2 and IL-15 resulted in a 4.3 ± 2.91 %, and 5.15 ± 1.12% increase in the number of NK cells expressing NKG2D, compared to resting NK cells, respectively. Although tumour-priming did not change 2B4 expression, cytokine-priming by IL-2 consistently increased the proportion of cells expressing 2B4 (mean increase, 23.5 ± 1.2%, n=3)
Figure 3.8 NKG2D expression by resting and primed NK cells

(A) Percentage of NKG2D⁺ NK cells; (B) representative FACS analysis of NKG2D⁺ NK cells from one donor. Freshly isolated resting NK cells (1x10⁶) were incubated alone or with CTV-1 cells (2x10⁶) or IL-2 (200IU/ml) overnight. Thereafter, cells were washed with HBSS and stained with anti-CD56 (clone B159), anti-CD3 (clone SK7), and anti-NKG2D (clone 1D11) mAbs. After electronic gating on the viable lymphocyte population and CD56⁺CD3⁺ NK cells NKG2D expression was analysed (MACS Quant Analyser).

FSC indicates forward-scattered light; rNK, resting NK cells; TpNK, tumour-primed NK cells with CTV-1; LAK-2,-7,-12 and-15, lymphokine-activated NK cell using IL-2, -7,-12 or -15; %#, percentage of NK cell population; *P <0.05; **P <0.005; ***P < 0.0005.
3.4.3 Homing receptors

Tumour-primed and cytokine-primed NK cells may traffic to distinct sites. Thus, following on from the investigation of the expression of CD62L (section 3.4.1.3), which serves as a differentiation marker as well as being involved in NK cell trafficking to lymph nodes (Frey et al. 1998), other receptors were investigated, including CD49d: extravasation (Berlin et al. 1995); CXCR4 and CXR7: homing to the bone marrow (Beider et al. 2003; Hartmann et al. 2008; Peled et al. 1999); α4β7 integrin: trafficking to the gut (Hamann et al. 1994; Kellersmann et al. 2002); and CXCR1: inflammatory sites (Maghazachi 2010). CXCR-1, -4 and -7 expression by resting and primed NK cells are shown in Figure 3.9. Tumour-primed NK cells upregulated CXCR1 (mean increase, 43.4 ± 9.25%, n=3) and CXCR7 (mean increase, 41.1 ± 3.45%, n=3) relative to resting NK cells. Resting NK cells constitutively expressed CXCR4 (mean, 88.7 ± 6.2%, n=3). Tumour-priming resulted in a significant reduction in CXCR4 expression (mean decrease, 54.6 ± 12.9%, n=3; p = 0.002). Similarly cytokine-priming by IL-2 (mean decrease, 84.05 ± 15.41%, n=3; p < 0.0001), IL-12 (mean decrease, 94.13 ± 7.86%, n=3; p <0.001) and IL-15 (mean decrease, 36.37 ± 2.32%, n=3; p = 0.001), but not IL-7 (mean decrease, xx ± xx%; p = 0.xx), resulted in the downregulation of CXCR4 expression. High levels of CD49d (mean, 99.88 ± 0.078%, n=3) and α4β7 (mean, 66.44 ± 4.37%, n=3) were expressed by resting NK cells. Neither tumour- nor cytokine-priming had any significant effect on the expression levels of CD49d (TpNK mean, 99.7 ± 0.32, n= 3; LAK-2 mean, 99.8 ± 0.11%, n=3; LAK-7 mean 99.9 ± 0.05 %, n=3; LAK-12 mean 99.8 ± 0.09 %, n=3; LAK-15 mean 99.7 ± 0.08%, n=3) or α4β7 (TpNK mean, 61.10 ± 5.2%, n= 3; LAK-2 mean, 69.08 ± 6.2 %, n=3; LAK-7 mean, 62.19 ± 4.34 %, n=3; LAK-12 mean, 69.67 ± 12.14 %, n=3; LAK-15 mean, 61.54 ± 8.73%, n=3).
Figure 3.9 CXCR-1, -4 and -7 expression by resting and primed NK cells

Freshly isolated resting NK cells (1x10^6) were incubated alone or with CTV-1 cells (2x10^6) or IL-2 (200IU/ml) overnight. Thereafter, cells were washed with HBSS and stained with anti-CD56 (clone B159), anti-CD3 (clone SK7), and anti-CXCR1 (clone 8F1/CXCR1), anti-CXCR4 (clone 12G5) or anti-CXCR7 (clone 8F11-M16) mAbs. After electronic gating on the viable lymphocyte population and CD56^+CD3^+ NK cells, CXCR-1, -4, and -7 expression was analysed (MACS Quant Analyser).

LAK-2,-7,-12 and -15 indicate lymphokine-activated NK cell using IL-2, -7,-12 or -15; rNK, resting NK cells; TpNK, tumour-primed NK cells with CTV-1; *P <0.05; **P <0.005; ***P < 0.0005
3.4.4 CD107a expression and different NK cell subsets

To investigate heterogeneity in NK cell responses associated with different phenotypes, the expression of CD107a, a degranulation marker (Alter et al. 2004), was used to analyse different subsets of resting, tumour-primed and cytokine-primed NK cells.

3.4.4.1 Correlation between CD107a expression and cytotoxicity

Firstly, correlation between NK cell cytotoxicity and CD107a expression was confirmed. Resting or primed NK cells were incubated with NK-resistant RAJI target cells in a 3h assay, in the presence of PE-conjugated anti-CD107a mAbs, with monensin added an hour after the start of incubation. Figure 3.10 shows that upregulation of CD107a expression correlated with an increase in killing capacity observed by primed NK cells (r=0.5, p=NS).
Figure 3.10 Correlation between NK cell cytotoxicity and CD107a expression

NK cell cytotoxicity against NK-resistant RAJI cells was measured by pre-labelling the target cells with PKH-67 (FITC) and co-incubating them in a 3 hr assay at 37°C. CD107a PE was added at the start of the assay and monensin was added after 1hr of incubation with target cells. After 3 h, the samples were washed and stained with anti-CD56 and CD3 mAbs. TO-PRO (APC) was added before acquisition of samples and percentage lysis was calculated after subtracting the value for the RAJI + no NK cells negative control. Upregulation of CD107a expression in primed NK cells correlated with acquired capacity for RAJI killing by primed NK cells.

LAK-2 indicates lymphokine-activated NK cell using IL-2; rNK, resting NK cells; TpNK, tumour-primed NK cells.
3.4.4.2 CD107a expression in resting and primed NK cells

Figure 3.11 shows that resting NK cells incubated with NK-sensitive K562 cells displayed higher CD107a expression (mean, 6.41 ± 0.14%, n=3) than NK cells incubated without target cells (mean, 0.33 ± 0.12%, n=3), or with NK-resistant RAJI cells (mean, 1.66 ± 0.47%, n=3). Tumour-primed NK cells and NK cells primed with IL-2 showed higher CD107a expression levels in a 3h cytotoxicity assay against RAJI cells, compared with resting NK cells (TpNK mean, 9.58 ± 1.55, n=3; LAK-2 mean, 6.79 ± 2.43, n=3).
Figure 3.11 CD107a expression in resting and primed NK cells

Freshly isolated resting NK cells were incubated alone or with tumour target cells after tumour- or cytokine-priming. (A) Co-incubation with NK-sensitive K562 tumour targets induces degranulation and upregulation of CD107a in NK cells compared with resting NK cells incubated without targets (B) NK cells primed with CTV-1 or IL-2, but not resting NK cells, degranulate after exposure to NK-resistant RAJI cells in a 4h assay, displaying elevated levels of CD107a. Data presented from FACS analysis of cells from one representative donor.

FSC indicates forward-scattered light; LAK-2, lymphokine-activated NK cell using IL-2; rNK, resting NK cells; TpNK, tumour-primed NK cells using CTV-1 cells.
3.4.4.3 CD107a expression in different NK cell subsets

CD107a expression was low in CD56\textsuperscript{bright} NK cells (mean, 0.91 ±0.57\%, n=3) (Figure 3.12), in accordance with previously published data (Grzywacz et al. 2007) in a degranulation assay against NK-sensitive K562 cells. Also consistent with previous findings, CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cells expressed higher levels of CD107a (mean, 12.91 ± 5.4\%, n=3) than CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells (mean, 1.71 ± 0.7\%, n=3) against K562 cells. Since resting CD56\textsuperscript{dim} NK cells shed CD16 in response to tumour-priming, CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells were positively selected before co-incubation with CTV-1 cells, and CD107a expression was analysed after co-incubation with NK-resistant RAJI cells. The CD56\textsuperscript{dim}CD16\textsuperscript{−} phenotype that emerged following tumour-priming, confirmed that the CD56\textsuperscript{dim}CD16\textsuperscript{+} subset is the source of NK cells with reduced CD16 expression and higher CD107a levels (mean, 13.91 ± 1.05, n=3) (Figure 3.13).
Figure 3.12 CD107a expression in different NK cell subsets

Freshly isolated NK cells from a normal healthy donor were co-incubated with K562 target cells (effector:target ratio, 2:1). NK cell subpopulations were defined by surface expression density of CD56 and CD16 (A). CD56<sup>dim</sup>CD16<sup>+</sup> NK cells (B) and CD56<sup>bright</sup> NK cells (D) showed little or no degranulation in response to target cell stimulation, whereas CD56<sup>dim</sup>CD16<sup>+</sup> NK cells displayed higher degranulation and expression of CD107 (C). FSC indicates forward scatter.
Figure 3.13 CD107a expression in different subsets of tumour-primed NK cells

Freshly isolated PBMCs from a normal healthy donor were positively selected for CD56dimCD16+ expression (1x10^6) and incubated with CTV-1 cells (2x10^6) overnight for priming. Immunophenotyping for surface expression of CD56 and CD16 was carried out pre- and post-sorting, as well as post tumour-priming the following day to verify CD16 shedding (A). The CD56dimCD16+ NK cell subpopulation that emerged following stimulation with CTV-1, expressed higher levels of CD107a than its CD56dimCD16+ counterpart in a 3h degranulation assay against NK-resistant RAJI cells.

FSC indicates forward scatter.
3.5 Discussion

The NK cell development model presented by Freud et al. (Freud et al. 2006; Freud and Caligiuri 2006) proposes that NK cells exhibit different maturational stages with distinct NK cell populations progressing from CD34⁺CD45RA⁺ HPCs to CD3⁻CD56bright NK cells. After further linear progression from CD56bright to CD56dim NK cells, CD56dimCD62L⁺ NK cells appear as an intermediary stage (Juelke et al. 2010), before CD62L expression is lost and expression of the terminal differentiation marker CD57 is acquired. The resulting CD56dimCD62L⁻CD57⁺ NK cell subpopulation is known to display high cytotoxicity against tumour targets and have a decreased proliferative capacity (Lopez-verges et al. 2010). In this Chapter, the antigen expression profile of tumour-primed NK cells was compared with that of cytokine-primed NK cells. Tumour-priming generated an increase in the overall number of CD56dimCD62L⁻CD57⁺ NK cells, supporting its role in promoting NK cell maturation toward a phenotype with enhanced effector functions.

The expansion of CD56bright NK cells was observed in response to cytokine- but not tumour-priming. Although this observation has been commonly reported in various conditions, such as during the reconstitution of the immune system after bone marrow transplantation or in patients who are treated with a daily dose of IL-2 (Carson and Caligiuri 1996), many questions remain unanswered regarding the mechanism of expansion. CD56bright cells have been postulated to play an important regulatory role by virtue of their ability to produce ample amounts of cytokines that modulate adaptive immune responses. It is possible that CD56bright cells are released in high numbers to serve as precursors for CD56dim NK cells, which have a high turnover. It is also possible that CD56bright NK cells are selectively expanded in response to certain stimuli, because of their distinct regulatory role involving cytokine production. Thus, the expansion of CD56bright NK cells observed here might be a specific NK cell response to cytokine stimulation. Of the four cytokines tested (IL-2, IL-7, IL-12 and IL-15), IL-12 was the only cytokine tested that resulted in expansion of the CD56bright population. Previous studies demonstrating selective
expansion of the CD56\textsuperscript{bright} sub-population in response to cytokine stimulation used combinations of IL-2, IL-12 and IL-15 (Takahashi et al. 2007). It is possible that the stimulating conditions used here, with exposure to individual cytokines over 20 hours was not strong enough to induce this effect.

Interestingly, the downregulation of CD16 in response to tumour-priming was only observed in the CD56\textsuperscript{dim} NK cell population. In contrast, CD56\textsuperscript{bright} NK cells responded to both cytokine- and tumour-priming by upregulating CD16 expression. This suggests that both priming mechanisms promote the progression of CD56\textsuperscript{bright}CD16\textsuperscript{−} NK cells towards the more mature CD56\textsuperscript{dim}CD16\textsuperscript{+} phenotype. NK cells that are further along the differentiation path, then go on to downregulate CD16 expression upon stimulation with CTV-1. This CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cell subpopulation was demonstrated in this Chapter to be the main subset responsible for cytotoxicity against tumour target cells, as measured by expression of the degranulation marker CD107a. When Penack et al. first introduced the CD107a assay as a method to investigate NK cell subsets responsible for target cell lysis, they reported CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cells as the main degranulating subset, following co-incubation with tumor target cells (Penack et al. 2005). This led to the conclusion that this NK cell subset was exclusively responsible for cytotoxicity against target cells given that other NK cell subsets (CD56\textsuperscript{bright}CD16\textsuperscript{−/+} and CD56\textsuperscript{dim}CD16\textsuperscript{+}) did not express CD107a, which seemed at odds with previous studies reporting CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells as the main cytotoxic subset in NK cells (Nagler et al. 1989). Zimmer et al. subsequently challenged this conclusion, by questioning the method of NK cell isolation used, and arguing that without careful exclusion of CD3\textsuperscript{+} cells, contamination by NKT cells (CD56\textsuperscript{+}CD3\textsuperscript{+}) is likely to account for this observation. Zimmer’s reasoning was that the majority of CD56\textsuperscript{dim}CD16\textsuperscript{−} cells in peripheral blood are CD3\textsuperscript{+} T cells, and it is these cells that are responsible for the observed cytotoxic activity (Zimmer et al. 2005). Following on from this study, Grzywacz et al. (Grzywacz et al. 2007) investigated the true nature of the CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cell population reported in these studies, by employing various NK cell purification methods, ensuring exclusion of CD3\textsuperscript{+} cells. Their results
confirmed that the contribution of CD56\text{dim}CD16^- cells to NK cytotoxicity is marginal. Upon culture with K562 cells however, a fraction of CD56\text{dim}CD16^- NK cells emerged, expressing CD107a. To confirm that the CD56\text{dim}CD16^+ subset was the source of cells with reduced CD16 expression, purified CD56^+CD16^+ cells were cultured with or without target K562 cells. In the absence of targets, these cells did not express CD107a, and retained CD16 expression. However, following co-culture with K562 cells, a proportion of CD56^+CD16^+ NK cells gained CD107a and downregulated CD16 expression. This finding was replicated in this Chapter in order to demonstrate that CD56\text{dim}CD16^+ NK cells shed CD16 upon target cell stimulation with CTV-1. The CD56\text{dim}CD16^- population that emerges following this exposure to CTV-1 tumour target cells is the main degranulating population upon exposure to subsequent targets, as demonstrated by tumour-primed NK cells in a cytotoxicity assay against NK-resistant RAJI cells. In accordance with other studies, CD107a expression wasn’t limited to the CD56\text{dim} subset, but CD107a expression was much lower (almost negligible in most cases) on CD56\text{bright} NK cells. Collectively, my results are aligned with Nagler \textit{et al.}, Penack \textit{et al.}, Grzywacz \textit{et al.} as well as previous work done by our group (North \textit{et al.} 2007; Sabry \textit{et al.} 2011), supporting the assertion that CD56\text{dim}CD16^+ NK cells are the main cytotoxic population in PB.

It has been postulated that the loss of CD16 expression in precursor CD56\text{dim}CD16^+ NK cells is probably mediated by matrix metalloproteases (MMPs) (Juelke \textit{et al.} 2010). A disintegrin and metalloprotease-17 (ADAM-17) was recently shown to specifically induce downregulation of CD16 and CD62L expression upon NK cell stimulation. The results in this Chapter also show downregulation of CD62L expression in response to both tumour- and cytokine-priming. The combined loss of CD16 and CD62L in response to CTV-1 stimulation was interpreted here as evidence for the dual role of tumour-priming in the promotion of NK cell progression toward a more mature differentiation stage and enhanced activation status. Loss of both those receptors may indeed play an important role in the synergistic release of molecules such as CD3-\zeta or other signaling proteins downstream from metalloproteases activation that result in better NK cell effector function. When Romee \textit{et al.}
investigated the expression of other NK cell activating receptors, including NCRs, CD2, DNAM-1, NKG2D and CD57, none of these were found to be downregulated following cytokine stimulation (Romee et al. 2013). The results presented here show that many of these activating receptors like are uniquely downregulated in response to CTV-1, which is suggestive of a highly specific response to target cell stimulation and perhaps priming with CTV-1. It is possible that receptors such as NKp46 or NKG2D are involved only in the priming stage of NK cell activation, and are thus shed following stimulation with CTV-1 and completion of priming.

Another NK cell activating receptor that is likely to be involved in the delivery of the tumour-priming signal is LFA-1. In addition to acting as a central mediator of NK cell adhesion to target cells, LFA-1 also plays an important role in the delivery of early signals in NK cell cytotoxicity (Barber et al. 2004). Moreover, it was recently identified as one of the signalling components that define the minimal requirements of NK cell-mediated natural cytotoxicity (Bryceson et al. 2009). LFA-1 binding induces changes in conformation and spatial distribution, such that receptor affinity and avidity are modified (Kim et al. 2004). Signals that enhance receptor affinity by inducing an open conformation in LFA-1 are known as ‘inside-out’ signals. Antibodies which selectively bind to open conformations of LFA-1 are not commercially available. Thus, although LFA-1 was observed here to be expressed at uniformly high levels on resting NK cells, with no change following tumour- or cytokine-mediated priming, it is likely that the conformation-independent mAbs used are a limitation of the experiments described in this Chapter. Further studies investigating the role of LFA-1 in tumour-mediated priming are explored in later chapters of this Thesis.

In conclusion, both tumour- and cytokine-priming led to a more mature NK cell profile, which is likely to be a requirement for enhanced effector functions. Both priming mechanisms also resulted in similar homing signatures and trafficking towards pro-inflammatory sites. Notable differences between the two stimulation profiles were observed, however, in the observed downregulation of important NK
cell activating receptors including CD16, NKG2D, NKp46, DNAM-1 and ICAM-1 by tumour-priming. The observation that tumour-primed NK cells exhibit enhanced effector functions, despite the loss of these important activating receptors, argues against NK cell impairment or loss of function. It is possible that the shedding of these receptors is an integral part of the mechanism of target cell stimulation of NK cell effector functions. It is also possible that these receptors are shed following stimulation to complete the priming stage of NK cell activation, with other receptors being involved in the ‘triggering’ stages. Different ligand combinations presented to NK cells through sensitive or insensitive targets are likely to induce different responses in NK cells. The secretory profiles of NK cells stimulated with different cytokines are compared in the next Chapter, to get a better understanding of the full picture of NK cell responses to different stimuli.
Chapter 4  Secretory profiles of tumour-primed NK cells

4.1 Introduction

NK cells were originally defined by their ability to lyse tumour cells ‘naturally’ without prior sensitisation, but it was later recognised that NK cell effector functions include both cytokine production and cytotoxicity (Vivier et al. 2008). NK cell responses are influenced by their cytokine microenvironment and interactions with stressed cells undergoing a pathogen infection or malignant transformation. Exposure to cytokines and other immune cells, such as DCs during inflammation can result in non-specific priming of NK cells, and the generation of more potent effector functions (Lucas et al. 2007). Target cells can also prime NK cells for greater effector potential through direct engagement of NK cell activating receptors (North et al. 2007). A variety of different priming mechanisms can trigger the secretion of a plethora of cytokines, which can eliminate target cells, relay the activation signals, and recruit other immune cells for a more directed and efficient immune response.

Factors released by NK cells have different activation thresholds and regulatory effects on the immune response. NK cells produce chemokines, such as MIP-1α and MIP-1β, RANTES, MCP-1, monokine induced by IFN-γ (MIG), eotaxin and IFN-γ-induced protein (IP)-10 (Cuturi et al. 1989; Roda et al. 2006). These chemokines have a lower stimulation threshold for their release compared with other cytokines secreted by NK cells and play a pivotal role in the recruitment of other immune cells to the target tissue (Bryceson et al. 2009; Fauriat et al. 2010a). Other prominent cytokines secreted by NK cells include TNF-α and IFN-γ, which have greater stimulatory requirements relative to chemokines, and contribute to host inflammatory responses (Bryceson et al. 2006a; Fauriat et al. 2010). Pro-inflammatory cytokines, such as IL-1, IL-6, IL-8 and IL-12 promote Th1 responses and stimulate the activation and proliferation of cytotoxic T cells, DCs and macrophages; whereas anti-inflammatory cytokines, such as IL-4 and IL-10, promote Th2 immune responses and humoral immunity, but suppress T cell and macrophage function (Elenkov and Chrousos 1999; Elenkov and Chrousos 2002). The balance between
Th1/Th2 and pro-/anti-inflammatory cytokines in the NK-target cell microenvironment can thus modulate immune responses toward activation or suppression, and influence the progression of disease.

NK cell responses to target cell stimulation vary according to the degree and combination of ligand expression on target cells for NK cell activating receptors (Bryceson et al. 2006; Bryceson et al. 2009). NK-sensitive tumour target cells like K562 provide ligands that engage NK cell activating receptors through the previously described priming and triggering stages (North et al. 2007), resulting in NK cell lysis. The NK-resistant CTV-1 tumour cell line has a markedly different ligand expression profile from that of K562 cells; it provides the NK cell priming signal only, without triggering cytotoxicity (North et al. 2007). The secretory responses of resting NK cells stimulated with K562 were recently reported (Fauriat et al. 2010) and NK cell responses to exogenous cytokines have been extensively studied (Fehniger et al. 1999). In Chapter 3, I showed that tumour-priming of resting NK cells through co-incubation with CTV-1 generates an NK cell antigen expression profile that is distinct from exogenous cytokine stimulation. In this Chapter, the effect of CTV-1 target cell stimulation on NK cell cytokine secretory responses was investigated and compared with the previously reported effects of K562 and exogenous cytokine stimulation.
4.2 Experimental Aims

The aim of this chapter was to define the cytokine secretion profiles of NK cells stimulated with NK-sensitive K562 cells, NK-resistant CTV-1 tumour cells, and exogenous cytokines IL-2, IL-7, IL-12, IL-15 and IL-21. The kinetics of cytokine secretion by NK cells were studied by quantifying cytokine secretion levels at 6 hours and 16 hours post-stimulation.

4.3 Methods

Human NK cells were isolated from the PBMCs of normal healthy donors by magnetic sorting for CD56+ cells. Resting NK cells (2 x 10^5) were washed twice and mixed with CTV-1 or K562 cells (4 x 10^5), IL-2 (200IU/ml) IL-7, IL-15, IL-21 (10 ng/ml), or IL-12 (20 ng/ml) for 16 hours at 37ºC, 5 % CO₂ in CM. Thereafter, supernatants were collected and stored at -80ºC pending measurement. The concentrations of cytokines were quantified by a multiplex immunoassay kit (Luminex 100 IS; Invitrogen) that detected 25 cytokines grouped into 4 different panels: pro-inflammatory (IL-1β, IL-1RA, IL-6, IL-8, GM-CSF and TNF-α), Th1/Th2 (IL-2, IL-2Rα, IL-4, IL-5, IL-10, IFN-γ), cytokines II (IL-7, IL-12p40, IL-13, IL15, IL-17 and IFN-α) and chemokines (eotaxin, IP-10, MCP-1, MIG, MIP-1α and MIP-1β). Statistical comparisons between pairs of unstimulated-stimulated NK cells were tested for significant differences in their means by multiple t test, corrected for multiple comparisons with the Holm-Sidak method. Results are presented as mean ± SD. Corrected p values < 0.05 were considered statistically significant. Unless stated otherwise, p values are indicative of significant difference relative to unstimulated NK cells at the same time point. Blank control values were subtracted from all samples before interpolation of X values (secretion at pg/ml) from absorbance. In the case of target cell stimulated NK cells, values of (blank controls + target cells incubated alone) were subtracted from samples before interpolation. Statistical analyses were performed with GraphPad Prism v.6 software.
4.4 Results

4.4.1 Inflammatory cytokines

Levels of the inflammatory cytokines IL-1β, IL-1 receptor antagonist (IL-1RA), IL-6, IL-8, granulocyte macrophage colony-stimulating factor (GM-CSF) and TNF-α secreted by resting, target cell-stimulated and cytokine-stimulated NK cells are shown in Figures 4.1 and 4.2, respectively. Secretion levels of inflammatory cytokines produced by CTV-1 and K562 target cells are shown in Figure 4.3. Inflammatory cytokine secretion by NK cells after 6 hours and 16 hours of stimulation are shown in Figure 4.4.

4.4.1.1 IL-1β

Resting NK cells produced low levels of the pro-inflammatory cytokine IL-1β, (mean at 6h, 6.03 ± 2.9 pg/ml; n = 4) (mean at 16h, 12.08 ± 1.8 pg/ml; n = 4). Target cell stimulation with CTV-1 or K562 cells for 6 hours induced a 7-fold (mean, 43.95 ± 28.36 pg/ml; n=4) and 4-fold (mean, 21.99 ± 3.53 pg/ml; n=4) increase in IL-1β levels, respectively, compared with resting NK cells. Overnight incubation with K562 cells resulted in an 8-fold increase in IL-1β (mean, 38.27 ± 15.29 pg/ml; n=3) compared with resting NK cells. By contrast, NK cells incubated with CTV-1 cells overnight secreted lower levels of IL-1β (mean, 16.44 ± 24.94 pg/ml; n=3) than those observed after a 6-hour incubation. Stimulation with IL-2 for 6 hours generated a 7-fold increase in IL-1β production (mean, 40.69 ± 40.56 pg/ml; n=3) compared with resting NK cells, and IL-2 levels increased a further 5-fold (mean, 192.1 ± 211.1 pg/ml; n=3) after overnight incubation. Overnight incubation with the activating cytokines IL-7, IL-12, or IL-15 induced a 2-fold (mean, 19.62 ± 25.47 pg/ml; n=3), 12-fold (mean, 142.81 ± 165.6 pg/ml; n=3) and 2-fold (mean, 22.56 ± 32.40 pg/ml; n=3) increase in IL-1β production, respectively, compared with resting NK cells. Stimulation with IL-21 showed no difference from resting NK cell secretion levels (mean, 14.77 ± 12.35 pg/ml; n=3). IL-1β was not detected in supernatants harvested from CTV-1 or K562 cells incubated alone.
4.4.1.2 IL-1RA

Low to intermediate levels of IL-1 receptor antagonist (IL-1RA), a natural inhibitor of the pro-inflammatory effect of IL-1β (Perrier et al. 2006) were secreted by resting NK cells (mean at 6h, 6.4 ± 2.3 pg/ml; n = 4) (mean at 16h, 43.21 ± 43.01 pg/ml; n = 4). Stimulation with CTV-1 or K562 induced a 7-fold increase (mean, 45.12 ± 45.48 pg/ml; n=4, and 43.30 ± 10.37 pg/ml; n=4, respectively) in IL-1RA production after 6 hours, compared with resting NK cells. Overnight incubation with K562 cells increased IL-1RA secretion 2-fold (mean, 88.75 ± 26.01 pg/ml; n=4), compared with resting NK cells. Conversely, overnight incubation with CTV-1 cells resulted in lower levels of IL-1RA secretion (mean, 35.75 ± 25.32 pg/ml; n=4) than those detected after a 6-hour incubation. IL-2 stimulation resulted in a 4-fold increase in IL-1RA secretion after 6 hours of incubation with NK cells (mean, 26.98 ± 23.07 pg/ml; n=4), which further increased 4-fold after overnight incubation (mean, 108.9 ± 78.95 pg/ml; n=3). An overnight incubation with IL-7, IL-12, IL-15 or IL-21 induced a 3-fold (mean, 142.5 ± 61.75 pg/ml; n=3), 4-fold (mean, 171.3 ± 127.4 pg/ml; n=3), 3-fold (mean, 119.2 ± 116.8 pg/ml; n=3), and 2-fold (mean, 95.63 ± 47.21 pg/ml; n=3) increase in IL-1RA production, respectively, compared with resting NK cells. K562 and CTV-1 cells incubated alone secreted low levels of IL-1RA (mean, 2.25 ± 1.3 pg/ml; n=3, and mean, 3.92 ± 8.2 pg/ml; n=3).

4.4.1.3 IL-6

Moderate levels of IL-6 were secreted by resting NK cells (mean at 6h, 25.7 ± 11.7 pg/ml, n = 4) (mean at 16h, 157.8 ± 148.6 pg/ml, n = 4). Stimulation with CTV-1 or K562 cells for 6 hours induced a 2-fold (mean, 41.86 ± 31.33 pg/ml; n=4) and 3-fold (mean, 83.15 ± 20.98 pg/ml; n=4) increase in IL-6 secretion compared with resting NK cells, respectively. These levels increased a further 2-fold (mean, 67.07± 46.73 pg/ml; n=3) and 3-fold (mean, 247.7 ± 111.1 pg/ml; n=3) after overnight incubation with CTV-1 or K562 cells, respectively. IL-6 levels detected in supernatants from NK cells stimulated with IL-2 for 6 hours were 4-fold higher (mean, 95.55 ± 102.9 pg/ml; n=3) than resting NK cells incubated alone. Overnight incubation with IL-2
increased IL-6 levels a further 9-fold (mean, 868 ± 976.4 pg/ml; n=3) after overnight incubation. NK cells incubated with IL-7, IL-12, IL-15 or IL-21 overnight showed a 4-fold (mean, 582.9 ± 769 pg/ml; n=3), 4-fold (mean, 665.4 ± 767.6 pg/ml; n=3), 3-fold (mean, 478.1 ± 715.5 pg/ml; n=3) and 2-fold (mean, 346.3 ± 421.2 pg/ml; n=3) increase in IL-6 secretion compared with resting NK cells, respectively. Supernatants from CTV-1 cells incubated alone did not show detectable levels of IL-6, whereas K562 cells secreted low levels of IL-6 (mean, 12.60 ± 2.29 pg/ml; n=3).

4.4.1.4 IL-8

Resting NK cells incubated without target cells or exogenous cytokines produced high amounts of IL-8 (mean at 6h, 215.09 ± 100.3 pg/ml; n = 4) (mean at 16h, 4171 ± 5347 pg/ml; n = 4). After 6 hours of stimulation, CTV-1 increased IL-8 production 3-fold (mean, 624.4 ± 467.5 pg/ml; n=4, p=0.0002), and K562 increased IL-8 production 12-fold (mean, 2570 ± 365.9 pg/ml; n=4, p<0.0001), compared with resting NK cells. IL-8 levels detected in supernatants from NK cells stimulated with IL-2 for 6 hours were 3-fold higher (mean 675.8 ± 630.8 pg/ml; n=3, p=0.001) than resting NK cells incubated alone. Overnight stimulation with K562, IL-7 or IL-21 resulted in IL-8 levels that were 3-fold (mean, 11509 ± 5799 pg/ml; n=3, p<0.0001), 3-fold (mean, 10956 ± 3006 pg/ml; n=3, p<0.0001) and 2-fold (mean, 8189 ± 4736 pg/ml; n=3, p=0.0067) higher than resting NK cells, respectively, whereas overnight incubation with CTV-1, IL-2, IL-12 or IL-15 resulted in the secretion of IL-8 at levels that were equal to or lower than those secreted by resting NK cells. Both CTV-1 and K562 cells secreted low levels of IL-8 (mean, 58.88 ± 33.24 pg/ml, n=3 and mean, 207.1 ± 37.96 pg/ml, n=3, respectively).
4.4.1.5 GM-CSF

GM-CSF was not detected in supernatants collected from resting or target cell-stimulated NK cells. Exogenous cytokine stimulation by IL-2 (mean, 73.45 ± 81.60 pg/ml; n=3), IL-7 (mean, 9.767 ± 14.24 pg/ml; n=3), IL-12 (mean, 33.12 ± 39.98 pg/ml; n=3) and IL-15 (mean, 78.42 ± 129.8 pg/ml; n=3), but not IL-21 induced GM-CSF secretion by NK cells after overnight incubation. GM-CSF was not detected in supernatants from CTV-1 or K562 cells incubated alone.

4.4.1.6 TNF-α

Trace amounts of TNF-α were detected in supernatants from resting NK cells incubated alone (mean at 6h, 1.92 ± 0.899 pg/ml; n = 6) (mean at 16h, 7.38 ± 4.7pg/ml; n = 6). Target cell stimulation for 6 hours with CTV-1 or K562 induced a 24-fold (mean, 46.51 ± 48.52 pg/ml; n=4) and 4-fold (mean, 8.231± 4.67 pg/ml; n=4) increase in TNF-α production compared with resting NK cells, respectively. Overnight incubation with CTV-1 resulted in lower levels of TNF-α (mean, 8.361 ± 15.03 pg/ml; n=3), than levels observed after a 6-hour incubation with the leukemic cell line. Conversely, NK cells co-incubated with K562 cells overnight showed a 2-fold increase (mean, 13.67 ± 3.284 pg/ml; n=3) in TNF-α production compared with that collected after 6 hours. IL-2 stimulated NK cells secreted moderate levels of (mean, 24.74 ± 33.57pg/ml; n=4) and higher levels after incubation for 16 hours (mean, 75.01 ± 55.57 pg/ml; n=6). Overnight stimulation with other exogenous cytokines lead to the secretion of moderate to high levels of TNF-α (LAK-7 mean, 58.95 ± 36.58; n=3; LAK-12 mean, 125.9 ± 146.38; n=3; LAK-15 mean, 51.25 ± 73.99; n=3). CTV-1 cells, but not K562 cells were shown to secrete low levels of TNF-α (mean, 2.821 ± 6.41 pg/ml; n=3).
Figure 4.1 Secretion of inflammatory cytokines by resting and target cell-stimulated NK cells

Freshly isolated resting NK cells (2 x 10⁵) were incubated alone, with CTV-1 (4 x 10⁵), or K562 (4 x 10⁵) target cells in 200 μl CM. After a 6-hour incubation at 37°C and 5% CO₂, supernatants were harvested and the cytokines IL-1β, IL-1Rα, IL-6, IL-8, GM-CSF and TNF-α were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 donors, p values < 0.05 were considered statistically significant and are indicative of significant difference relative to unstimulated NK cells at the same time point.

rNK indicates resting NK cells; *P<0.05; **P<0.005; ***P < 0.0005.
Resting NK cells (2 x 10^5) were washed twice and mixed with IL-2 (200IU/ml) IL-7, IL-15, IL-21 (10 ng/ml), or IL-12 (20 ng/ml). After a 16-hour incubation at 37°C, 5 % CO_2 in CM, supernatants were harvested and the cytokines IL-1β, IL-1Rα, IL-6, IL-8, GM-CSF and TNF-α were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 donors, p values < 0.05 were considered statistically significant and are indicative of significant difference relative to unstimulated NK cells at the same time point.

LAK-2, -7, -12 and -15 indicate lymphokine-activated NK cells using IL-2, -7, -12 or -15; *P<0.05; **P<0.005; ***P < 0.0005.

Figure 4.2 Secretion of inflammatory cytokines by cytokine-stimulated NK cells.
Figure 4.3 Secretion of inflammatory cytokines by tumour cells.

CTV-1 or K562 cells (2 x $10^5$ in 200 μl CM) were incubated at 37°C and 5% CO₂ for 16 hours. Supernatants were harvested and the cytokines IL-1β, IL-1Ra, IL-6, IL-8, GM-CSF and TNF-α were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 replicates.
Figure 4.4 Kinetics of NK cell secretion of inflammatory cytokines upon stimulation with CTV-1, K562 or IL-2.

Freshly isolated resting NK cells (2 x 10^5) were incubated with CTV-1 (4 x 10^5), K562 (4 x 10^5) or IL-2 (200IU/ml) in 200 μl CM at 37°C and 5% CO₂. Supernatants were harvested at 6 hours and 16 hours and the concentrations of IL-1β, IL-1RA, IL-6, IL-8, GM-CSF and TNF-α were determined by a multiplex immunoassay. Values represent the mean of 3–6 donors.
4.4.2 Th1/Th2 cytokines

Levels of Th1/Th2 cytokines (IL-2, IL-2R, IL-4, IL-5, IL-10, IFN-γ) secreted by resting, target cell-stimulated and cytokine-stimulated NK cells are shown in Figures 4.5 and 4.6, respectively. Th1/Th2 cytokine secretion levels of CTV-1 and K562 target cells are shown in Figure 4.7. Th1/Th2 cytokine secretion by NK cells after 6 hours and 16 hours of stimulation are shown in Figure 4.8.

4.4.2.1 IFN-γ

Resting NK cells did not secrete detectable levels of IFN-γ after 6 hours of incubation alone and secreted trace amounts after overnight incubation (mean, 1.72 ± 0.8 pg/ml; n = 4). Short incubations with the target cell lines CTV-1 or K562 induced the release of low amounts of IFN-γ production (mean, 11.61 ± 13.64 pg/ml; n=4) and (mean, 1.6 ± 1.3 pg/ml; n=4), respectively. Overnight incubation with K562 led to a slight increase in IFN-γ levels (mean, 2.3 ± 1.72 pg/ml; n=3), whereas co-incubation with CTV-1 overnight did not (mean, 6.7 ± 9.19 pg/ml; n=3). IL-2 stimulation for 6 hours induced a 60-fold increase in IFN-γ secretion (mean, 68.8 ± 80.34 pg/ml; n=3) compared with resting NK cells, which increased a further 15-fold after an overnight incubation (mean 928.6, ± 559.9 pg/ml; n=3, p<0.0001). Overnight exposure to the cytokines IL-7, IL-12, IL-15 or IL-21 induced a 25-fold (mean, 43.1 ± 67.7 pg/ml; n=3), 300-fold (mean, 513.2 ± 609.5 pg/ml; n=3, p=0.0001), 326-fold (mean, 554.7 ± 822.2 pg/ml; n=3, p=0.001) and 7-fold (mean, 12.5 ± 14.33 pg/ml; n=3) increase in IFN-γ production compared with resting NK cells, respectively. CTV-1 and K562 cells secreted undetectable levels of IFN-γ.

4.4.2.2 IL-2R

IL-2R was weakly secreted by resting NK cells (mean, 0.64 ± 2.4 pg/ml; n = 4). After 6 hours of co-incubation with CTV-1, IL-2R levels were significantly higher than levels detected in supernatants from resting NK cells (mean, 65.35 ± 80.42 pg/ml; n=4, p=0.001). Overnight incubation with CTV-1 cells resulted in lower levels of IL-2R compared with that in supernatants harvested after 6 hours (mean,
52.05 ± 35.07 pg/ml; n=4). Co-incubation with K562 cells induced a moderate increase in IL-2R production after 6 hours compared with resting NK cells (mean, 13.33 ± 1.35 pg/ml; n=4), and secretion levels further increased 6-fold after overnight incubation (mean, 82.61 ± 35.91 pg/ml; n=3). Similarly, IL-2 stimulation increased IL-2Ra production 33-fold after 6 hours compared with resting NK cells (mean, 33.48 ± 41.45 pg/ml; n=3, p=0.006), and levels detected after overnight incubation were a further 4-fold higher (mean, 147.16 ± 130.7 pg/ml; n=3). The activating cytokines IL-7, IL-12, IL-15 and IL-21 resulted in a 4-fold (mean, 129.38 ± 140.7 pg/ml; n=3, p=0.002), 6-fold (mean, 182.11 ± 217.6 pg/ml; n=3), 4-fold (mean, 121.31± 156.4 pg/ml; n=3) and 3-fold (mean, 81.85 ± 78.94pg/ml; n=3, p=0.004) increase in IL-2R secretion after overnight incubation, respectively, compared with resting NK cells. Supernatants collected from CTV-1 and K562 cells did not contain detectable amounts of IL-2R.

4.4.2.3 IL-2, IL-4, IL-5 and IL-10

IL-2, IL-4 and IL-5 were secreted at undetectable levels by resting NK cells, target cell-stimulated or cytokine-stimulated NK cells. IL-10 secretion by resting NK cells and target cell-stimulated NK cells was undetectable. However, low levels of IL-10 were detected in supernatants from NK cells incubated with the activating cytokines IL-2 (mean, 19.35 ± 2.15 pg/ml; n=3), IL-7 (mean, 8.89 ± 2.78 pg/ml; n=3), IL-12 (mean, 12.93 ± 5.09 pg/ml; n=3), IL-15 (mean, 6.87 ± 1.51 pg/ml; n=3), and IL-21 (mean, 6.26 ± 1.55 pg/ml; n=3). The target cells CTV-1 and K562 did not secrete detectable levels of the cytokines IL-2, IL-4, IL-5 or IL-10.
Figure 4.5 Secretion of Th1/Th2 cytokines by resting and target cell-stimulated NK cells.

Freshly isolated resting NK cells (2 x 10^5) were incubated alone, with CTV-1 (4 x 10^5), or K562 (4 x 10^5) target cells in 200 μl CM. After a 6-hour incubation at 37°C and 5% CO_2, supernatants were harvested and the cytokines IFN-γ, IL-2, IL-2R, IL-4, IL-5, and IL-10 were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 donors, p values < 0.05 were considered statistically significant and are indicative of significant difference relative to unstimulated NK cells at the same time point.

rNK indicates resting NK cells; *P<0.05; **P<0.005; ***P < 0.0005.
Figure 4.6 Secretion of Th1/Th2 cytokines by cytokine-stimulated NK cells.

Resting NK cells (2 x 10^5) were washed twice and mixed with IL-2 (200IU/ml) IL-7, IL-15, IL-21 (10 ng/ml), or IL-12 (20 ng/ml). After a 16-hour incubation at 37°C, 5 % CO\textsubscript{2} in CM, supernatants were harvested and the cytokines IFN-γ, IL-2, IL-2R, IL-4, IL-5, and IL-10 were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 donors, p values < 0.05 were considered statistically significant and are indicative of significant difference relative to unstimulated NK cells at the same time point.

LAK-2, -7, -12 and -15 indicate lymphokine-activated NK cells using IL-2, -7, -12 or -15; *P<0.05; **P<0.005; ***P < 0.0005.
Figure 4.7 Secretion of Th1/Th2 cytokines by target cells.

CTV-1 or K562 cells (2 x 10^5 in 200 μl CM) were incubated at 37°C and 5% CO₂ for 16 hours. Supernatants were harvested and the cytokines IFN-γ, IL-2, IL-2R, IL-4, IL-5 and IL-10 were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 replicates.
Figure Kinetics of NK cell secretion of Th1/Th2 cytokines upon stimulation with CTV-1, K562 or IL-2.

Freshly isolated resting NK cells (2 x 10^5) were incubated with CTV-1 (4 x 10^5), K562 (4 x 10^5) or IL-2 (200IU/ml) in 200 μl CM at 37°C and 5% CO₂. Supernatants were harvested at 6 hours and 16 hours and the concentrations of IFN-γ and IL-2R were determined by a multiplex immunoassay. Values represent the mean of 3–6 donors.
4.4.3 Cytokines II

Levels of the cytokines IFN-α, IL-7, IL-12p40, IL-13, IL-15 and IL-17 secreted by resting, target cell-stimulated and cytokine-stimulated NK cells are shown in Figures 4.9 and 4.10, respectively. Cytokines II secretion levels of CTV-1 and K562 target cells are shown in Figure 4.11. Cytokines II secretion by NK cells after 6 hours and 16 hours of stimulation are shown in Figure 4.12.

4.4.3.1 IFN-α

IFN-α was not detected in supernatants from resting NK cells or target cell stimulated NK cells. Exposure to exogenous cytokines induced the secretion of trace amounts of IFN-α; IL-2 (mean, 1.049 ± 0.91 pg/ml; n=3), IL-7 (mean, 1.573 ± 0.4 pg/ml; n=3), IL-12 (mean, 1.53 ± 0.78 pg/ml; n=3), IL-15 (mean, 1.41 ± 0.44 pg/ml; n=3) or IL-21 (mean, 0.52 ± 0.34 pg/ml; n=3) of IFN-α. CTV-1 and K562 cells did not secrete detectable levels of IFN-α.

4.4.3.2 IL-7

IL-7 was secreted at undetectable levels by resting NK cells. Supernatants collected after 6 hours of target cell stimulation by CTV-1 (mean, 2.159 ± 7.16 pg/ml; n=3) or K562 (mean, 2.466 ± 5.801 pg/ml; n=3) showed low levels of IL-7. Supernatants collected after overnight incubation with CTV-1 did not show detectable levels of IL-7, whereas low levels (mean, 3.59 ± 6.63 pg/ml; n=3) were detected in the case of overnight incubation with K562. Overnight incubation with IL-12 (mean, 10.49 ± 15.30 pg/ml; n=3) or IL-15 (mean, 21.76 ± 10.82 pg/ml; n=3) induced the highest IL-7 secretion levels by NK cells. IL-2 and IL-21 had no effect on IL-7 production by NK cells. CTV-1 and K562 target cells incubated alone did not produce IL-7.
4.4.3.3 IL-12p40/p70

IL-12p40/p70 was secreted at low levels by resting NK cells (mean at 6h, 1.137 ± 0.81 pg/ml; n=4) (mean at 16h, 10.01 ± 1.04 pg/ml; n=4) by resting NK cells. Target cell stimulation with CTV-1 or K562 for 6 hours increased IL-12p40/p70 secretion 8-fold (mean, 9.34 ± 2.69 pg/ml; n=3) and 4-fold (mean, 4.253 ± 1.084 pg/ml; n=3), respectively, compared with resting NK cells. Overnight incubation with CTV-1 resulted in IL-12p40/p70 levels similar to those secreted by resting NK cells (mean, 11.84 ± 3.18 pg/ml; n=3), whereas overnight incubation with K562 resulted in secretion levels that were slightly higher than resting NK cells (mean, 16.69 ± 4.16 pg/ml; n=3). IL-2 stimulation for 6 hours induced a 7-fold increase (mean, 8.142 ± 2.97 pg/ml; n=3, p=0.001) in IL-12p40/p70 levels compared with resting NK cells, which increased a further 3-fold after overnight incubation (mean, 27.31 ± 2.84 pg/ml; n=3, p=0.004). Overnight exposure to IL-7, IL-12, IL-15 or IL-21 induced a 6-fold (mean, 59.25 ± 21.90 pg/ml; p=0.003), 20-fold (mean, 204.8 ± 51.84 pg/ml; n=3), 6-fold (mean, 63.20 ± 86.93 pg/ml; n=3), and 3-fold (mean, 29.94 ± 35.57 pg/ml; n=3, p=0.007) increase in IL-12p40/p70 production compared with resting NK cells. Supernatants from CTV-1 and K562 cells incubated alone did not show IL-12p40/p70 secretion.

4.4.3.4 IL-13 and IL-17

IL-13 was not detected in the supernatants of resting, target cell-stimulated, or cytokine-stimulated NK cells. IL-17 was secreted in low amounts by IL-12-activated NK cells (mean, 6.13 ± 7.218 pg/ml; n=3). CTV-1 and K562 cells secreted undetectable levels of IL-13 and IL-17.
4.4.3.5 IL-15

Resting NK cells secreted undetectable levels of IL-15 after 6 hours and trace levels after overnight incubation (mean, 3.958 ± 4.764 pg/ml; n=3). Stimulation with CTV-1 for 6 hours induced IL-15 secretion (mean, 3.816 ± 6.66 pg/ml; n=3), but supernatants collected after overnight incubation were similar to those of resting NK cells (mean, 4.09 ± 5.04 pg/ml; n=3). A 6-hour incubation with K562 induced moderate levels of IL-15 secretion (mean, 95.77 ± 36.54 pg/ml; n=3, p<0.0001), which increased a further 3-fold (mean, 285.1 ± 135 pg/ml; n=3, p<0.0001) after an overnight incubation. IL-2 stimulation induced low levels of IL-15 production (mean, 2.75± 5.15 pg/ml; n=3) after 6 hours of incubation with NK cells, and levels were a further 8-fold higher (mean, 16.85 ± 17.11 pg/ml; n=3) after overnight incubation. Overnight incubation with IL-7, IL-12, or IL-21 resulted in a 6-fold (mean, 24.10 ± 10.39 pg/ml; n=3), 6-fold (mean, 22.63 ± 22.33 pg/ml; n=3), and 3-fold (mean, 12.94 ± 3.279 pg/ml; n=3) increase in IL-15 secretion, respectively, compared with resting NK cells. CTV-1 and K562 cells did not secrete detectable levels of IL-15.
Figure 4.8 Secretion of cytokines II by target cell-stimulated NK cells.

Freshly isolated resting NK cells (2 x 10^5) were incubated alone, with CTV-1 (4 x 10^5), or K562 (4 x 10^5) target cells in 200 μl CM. After a 6-hour incubation at 37°C and 5% CO₂, supernatants were harvested and the cytokines IFN-α, IL-7, IL-12p40, IL-13, IL-15 and IL-17 were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 donors, p values < 0.05 were considered statistically significant and are indicative of significant difference relative to unstimulated NK cells at the same time point.

rNK indicates resting NK cells; *P<0.05; **P<0.005; ***P < 0.0005.
Figure 4.9 Secretion of cytokines II by cytokine-stimulated NK cells.

Resting NK cells ($2 \times 10^5$) were washed twice and mixed with IL-2 (200IU/ml) IL-7, IL-15, IL-21 (10 ng/ml), or IL-12 (20 ng/ml). After a 16-hour incubation at 37°C, 5 % CO$_2$ in CM, supernatants were harvested and the cytokines IFN-α, IL-7, IL-12p40, IL-13, IL-15 and IL-17 were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 donors, p values < 0.05 were considered statistically significant and are indicative of significant difference relative to unstimulated NK cells at the same time point.

LAK-2, -7, -12 and-15 indicate lymphokine-activated NK cells using IL-2, -7, -12 or -15; *P<0.05; **P<0.005; ***P< 0.0005.
Figure 4.10 Secretion of cytokines II by target cells.

CTV-1 or K562 cells (2 × 10^5 in 200 µl CM) were incubated at 37°C and 5% CO₂ for 16 hours. Supernatants were harvested and the cytokines IFN-α, IL-7, IL-12p40, IL-13, IL-15 and IL-17 were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 replicates.
Figure 4.11 Kinetics of NK cell secretion of cytokines II upon stimulation with CTV-1, K562 or IL-2.

Freshly isolated resting NK cells (2 x 10^5) were incubated with CTV-1 (4 x 10^5), K562 (4 x 10^5) or IL-2 (200IU/ml) in 200 μl CM at 37°C and 5% CO₂. Supernatants were harvested at 6 hours and 16 hours and the concentrations of IFN-α, IL-7, IL-12p40, IL-13, IL-15 and IL-17 were determined by a multiplex immunoassay. Values represent the mean of 3–6 donors.
4.4.4 Chemokines

Levels of chemokines eotaxin, IP-10, MCP-1, MIG, MIP-1α and MIP-1β secreted by resting, target cell-stimulated and cytokine-stimulated NK cells are shown in Figures 4.13 and 4.14, respectively. Chemokine secretion levels of CTV-1 and K562 target cells are shown in Figure 4.15. Chemokine secretion by NK cells after 6 hours and 16 hours of stimulation are shown in Figure 4.16.

4.4.4.1 Eotaxin and MIG

Eotaxin and MIG were not detected in supernatants from resting or target cell-stimulated NK cells. Overnight stimulation with IL-2, IL-7, IL-12 or IL-21 induced NK cell secretion of trace amounts of MIG, but none of these cytokines induced detectable levels of eotaxin. IL-15 stimulation resulted in the production of moderate MIG levels by NK cells (mean, 13.66 ± 7.73 pg/ml; n=3), but did not induce eotaxin secretion. Supernatants from CTV-1 and K562 cultures showed undetectable levels of both chemokines.

4.4.4.2 IP-10

Trace amounts of IP-10 were detected in supernatants from overnight cultures of resting NK cells (mean, 7.78 ± 1.88 pg/ml; n=4). Target cell stimulation with K562 or CTV-1 for 6 hours resulted in moderate (mean, 12.48 ± 6.94 pg/ml; n=4) and high (mean, 1060 ± 550.7 pg/ml; n=3, p<0.0001) levels of IP-10 production, respectively. Overnight stimulation with target cells resulted in a further 1.5-fold increase in IP-10 secretion in the case of K562 (mean, 1541 ± 240 pg/ml; n=3, p<0.0001), whereas levels of IP-10 detected after overnight stimulation with CTV-1 were similar to those after 6 hours (mean, 12.87 ± 5.96 pg/ml; n=3). Exogenous cytokine stimulation with IL-2, IL-7, IL-12, IL-15 or IL-21 induced a 5-fold (mean, 40.74 ± 7.98 pg/ml; n=3), 3-fold (mean, 25.1 ± 6.34 pg/ml; n=3), 7-fold (mean, 51.46 ± 12.22 pg/ml; n=3), 22-fold (mean, 172.51 ± 63.12 pg/ml; n=3) and 5-fold (mean, 37.3 ± 21.11 pg/ml; n=3) increase in IP-10 secretion, respectively, compared with resting NK cells. IL-2-induced secretion of IP-10 was 4-fold higher after overnight incubation with NK
cells compared with levels detected at the 6-hour timepoint (mean, 10.9 ± 4.72 pg/ml; n=3). IP-10 was not secreted by CTV-1 or K562 cells cultured alone.

4.4.4.3 MCP-1

Trace levels of MCP-1 were detected in supernatants from resting NK cells after overnight incubation (mean, 1.75 ± 2.02 pg/ml; n=3). Target cell stimulation by K562 (mean, 10.38 ± 6.55 pg/ml; n=3), but not CTV-1, induced the secretion of higher amounts of MCP-1. Stimulation with IL-2, IL-7, IL-15 or IL-21 induced a 2-fold (mean, 4.02± 4.5 pg/ml; n=3), 16-fold (mean, 27.99 ± 42.45 pg/ml; n=3), 7-fold (mean, 11.91 ± 20.45 pg/ml; n=3) and 9-fold (mean, 16.55 ± 22.87 pg/ml; n=3) increase in MCP-1, respectively, compared with resting NK cells. Exposure to the activating cytokine IL-12 had no effect on MCP-1 production by NK cells (mean, 1.54 ± 1.73 pg/ml; n=3). Trace or no amounts of the cytokine were detected in supernatants from CTV-1 or K562 cell cultures.

4.4.4.4 MIP-1α

MIP-1α was secreted in low or moderate amounts by resting NK cells (mean at 6h, 2.37 ± 1.4 pg/ml; n = 4) (mean at 16h, 87.04 ± 147.7 pg/ml; n = 4). Co-incubation with CTV-1 or K562 cells for 6 hours, resulted in a 107-fold (mean, 253.2 ± 81.2 pg/ml; n=4) and 14-fold (mean, 34.3 ± 32.76 pg/ml; n=4) increase in MIP-1α production, respectively, compared with resting NK cells. Overnight incubation with K562 resulted in a 4-fold increase in MIP-1α secretion by NK cells (mean, 137.6 ± 109 pg/ml; n=3), compared with supernatants collected after 6 hours. In the case of CTV-1, however, lower levels were detected in supernatants from overnight co-cultures (mean, 108.9 ± 147.7 pg/ml; n=3), compared with the 6-hour time point. IL-2 stimulation of NK cells resulted in a 41-fold increase in MIP-1α secretion compared to resting NK cells after 6 hours (mean, 97.8 ± 155.5 pg/ml; n=3) and a 10-fold increase after overnight incubation (mean, 894.9 ± 1040 pg/ml; n=3, p=0.0002). Overnight exposure to IL-7, IL-12, IL-15 or IL-21 resulted in 9-fold (mean, 780.1 ± 1217 pg/ml; n=3, p=0.002), 31-fold (mean, 2722 ± 3324 pg/ml; n=3,
p<0.0001), 11-fold (mean, 920 ± 1555 pg/ml; n=3) and 5-fold (mean, 425.4 ± 639.5 pg/ml; n=3) increase in MIP-1α secretion by NK cells, respectively, compared to resting NK cells.

4.4.4.5 MIP-1β

MIP-1β was detected in moderate amounts in supernatants from resting NK cells (mean at 6h, 8.4 ± 4.2 pg/ml; n = 4) (mean at 16h, 49.77 ± 68.69 pg/ml; n = 4). Target cell stimulation induced a 124-fold (mean, 1042 ± 1367 pg/ml; n=4, p=0.0005) and 15-fold (mean, 125.1 ± 114.1 pg/ml; n=4) increase in MIP-1β secretion by CTV-1 and K562 cells, respectively, after 6 hours of co-incubation with NK cells. Lower levels of MIP-1β were detected in supernatants from overnight co-cultures with CTV-1 (mean, 319.2 ± 438.9 pg/ml; n=4, p=0.001), compared with supernatants collected after 6 hours. In contrast, overnight NK cell co-cultures with K562, showed higher levels of MIP-1β (mean, 152.9 ± 145 pg/ml; n=4) than the 6-hour timepoint. Cytokine stimulation with IL-2, IL-7, IL-12, IL-15 or IL-21 induced a 14-fold (mean, 690.2 ± 709.6 pg/ml; n=3, p=0.002), 11-fold (mean, 555.8 ± 720.4 pg/ml; n=3), 33-fold (mean, 1632 ± 1911 pg/ml; n=3), 17-fold (mean, 852.6 ± 1249 pg/ml; n=3) and 7-fold (mean, 328.3 ± 383.3 pg/ml; n=3) increase in MIP-1β production compared with resting NK cells

4.4.4.6 RANTES

Low levels of RANTES were secreted by resting NK cells (mean at 6h, 4.5 ± 5.615 pg/ml; n=3) (mean at 16h, 11.38 ± 5.88 pg/ml; n=3). Target cell stimulation induced a 22-fold (mean, 98.54 ± 108.3 pg/ml; n=3) and 3-fold (mean, 13.02 ± 6.16 pg/ml; n=3) increase in RANTES secretion by NK cells, after co-culturing for 6 hours with CTV-1 or K562 cells, respectively. Supernatants collected from NK cells co-incubated with CTV-1 overnight, showed lower secretion levels of RANTES (mean, 47.3 ± 64.82 pg/ml; n=3) than that detected after 6 hours. However, NK cells co-incubated with K562 cells overnight, secreted higher levels of RANTES (mean, 18.19 ± 16.82 pg/ml; n=3) compared with the 6-hour time point. Overnight exposure
to IL-2, IL-7, IL-12, IL-15 or IL-21 induced a 9-fold (mean, 102.54 ± 103.1 pg/ml; n=3), 3-fold (mean, 38.23 ± 48.58 pg/ml; n=3), 19-fold (mean, 220.37 ± 250.3 pg/ml), 10-fold (mean, 116.22 ± 156.7 pg/ml; n=3) and 3-fold (mean, 33.92 ± 40.70 pg/ml; n=3) increase in RANTES production by NK cells, respectively, compared with resting NK cells.
Figure 4.12 Secretion of chemokines by resting and target cell-stimulated NK cells.

Freshly isolated resting NK cells (2 x 10^5) were incubated alone, with CTV-1 (4 x 10^5), or K562 (4 x 10^5) target cells in 200 μl CM. After a 6-hour incubation at 37°C and 5% CO₂, supernatants were harvested and the cytokines eotaxin, IP-10, MCP-1, MIG, MIP-1α, MIP-1β were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 donors, p values < 0.05 were considered statistically significant and are indicative of significant difference relative to unstimulated NK cells at the same time point.

rNK indicates resting NK cells; *P<0.05; **P<0.005; ***P < 0.0005.
Figure 4.13 Secretion of chemokines by cytokine-stimulated NK cells.

Resting NK cells (2 x 10^5) were washed twice and mixed with IL-2 (200IU/ml) IL-7, IL-15, IL-21 (10 ng/ml), or IL-12 (20 ng/ml). After a 16-hour incubation at 37°C, 5% CO_2 in CM, supernatants were harvested and the cytokines eotaxin, IP-10, MCP-1, MIG, MIP-1α, MIP-1β and RANTES were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 donors, p values < 0.05 were considered statistically significant and are indicative of significant difference relative to unstimulated NK cells at the same time point.

LAK-2, -7, -12 and -15 indicate lymphokine-activated NK cells using IL-2, -7, -12 or -15; *P<0.05; **P<0.005; ***P < 0.0005.

128
Figure 4.14 Secretion of cytokines by target cells.

CTV-1 or K562 cells (2 x 10^5 in 200 μl CM) were incubated at 37°C and 5% CO₂ for 16 hours. Supernatants were harvested and the cytokines IP-10, MCP-1, MIG, MIP-1α, MIP-1β and RANTES were quantified using a multiplex immunoassay. Graph represents mean values ± SD of 3-6 replicates.
Figure 4.15 Kinetics of NK cell secretion of chemokines upon stimulation with CTV-1, K562 or IL-2.

Freshly isolated resting NK cells (2 x 10^5) were incubated with CTV-1 (4 x 10^5), K562 (4 x 10^5) or IL-2 (200IU/ml) in 200 μl CM at 37°C and 5% CO₂. Supernatants were harvested at 6 hours and 16 hours and the concentrations of IP-10, MIP-1α, MIP-1β and RANTES were determined by a multiplex immunoassay. Values represent the mean of 3–6 donors.
4.5 Discussion

NK cells play an instrumental role in generating rapid immune responses upon recognition of infected or neoplastic cells. These responses are not limited to cytotoxic effector mechanisms, but also involve the secretion of immune modulatory cytokines and chemokines, which aid in the clearance of altered cells and the development of an adaptive immune response (Vivier et al. 2008). The set of experiments described in this Chapter aimed to define the cytokine and chemokine profiles of peripheral blood NK cells upon stimulation with tumour target cells or exogenous cytokines.

NK cell effector responses are determined by the engagement of a multitude of germline-encoded receptors with specific ligands on the cell membrane of target cells. The combination and intensity of ligand expression dictate the degree of NK cell cytotoxic activity and the susceptibility of target cells to NK cell lysis (Bryceson et al. 2005; Bryceson et al. 2009; Lanier 2003). A similar hierarchy is observed for cytokine release upon NK cell interactions with target cells depending on variations in the degree of activating NK cell receptor engagement (Fauriat et al. 2010). Recent evidence suggests that NK cell cytokine secretion is distinct from cytotoxicity, as indicated by different signalling requirements (Bryceson et al. 2009), trafficking and secretion pathways, dependency on recycling endosomes (RE) and non-polarized release (Reefman et al. 2010). Thus, CTV-1 target cells expressing ligand combinations that are insufficient to trigger NK cell cytotoxicity can still induce ample production of cytokines in NK cells.

An early feature of the NK cell immune response is the production of chemokines, which play a pivotal role in activating and directing other activated NK cells and leukocytes to target sites. Temporally, the secretion of chemokines has been shown to precede other cytokines and require less stimulation than cytokine production or degranulation (Bryceson et al. 2005; Fauriat at al. 2010). Engagement of individual activating receptors 2B4 or NKG2D on resting NK cells is sufficient to trigger
chemokine secretion and the co-engagement of additional receptors can accelerate and enhance the level of chemokines secreted (Fauriat et al. 2010). The results in this Chapter show NK cells that have completed the priming stage via stimulation with CTV-1 or exogenous cytokines demonstrate chemokine profiles that are distinct from NK cells that have completed the priming and triggering stages through target cell recognition of K562 cells. NK cells incubated with CTV-1 or exogenous cytokines produced ample amounts of MIP-1α, MIP-1β, and RANTES, but very low amounts of IP-10. Conversely, K562-stimulated NK cells produced high levels of IP-10, and low levels of MIP-1α, MIP-1β, and RANTES. This suggests that it is the activation stage, rather than the type of stimulus that is influencing the chemokine secretion profile observed. Notably however, CTV-1 stimulation consistently induced higher levels of chemokine production than IL-2 stimulation after 6 hours of co-incubation with NK cells, demonstrating that the kinetics of chemokine secretion is dependent on the type of stimulus.

Secretion of the cytokines TNF-α and IFN-γ were previously reported to require greater receptor cooperation and occur at later time points after stimulation, relative to chemokine secretion (Bryceson et al. 2006; Fauriat et al. 2010). TNF-α secretion can be induced by the co-engagement of the activating receptors 2B4 and NKG2D, whereas IFN-γ secretion requires additional LFA-1-dependent signalling (Fauriat et al. 2010). In this Chapter, CTV-1 stimulation induced production of both cytokines, which suggests that the minimal requirements for receptor engagement are met. These results are inconsistent with studies by Bryceson et al. demonstrating that the minimal requirements for IFN-γ secretion are the same as natural cytotoxicity, since CTV-1 stimulation induces IFN-γ production but does not trigger cytotoxicity in NK cells. Strikingly, CTV-1 stimulated the release of TNF-α and IFN-γ at levels that were approximately 7-fold higher than K562 stimulation after a 6-hour incubation with NK cells. Co-stimulation of receptors by engagement of LFA-1 has been shown to accelerate and increase cytokine secretion in NK cells (Fauriat et al. 2010). CTV-1 cells express the intracellular adhesion molecule (ICAM)-1, the natural ligand for LFA-1, whereas K562 cells do not. It is possible that CTV-1 has a better ligand
profile for the induction of cytokine secretion. It is also possible that NK cells that have been primed are more responsive in terms of cytokine secretion than NK cells that have gone through target cell priming and killing, as in the case of K562 stimulation.

Previous studies assessing the relationship between the production of chemokines and cytokines revealed that the production of TNF-α and IFN-γ were contained within the chemokine-producing NK cell subset (Fauriat et al. 2010). The majority of IFN-γ producing NK cells were also shown to secret TNF-α, but not vice versa. This study showed the graded response in factor release by NK cells and established a hierarchy based on the different activation thresholds required for chemokine < TNF-α < IFN-γ release. Engagement of individual activating receptors on resting NK cells suffices for chemokine secretion, to alert and recruit other immune cells, whereas more complex interactions are required to induce TNF-α and IFN-γ secretion. Results in this Chapter show that the engagement of ligands on CTV-1 target cells by activating NK cell receptors was sufficient to induce chemokine, TNF-α and IFN-γ secretion. It is important to note that intracellular expression of cytokines can be induced with less stringent activating signals (i.e. individual receptor engagement) without detection of secretion (Fauriat et al. 2010a). This level of NK cell regulation that precedes cytokine exocytosis was not explored here, but deserves further investigation.

When the relationship between degranulation and cytokine production was investigated previously, CD107a expression did not correlate with IFN-γ or TNF-α production (Fauriat et al. 2010a). Although this was justified by differences in response kinetics, accumulating evidence on the differential mechanisms of cytokine secretion and cytotoxicity in NK cells as well as the data highlighted here, suggest the need to view the two NK cell functions independently (Reefman et al. 2010). It is important to note that in contrast to other activating receptors, the engagement of CD16 is sufficient to induce the secretion of chemokines, IFN-γ and TNF-α and degranulation (Bryceson et al. 2006; Bryceson, et al. 2009; Fauriat et al. 2010). The strong propensity of CD16 to induce cytotoxicity and cytokine secretion in NK cells
is based on antibody-dependent mechanisms. Evidence that these effector functions are independently regulated, and that in some cases the amount of cytokine secretion does not relate to cytolytic activity of NK cells, suggests that it is possible to extend our view of antibody-dependent cellular cytotoxicity as distinct from antibody-dependent cytokine secretion.

Activation and proliferation of NK cells is influenced by direct contact with target cells as well as the local cytokine milieu. These cytokines provide a means of amplifying the effect of NK cells in the early phase of the immune response and act as co-stimulators with target cells to initiate NK cell cytotoxicity and further cytokine production. The Th1/Th2 and pro-/anti-inflammatory balance in the NK-target cell microenvironment can modulate NK cell secretory responses toward tolerance or immunity (Elenkov and Chrousos 1999; Elenkov and Chrousos 2002). Type 1 cytokines such as IL-2, IL-12 and IL-15 are involved in Th1 immune responses and induce cell-mediated immunity. By contrast, type 2 cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 are involved in Th2 immune responses, which promote humoral immunity against tumours and/or immune tolerance. Consistent with previous findings, resting and target cell-stimulated NK cells did not secrete the cytokines IL-4, IL-5, IL-7, IL-10, IL-13, IL-17 or GM-CSF (Fauriat et al. 2010a). Some of these cytokines have previously been reported to be secreted by NK cells upon stimulation with exogenous cytokines (Warren et al. 1995; Warren et al. 1996), and the findings presented here demonstrate secretion of IL-7, IL-10, IL-17 and GM-CSF, but not IL-4, IL-5 or IL-13, in response to exogenous cytokine stimulation. Other pro-inflammatory/Th1 cytokines such as IL-1β, IL-1RA, IL-6, IL-8, IL-12 and IL-15 were secreted by resting NK cells and augmented by target cell or exogenous cytokine stimulation. Notably, NK cells stimulated with CTV-1 produced the highest levels of IL-1β, IL-1RA and IL-12 after a short incubation, which suggests the specific induction of early-phase inflammatory signalling.

Other than its role in enhancing in vivo anti-tumour inflammatory responses and promoting IFN-γ secretion (Trinchieri 1995a; Trinchieri 1995b), IL-12 is central to the generation of NK cells with memory-like features (Sun et al. 2012). NK cells can
be altered after *in vitro* priming to respond more robustly on subsequent stimulation. Evidence that NK cells can undergo clonal expansion and mediate secondary immune responses has been demonstrated following priming with viral targets or exogenous cytokines, as well as after sensitization with haptens (Min-Oo et al. 2013; Sun et al. 2011). The precise signals that promote the generation of long-lived memory NK cells are not clearly defined and the mechanisms by which NK cells are modified after an initial activation to respond more vigorously on subsequent stimulation are not understood. NK cells primed with CTV-1 have been shown to maintain their primed status longer than cytokine-primed NK cells (North et al. 2007; Sabry et al. 2011). In this chapter, CTV-1 stimulation was shown to induce the highest secretions of IL-12 after short incubation. The role of IL-12 in NK cell priming for the maintenance of tumour-activation signals and the development of NK memory-like features should be investigated in future experiments.

Any immune challenge that threatens the stability of the internal milieu can be regarded as a ‘stressor’, which can activate the stress system to further modulate the local pro-/anti-inflammatory cytokine balance (Elenkov and Chrousos 1999; Elenkov and Chrousos 2002). As such, the presence of tumour cells can induce the release of stress hormones like glucocorticoids and catecholamines to directly influence *in vivo* NK cell responses toward suppression or activation. Alternatively, tumour cells can release cytokines to activate components of the stress system and indirectly shape NK cell cytokine secretion. NK cells primed by CTV-1 secreted high levels of IL-1β, IL-6 and TNF-α, which are the main cytokines associated with stress system activation. Tumour target cells used in these experiments were also observed to release several soluble factors like IL-1β, IL-6, IL-8, IL-12, IL-15 and IP-10. In most cases, K562 cells showed higher cytokine secretion than CTV-1, which can partly explain their greater susceptibility to NK cell-mediated immune responses. However, evidence that the stress system can shift the equilibrium between anti-inflammatory and pro-inflammatory cytokines in either direction makes it difficult to determine the effect of target cell cytokine secretion on NK cell activity.
Recent studies have shown CD56^{dim} rather than CD56^{bright} NK cells to be more prominent producers of cytokines upon target cell recognition (Fauriat et al. 2010). The results described in this Chapter are mainly representative of CD56^{dim} NK cell responses, since they comprise the majority of PB NK cells. Data here further support evidence that CD56^{dim} NK cells are an important source of pro-inflammatory cytokines during early immune responses and can respond vigorously in terms of cytokine and chemokine production after contact with target cells or exposure to cytokines. CD56^{dim}CD16^{-} NK cells were previously reported as the main subset exhibiting anti-tumoural activity (Fauriat et al. 2010; Penack et al. 2005, Chapter 3). NK cells primed with CTV-1 were shown in Chapter 3 to downregulate CD16 expression. Thus, future investigations should aim to define the cytokine secretion profile of NK cell CD56^{dim}CD16^{+/−} subsets.

In conclusion, the results presented in this Chapter provide insight into the regulation of cytokine secretion in resting NK cells after activation with different stimulating mechanisms. NK cell secretory responses enable them to act as primary initiators of the immune response by relaying activating signals from other activated immune or stressed cells. Fauriat et al. previously showed that in contrast to cytokine stimulation, target cell recognition by resting NK cells induces a pro-inflammatory cytokine profile characterized by MIP-1α, MIP-1β, RANTES, TNF-α and IFN-γ. Interestingly, stimulation with CTV-1 cells consistently induced the secretion of these cytokines at a much higher rate than that of K562 cells after a short incubation. A possible explanation for enhanced CTV-1 stimulation of cytokine secretion is that CTV-1-stimulated NK cells that are primed but have yet to initiate the process of cytotoxic killing are more responsive than K562-stimulated NK cells that have gone through the full activation cycle of priming and triggering stages. Another explanation lies in the ligand expression profile of CTV-1 cells, which might hold the code for better synergy among NK cell activating receptors for optimal cytokine secretion. NK cell functional responses can be anti-viral, anti-tumoural or immunomodulatory, depending on the type of threat faced by the immune system and the activating NK cell signals received. The results in this Chapter demonstrate
significant differences in NK cell cytokine secretory responses to tumour target cells and exogenous cytokines. This should be taken into consideration in the development of strategies involving *ex vivo* stimulation of NK cells to enhance NK cell functional properties for use in cellular immunotherapy.
Chapter 5  Dissecting the tumour-priming signal

5.1 Introduction

NK cells express a wide array of activating cell surface receptors that can be engaged by various ligands on tumour cells to induce cytokine secretion and target cell lysis. Resting NK cells require the co-engagement of several activating receptors to progress through the priming and triggering stages of NK cell activation (Bryceson et al. 2006). Upon contact with a susceptible target cell, an NK cell goes through discrete events of cell adhesion, conjugate formation, lytic granule polarization and release (Orange 2008).

Strong adhesion to target cells is mediated by the β2 integrin, lymphocyte function-associated antigen (LFA)-1, which is a heterodimer of CD11a and CD18 (Carpen et al. 1991; Helander and Timonen 1998). Signaling through LFA-1 also contributes to early events in NK cell cytotoxicity, leading to granule polarization (Barber et al. 2004; Bryceson et al. 2005). CTV-1 cells express the intercellular adhesion molecule (ICAM)-1, the natural ligand for LFA-1. Engagement of LFA-1 by ICAM-1 has been shown to be important for the formation of stable conjugates between NK cells and target cells, in order to create the synergy between activating receptors that is necessary for efficient target cell lysis (Barber et al. 2004; Bryceson et al. 2009). CTV-1 cells also express several other ligands for NK cell co-activation, including those for CD2 and NKG2D. Both these receptors have been shown to synergize with LFA-1 to enhance resting NK cell activation (Bryceson et al. 2006; Bryceson et al. 2009; Katodritou et al. 2011; North et al. 2007).

In this Chapter, the critical priming ligands provided by CTV-1 for NK cell activation were investigated. In addition, a bead-based strategy to replicate the CTV-1 priming signal was devised, with the objective of achieving activation of NK cells in the absence of tumour target cells.
5.2 Experimental Aims

The aims of the set of experiments described in this Chapter were: 1) to identify the critical ligands provided by CTV-1 cells for the tumour-priming of resting NK cells; and 2) to devise methods to replace CTV-1-mediated priming of NK cells with bead-mediated priming. Ligands for LFA-1, CD2 and NKG2D NK cell receptors were investigated to confirm their role in the delivery of the tumour-priming signal. Thereafter, methods of microbead-mediated stimulation targeting LFA-1, CD2 and NKG2D were optimized in order to replicate the tumour-priming signal in the absence of CTV-1. Parameters optimized included the type of bead, bead:NK cell ratios, ligand:ligand ratios and the co-incubation period.
5.3 Methods

5.3.1 Blockade studies

To investigate the involvement of LFA-1, CD2 and NKG2D in CTV-1-mediated NK cell priming, purified CD56+ NK cells from normal donors were pre-incubated for 30 mins at room temperature in the presence or absence of saturating concentrations of antibodies. After washing with HBSS, and overnight co-incubation of NK cells (1 x 10^6 cells) with CTV-1 (2 x 10^6 cells), immunophenotyping was carried out for the analysis of antigen expression (MACS Quant Analyzer) with isotype controls used for every experiment. NK cell activation was also determined by evaluating NK cell activity against NK-resistant RAJI cells in a 4-hour cytotoxicity assay. Statistical comparisons between treatments were performed using the multiple t-test, corrected for multiple comparisons with the Holm-Sidak method. Results are presented as mean ± SD. Corrected P values < 0.05 were considered statistically significant. Statistical analyses were performed with GraphPad Prism v.6 software.

5.3.2 Bead Stimulation

5.3.2.1 Dynabeads Sheep anti-mouse IgG

Dynabeads Sheep anti-Mouse IgG have a diameter of 4.5μm. They are uniform superparamagnetic polystyrene beads coated with polyclonal Sheep anti-Mouse IgG antibodies. Dynabeads were washed before use by resuspending in the vial, transferring 50 μl to a tube and adding 1ml of PBS buffer with 0.1% BSA, pH 7.4. The tube was placed in a magnet for 1 minute and the supernatant was discarded. After removing the tube from the magnet, the Dynabeads were resuspended in the same volume of buffer as the initial volume of Dynabeads used (50 μl). Thereafter, washed beads were transferred to a tube and 1.5 μg of each primary monoclonal antibody (mAb) added: anti-LFA-1 (R7.1), anti-CD2 (RPA 2.11) and anti-NKG2D (149810). After incubation for 1 hour at 4 °C with rotation, the antibody-beads
complex was washed again by placing in a magnet for 1 min and discarding the supernatant. The beads were washed twice using 1ml of buffer. After removing the tube from the magnet, Dynabeads were resuspended in 50 μl PBS buffer with 0.1% BSA, pH 7.4. Beads were then used to stimulate NK cells at a ratio of 8 beads per cell. Dynabeads (4 × 10⁶) coated with saturating amounts of mAb, were incubated with resting NK cells (5 × 10⁵) in 500 μl RPMI supplemented with 10% human serum for the indicated duration at 37°C. The cultures were rotated end-over-end during the stimulation. For each primary mAb, coating of Dynabeads was ≥ 98% efficient as determined by flow cytometry.

5.3.2. 2 Dynabeads Protein G

Dynabeads coupled to Protein G were then used to generate an artificial priming signal so that cognate ligands, instead of mAbs, engaged activating NK cell receptors. Dynabeads Protein G have a diameter of 2.5μm. Beads were resuspended by vortexing for >30 seconds and then transferred to a fresh tube. The tube was placed on a magnet to separate the beads from the solution, and the supernatant was removed. The tube was removed from the magnet, and 1.5 μg of recombinant Human ICAM-1 Fc Chimera, recombinant Human MICA Fc Chimera, or anti-CD2 (RPA 2.11) were added to the Dynabeads. CD15 was not used as it is not commercially available. The beads were then incubated for 30 minutes at room temperature with rotation. Thereafter, the tube was placed on a magnet and the supernatant removed. Beads were then used to stimulate NK cells at a ratio of 8 beads per cell. Dynabeads (4 × 10⁶) coated with saturating amounts of mAb, were incubated with resting NK cells (5 × 10⁵) in 500 μl RPMI supplemented with 10% human serum for the indicated duration at 37°C. The cultures were rotated end-over-end during the stimulation. Coating of Dynabeads was ≥ 98% efficient as determined by flow cytometry.
5.4 Results

5.4.1 Critical ligands provided by CTV-1 for tumour-priming

5.4.1.1 LFA-1-mediated tumour priming of NK cells

LFA-1 provides early signals in NK cell cytotoxicity leading up to granule polarization (Barber et al. 2004; Bryceson et al. 2005). Ligation of LFA-1 on NK cells to its ligand ICAM-1 on target cells specifically contributes to the stability of the immunological synapse with target cells (Bryceson et al. 2006; Bryceson et al. 2009). In the first set of experiments, the ability of anti-ICAM-1 mAbs to block LFA-1-mediated tumour priming of NK cells was investigated.

Figure 5.1 shows that ICAM-1 blockade reduced CTV-1-mediated priming of NK cells, as measured by CD69 expression (mean decrease, 27.4 ± 5.48%, n=4; p = 0.014) and CD25 expression (mean decrease, 34.1 ± 3.14%, n=4; p = 0.03). In addition, as shown in Figure 5.2, pre-incubation with anti-ICAM-1 mAbs significantly reduced the lytic activity of CTV-1-primed NK cells (mean decrease, 24.0 ± 10.5%, n=4; p = 0.019).
Figure 5.1 Effect of ICAM-1 blockade on CTV-1-mediated priming of NK cells

CTV-1 cells were pre-incubated with saturating concentrations of anti-ICAM-1 or anti-CD56 mAbs and co-cultured with resting NK cells from normal volunteer donors for 16 hours. Resting and tumour-primed NK cells were then tested for: (A) CD69 expression; and (B) CD25 expression by flow cytometry. Data presented are the mean (± SD) percentage of cells expressing CD69 or CD25, respectively.

rNK indicates resting NK cells; TpNK, tumour-primed NK cells by co-incubation with CTV-1; *p < 0.05.
Figure 5.2 Effect of ICAM-1 blockade on lytic activity of CTV-1-primed NK cells.

CTV-1 cells were pre-incubated with saturating concentrations of anti-ICAM-1 or anti-CD56 antibodies and co-cultured with resting NK cells from normal volunteer donors for 20 hours. Tumour-primed NK cells were then tested for lysis of NK-resistant RAJI cells in a 4-hour cytotoxicity assay. The data presented are the mean (± SD) percentage of RAJI cells lysed.

rNK indicates resting NK cells; TpNK, tumour-primed NK cells; *p < 0.05.
5.4.1.2 CD2-mediated tumour priming of NK cells

CD2 receptor-mediated activation of NK cells through engagement of its ligand CD15 was previously demonstrated by Warren et al., who showed that some anti-CD15 mAbs that sterically block the CD2L site are able to block NK killing of otherwise susceptible target cells, including the prototypical NK cell target K562 (Warren et al. 1996). CTV-1 cells express CD15, so it was hypothesized that CD2-CD15 interactions contribute to the delivery of the tumour-priming signal.

Figure 5.3 shows that pre-incubation of CTV-1 with anti-CD15 mAbs inhibited priming of NK cells as measured by CD69 expression (mean decrease, 30.1 ± 13.3%, n=4) and CD25 expression (mean decrease, 57.7 ± 7.78%, n=4; p = 0.03). The role of CD2 as the NK-associated ligand for CD15 was confirmed by the blockade of NK priming by anti-CD2 mAbs, which resulted in the reduced expression of CD25 (mean decrease, 39.4 ± 5.69%, n=4). Combined blockade of CD2 and CD15 resulted in a decrease in expression of both activation markers (CD69: mean decrease, 46.3 ± 14.4%, n=4). CD25: mean decrease, 88.3 ± 2.25%, n=4; p = 0.02). As shown in Figure 5.4, pre-incubation with CD15 inhibited lytic activity of tumour-primed NK cells against RAJI cells (mean decrease, 79.6 ± 4.1%; p = 0.007). CD2 blockade also resulted in a reduction of tumour-primed lytic activity (mean decrease, 59.1 ± 8.04%, n=4). Finally, combined blockade of CD2-CD15 interactions significantly reduced lysis of RAJI cells (mean decrease, 87.4 ± 3.03%, n=4; p = 0.01).
Figure 5.3 Effect of CD15 or CD2 blockade on CTV-1-mediated priming of NK cells.

Resting human NK cells from healthy volunteer donors were incubated overnight with or without CTV-1 cells in the presence or absence of the indicated mAbs at saturating concentrations. After 20 hours, the cells were analyzed for expression of: (A) CD69 expression; and (B) CD25 expression by flow cytometry. Data shown are the mean (± SD) percentage of CD69⁺ or CD25⁺ cells, respectively.

rNK indicates resting NK cells; TpNK, tumour-primed NK cells; *p < 0.05.
Figure 5.4 Effect of CD15 or CD2 blockade on lytic activity of CTV-1-primed NK cells.

CTV-1 cells were incubated with saturating concentrations of anti-CD15 (clone MEM158), anti-CD2 (OKT11), or anti-CD56 (clone NCAM16.2) mAbs and co-cultured with resting NK cells from normal volunteer donors (n = 5) for 20 hours. Tumour-primed NK cells were then tested for their ability to lyse NK-resistant RAJI cells in a 4-hour cytotoxicity assay. Data shown are the mean (± SD) percentage lysis of RAJI cells.

rNK indicates resting NK cells; TpNK, tumour-primed NK cells; *p < 0.05; **p < 0.005.
5.4.2 Microbead-mediated priming of NK cells

5.4.2.1 NK cell priming with antibody-coated microbeads

In the next set of experiments, I set out to replace the CTV-1-mediated NK cell priming signal with an artificial priming system involving antibody-coated microbeads. Dynabeads coated with anti-LFA-1, anti-CD2 and anti-NKG2D mAbs were co-incubated with resting NK cells for 20 hours. NK cells were then assayed for priming using CD25 and CD69 activation markers. A modest up-regulation in CD69 expression (mean increase, 7.51 ±7.2%, n=3) and CD25 expression (mean increase, 7.02% ± 3.6; n=3) was observed compared with resting NK cells (Figure 5.5). Enhanced killing was not observed by NK cells co-incubated with the antibody-coated beads (Figure 5.6).
Figure 5.5 Effect of antibody-coated microbeads on NK cell priming.

Resting human NK cells from healthy volunteer donors were incubated overnight with CTV-1 cells, microbeads coated with anti-(LFA-1+NKG2D+CD2) mAbs or uncoated beads. After 20 hours, NK cells were analyzed for (A) CD69 and (B) CD25 expression by flow cytometry. Data shown are the mean (± SD) percentage of CD69⁺ cells and CD25⁺ cells, respectively.

rNK indicates resting NK cells; TpNK, tumour-primed NK cells.
Figure 5.6 Effect of antibody-coated microbeads on the lytic activity of NK cells.

Resting human NK cells from healthy volunteer donors (n = 3) were incubated overnight with CTV-1 cells, microbeads coated with anti-(LFA-1+NKG2D+CD2) mAbs or uncoated beads. After 20 hours, NK cells were tested for lysis of NK-resistant RAJI cells in a 4-hour cytotoxicity assay. Data shown are the mean (± SD) percentage lysis of RAJI cells.

rNK indicates resting NK cells; TpNK, tumour-primed NK cells.
5.4.2.2 NK cell priming with CTV-1 ligand-coated microbeads

Dynabeads pre-coated with recombinant Human ICAM-1 Fc Chimera, recombinant Human MICA Fc Chimera and anti-CD2 (RPA 2.11) were co-incubated with resting NK cells for 20 hours. NK cells were then assayed for priming using CD25 and CD69 activation markers. A modest upregulation in CD69 expression (mean increase, 10.6 ± 10.95%, n=3) and CD25 expression (mean increase, 14.7 ± 4.08%, n=3) was observed compared with resting NK cells (Figure 5.7). Beads coated with ICAM-1 formed a higher number of conjugates with NK cells compared with beads coated with anti-LFA-1 (mean increase, 74.1 ± 2.08%, n=3; p = 0.003) or anti-NKG2D (mean increase, 84.7 ± 4.04%, n=3; p = 0.003). Enhanced killing was not observed by resting NK cells co-incubated with ligand-coated microbeads (Figure 5.8).
Figure 5.7 Effect of CTV-1 ligand-coated microbeads on NK cell priming.

Resting human NK cells from healthy volunteer donors (n = 3) were incubated overnight with CTV-1 cells, microbeads coated with recombinant Human ICAM-1 Fc Chimera, recombinant Human MICA Fc Chimera and anti-CD2 (RPA 2.11) or uncoated beads. After 20 hours, NK cells were analyzed for (A) CD69 and (B) CD25 expression by flow cytometry. Data shown are the mean (± SD) percentage of CD69⁺ cells and CD25⁺ cells, respectively.

rNK indicates resting NK cells; TpNK, tumour-primed NK cells.
Resting human NK cells from healthy volunteer donors (n = 3) were incubated overnight with CTV-1 cells, microbeads coated with recombinant Human ICAM-1 Fc Chimera, recombinant Human MICA Fc Chimera and anti-CD2 (RPA 2.11) or uncoated beads. After 20 hours, NK cells were tested for lysis of NK-resistant RAJI cells in a 4-hour cytotoxicity assay. Data shown are the mean (± SD) percentage lysis of RAJI cells.

rNK indicates resting NK cells; TpNK, tumour-primed NK cells.

Figure 5.8 Effect of CTV-1 ligand-coated microbeads on lytic activity of NK cells.
5.5 Discussion

NK cell-mediated natural cytotoxicity against tumour targets involves a wide array of activating NK cell receptors. Upon contact with a susceptible target cell, an NK cell undergoes discrete activation events, starting with LFA-1-dependent adhesion and culminating in the polarised release of cytotoxic granules into the immunological synapse (Bryceson et al. 2011). Conversely, an encounter with an NK-resistant target cell that fails to trigger NK cell cytotoxicity can be due to a deficiency in one or more of the signalling requirements for NK cell activation. CTV-1 is a leukemic cell line that primes resting NK cells to display enhanced effector functions, but fails to trigger NK cell cytotoxicity (North et al. 2007). In this Chapter, the CTV-1 ligands responsible for the delivery of the tumour-priming signal to NK cells were examined. The results presented indicate that the engagement of ICAM-1 and CD15 with their respective receptors on resting NK cells was shown to be critical for the NK cell priming process.

LFA-1-dependent NK cell adhesion to target cells occurs through the activation of inside-out signals, which induce an open conformation in LFA-1 to promote ligand affinity and avidity (Barber and Long 2003). In T cells, LFA-1 does not signal on its own in the absence of TCR engagement (Luo et al. 2007), so it is not possible to dissect the relative contributions of TCR and LFA-1 signals, which collectively result in efficient granule polarization (Anikeeva et al. 2005). By contrast, inside-out signalling by other receptors for the binding of LFA-1 is not required in NK cells. Thus, the outcome of signalling by LFA-1 alone can be examined. Studies have shown that LFA-1 binding to purified ICAM-1 on beads or to ICAM-1 expressed on insect cells is sufficient to induce granule polarization in NK cells (Barber et al. 2004; Bryceson et al 2005). A variety of activating receptors, including 2B4, NKG2D, DNAM-1, CD2 or LFA-1 itself, can rapidly induce inside-out signals for activation of LFA-1 in freshly isolated human NK cells to promote adhesion (Bryceson et al. 2009). Co-engagement of some of these co-activation receptors with ICAM-1 has been shown to enhance signalling in NK cells (Bryceson et al 2005; Bryceson et al. 2009). Blockade of ICAM-1 on CTV-1 cells was shown here to
inhibit tumour-priming of resting NK cells as evidenced by downregulation of activation marker expression and reduced cytotoxicity against RAJI cells. It is possible that ICAM-1 blockade results in a decrease in the number of NK cells that overcome the signalling threshold for granule polarization. ICAM-1 is also required for the formation of organized natural cytotoxicity synapses (Liu et al. 2009). Thus, another explanation for the blockade of NK cell priming in the absence of ICAM-1 signalling could be a decrease in the number of stable NK-target cell conjugates required for efficient killing by NK cells. Indeed, my observations confirmed a higher number of bead-NK cell conjugates was achieved when beads were coated with ICAM-1, compared to any other ligand or antibody.

When screening for potential NK-activating ligands on CTV-1, our group had previously identified four other candidate molecules: CD58, CD48, CD38 and CD15 (Sabry et al. 2011). Only CD15 was shown to have a significant effect on NK cell priming as measured by CD25 and CD69 expression (Sabry et al. 2011). Warren et al. had previously reported CD15 as a novel ligand for CD2, and CD2 is a known mechanism by which NK cell activation occurs (Bryceson et al. 2006). Therefore, I hypothesized that CD15 on CTV-1 engages CD2 on NK cells to deliver part of the tumour-priming signal. I used the anti-CD15 mAb clone MEM158, instead of clone LeuM1, because the latter was previously reported to bind a site within CD15 that excludes the CD2L site (Warren et al. 1996). Pre-incubation of CTV-1 with anti-CD15 mAb clone MEM158 significantly reduced the lytic activity of tumour-primed NK cells. Further work by our group confirmed that CD15+ tumour cell lines such as ‘leukemic’ MV-411 and SEM, but not CD15– cell lines MOLT-16 and PF-382 are capable of priming NK cells. Induced expression of CD15 on the NK-resistant RAJI, through transfection with cDNA for fucosyltransferase (FUT)-4, the CD15 synthesizing enzyme, renders it susceptible to lysis by resting NK cells (Sabry et al. 2011). All of this confirms the involvement of CD15 in the priming of NK cells and suggests that CD15 is an important constituent of the NK-CTV-1 immune synapse.
When I set out to replicate the tumour-priming signal delivered to resting NK cells in the absence of CTV-1 cells with antibody-coated microbeads, anti-NKG2D mAbs were added to the stimulation cocktail (which also included anti-CD2 and LFA-1 mAbs). NKG2D is an activating NK cell receptor that was consistently downregulated when I investigated the phenotypic properties of tumour-primed NK cells in Chapter 3. CTV-1 cells express the stress ligands for NKG2D, MICA and MICB. In addition, previous work by our group had shown that blockade of NKG2D suppresses the proportion of CD69+ NK cells and the intensity of CD69 expression (Sabry et al. 2011). First, beads of a diameter 4.5 µm were coated with antibodies against the three NK cell receptors: LFA-1, CD2 and NKG2D. The NK cell activation achieved following bead stimulation was minimal as measured by activation marker expression. Since mAbs don’t recapitulate activation processes induced by physiological ligands, I sought to replace the artificial priming system with Protein-G coupled beads bound to fusion proteins of ICAM-1 and ULBP-1; the respective ligands for LFA-1 and NKG2D. Ideally, those beads would have been coated with CD15 as well, but since it isn’t commercially available, I used anti-CD2 mAbs. These experiments again resulted in some upregulation of NK cell activation markers, but it is clear that the methodology remains to be optimized such that priming equivalent to cell contact is achieved. It is possible that simultaneous co-engagement of receptors is a crucial requirement for the replacement of target cell stimulation as previously suggested (Bryceson et al. 2009). It is also possible that other receptor combinations are more optimal for NK cell activation synergy (Bryceson et al. 2006).
Chapter 6    General Discussion

6.1   NK Cells and tumours: a few burning questions

With a wide array of cell surface receptors designed for robust immune surveillance and protection against the wide variety of threats faced by the immune system, NK cells are strongly equipped to act as initiators of immune responses upon recognition of stressed or aberrant cells. The innate ability of NK cells to mediate cytotoxicity against tumour cells co-evolved alongside tumour escape mechanisms, by which tumour growth and development can take place without triggering the NK cell threshold for full activation. Tumour resistance to lysis by NK cells may be overcome by priming NK cells with cytokines or by binding NK activating receptors to ligands expressed on target cells. In this Thesis, the activation profiles of NK cells upon tumour-priming with the NK-resistant leukaemia cell line CTV-1 were defined, and the NK cell receptors involved in the delivery of the tumour-priming signal were identified. The results presented here demonstrate that NK cell responses to CTV-1 tumour-priming are distinct from those induced by exogenous cytokine-priming, and from those induced by the NK-sensitive target cell line K562. This supports the hypothesis that NK cell responses differ according not only to the type of stimulus, be it an exogenous cytokine or a target cell, but also depending on the ligand combination presented by the target cell and the threshold for NK cell activation achieved.

In contrast to inhibitory receptor expression, which is to a great extent genetically determined, recent evidence suggests that activation receptor expression is heavily influenced by the environment (Horowitz et al. 2013). Therefore, NK cells may be reliant on inhibitory receptor expression for mechanisms of self-tolerance, while using adaptable expression patterns of activating receptors and strict requirements for co-activation to regulate responses to other immune threats such as tumourigenesis. It is also possible that regulation of NK cell responses is exerted on multiple levels that include the presence of inhibitory receptors and the specificity of activation
requirements. In any case, the exact mechanisms regulating NK cell effector responses remain poorly understood and the signalling pathways are still undefined. The experiments described in this Thesis aimed to improve our understanding of how NK cells regulate their effector responses against tumours, in order to facilitate the clinical use of NK cells for immunotherapy. With this in mind, a few burning questions pertaining to NK cell biology remain, and these are discussed in the following sections.

6.2 Tumour-associated NK Cells: impaired or tumour-primed?

It is widely thought that cancer patients have NK cell dysfunctions that are secondary to the presence and progression of cancer (Kiessling et al. 1999). Commonly reported traits of tumour-associated NK cells include decreased cytotoxic activity (Bauernhofer et al. 2003; Campos et al. 1998; Carrega et al. 2008; Frassanito et al. 1997; Jinushi et al. 2005; Katrinakis et al. 1996; Koda et al. 2003; LeFever and Funahashi 1991; Lutgendorf et al. 2005; Mocchegiani et al. 1999; Rajaram et al. 1990; Tajima et al. 1996; Taketomi et al. 1998; Wakiguchi et al. 1994), downregulation of activating receptor expression (Costello et al. 2002; El-Sherbiny et al. 2007; Fauriat et al. 2007; Jinushi et al. 2005; Konjevic et al. 2007) and intracellular signalling molecules (Buggins et al. 1998; Healy et al. 1998; Kono et al. 1996; Lai et al. 1996; Nakagomi et al. 1993), defective proliferation (Gati et al. 2004; Pierson and Miller 1996; Tsukuda et al. 1993), poor infiltration, decreased cell counts and defective cytokine production (Chang et al. 1989; Tajima et al. 1994). It is important to note that studies since the late 1980s have demonstrated the reversal of these tumour-associated NK cell traits after a few days of ex vivo culturing alone or with IL-2, suggesting the absence of any inherent NK cell defect per se (Lotzova et al. 1987). Instead, I propose based on the data presented here regarding the antigen expression and secretory profiles of tumour-primed NK cells that these observations are indicative of a tumour-specific NK cell response. This is proposed bearing in mind that the tumour must have undergone selective pressure to downregulate NK cell functions in order to grow within an immunocompetent setting (Dunn et al. 2002).
The weakened capacity of NK cells to kill tumour targets has previously been shown
to be ‘corrected’ with the addition of activating stimuli, blockade of inhibitory
factors, or when tested against an allogeneic tumour (Gati et al. 2004;Pierson et al.
1996). The observation that NK cell-mediated killing of tumour target cells occurs
without having undergone any restorative measures is in itself evidence against NK
cell functional impairment or incapacity. Loss of CD3-ζ expression is the most
frequently cited example of a defective NK cell phenotype (Buggins et al.
1998;Healy et al. 1998;Kono et al. 1996;Lai et al. 1996;Nakagomi et al. 1993) and
since some of the most important NK cell activating receptors involved in tumour
killing are associated with CD3-ζ, including CD16 (Wirthmueller et al. 1992) and
several NCRs(Nagakomi et al. 199;Anderson et al. 1990;Vitale et al. 1998), a
generalized loss of function is expected. However, tumour-primed NK cells, which
were shown here and elsewhere (North et al. 2007) to have enhanced effector
functions relative to resting NK cells, also exhibit marked downregulation of
numerous activating receptors. Specifically, changes in NK cell phenotype after
tumour-priming included a significant loss of CD16, NKG2D, NKp46 and DNAM-1.
Several studies have reported better killing of tumour targets by NK cell subsets with
downregulated receptors such as CD16 or NKp46 compared with their counterparts
with normal expression (Gati et al. 2004;Penack et al. 2005). My data supported this
observation by demonstrating that the CD56^dimCD16^− NK cell subset that arises in
response to tumour-priming is the main degranulating subset in a 4-hour cytotoxicity
assay against tumour cell targets (Chapter 3). This argues that ligand-induced
downregulation of NK cell activating receptors is part of a specific NK cell response
to tumour stimulation, as has been previously reported (Groh et al. 2002;Huard and
Karlsson 2000;Linsley et al. 1993;Valitutti et al. 1997), rather than a marker of
dysfunction. When Romee et al. (Romee et al. 2013) investigated the expression of
NK cell activating receptors following cytokine stimulation, they observed that none
of the NCRs, or co-activation NK cell receptors CD2, DNAM-1, NKG2D and CD57
were down-regulated in response to cytokine-priming (Romee et al. 2013). The
results shown here confirm this observation, demonstrating that many of these
activating receptors are uniquely down-regulated in response to CTV-1, but not after activation with IL-2, IL-7, IL-12 or IL-15. This further supports the specificity of the NK cell response to target cell stimulation and more specifically priming with CTV-1. It is likely that target cell stimulation specifically induces NK cell responses through shedding of these receptors. It is also possible that these receptors are shed following stimulation to complete the tumour-priming stage of NK cell activation, with other receptors being involved in the ‘triggering’ stages. In any case, based on these observations, it would be inaccurate to link this change in phenotype following stimulation with tumour cells with NK cell impairment.

Another commonly reported observation linked to NK cell impairment in cancer is a weakened capacity for IFN-γ release (Chang et al. 1989; Tajima et al. 1996; Wakiguchi et al. 1994). Recent studies have highlighted hierarchies in the strength of the activating stimuli required for different NK cell responses (Bryceson et al. 2005; Bryceson et al. 2009; Fauriat et al. 2010). Signals for LFA-1-dependent NK cell adhesion and release of chemokines such as MIP-1β, exhibit a low threshold for activation, which in some cases can be met through the engagement of a single NK cell activating receptor. Degranulation and the release of other cytokines such as TNF-α require stronger activating stimuli. IFN-γ release has the highest activation threshold for NK cell receptor cooperation (Bryceson et al. 2011a). Thus, defective cytokine production by tumour-associated NK cells, which is often reported as a decrease in INF-γ release, can be explained by the absence of sufficient activating signals necessary for its secretion. As shown in Chapter 4, in the case of CTV-1 stimulation, the ligand combination presented by the tumour cells is sufficient to meet the activation threshold for chemokine, TNF-α and INF-γ secretion, as demonstrated by the secretory profiles of tumour-primed NK cells, but not for degranulation. Fauriat et al. (Fauriat et al. 2010) previously showed that in contrast to cytokine stimulation, target cell recognition of K562 cells by resting NK cells induces a pro-inflammatory cytokine profile characterized by MIP-1α, MIP-1β, RANTES, TNF-α and IFN-γ. Interestingly, stimulation with CTV-1 cells consistently induced the secretion of these cytokines at a much higher rate compared with K562
cells after a short incubation period (Chapter 4). A possible explanation for the enhanced cytokine secretion following CTV-1 stimulation is that NK cells that are primed but have yet to progress through the triggering stages are more responsive than NK cells that have gone through the full activation cycle. Another explanation lies in the ligand expression profile of CTV-1 cells, which might hold the code for better synergy among NK cell activating receptors for optimal cytokine secretion. Either way, using a cytokine that has the highest requirement for NK cell receptor cooperation after degranulation, as a measure of defective cytokine production by NK cells can be misleading.

In a similar manner to NK cells, tumour-associated T lymphocytes can recognize and eliminate autologous tumours after ex vivo culture with IL-2 (Lai et al. 1996; Matsuda et al. 1995; Mulder et al. 1997), or anti-CD28 and anti-CD3 mAbs (Renner et al. 1996), despite their inability to kill those targets in situ. Chronic stimulation of T cells, in the absence of a second activation signal, has also been shown to decrease T cell receptor expression, proliferative capacity and responsiveness (Correa et al. 1997). It can be easily envisaged that chronic stimulation of NK cells by tumours or any other inflammatory setting, results in a similar reduction in proliferation and response. Collectively, the observations discussed above argue against the commonly reported conclusion of NK cell dysfunction. Instead, these observations argue for a tumour-specific NK cell response, and suggest the absence of sufficient activating signals for full NK cell effector function in the tumour microenvironment. A tumour-primed NK cell waiting for the second signal to trigger killing, is likely to have down-regulated receptors involved in the priming stage, but is still functional and ready for killing upon receipt of secondary stimulation. In the following section, the NK cell receptors involved in the tumour-priming stage are elucidated.
6.3 What are the activating signals involved in tumour-priming?

Upon contact with a susceptible target cell, resting NK cells progress through the discrete activation events of adhesion, stable conjugate formation, granule polarization and exocytosis in order to mediate effective natural cytotoxicity. Resistance to NK cell-mediated killing can be attributable to a deficiency in any of the signalling requirements for the events leading to granule exocytosis. Activating NK cell receptors contain highly divergent cytoplasmic signalling domains and belong to a number of different receptor families, many of whose signalling pathways are not well defined (Bryceson et al. 2006a; Watzl and Long 2010). Tumour-priming data from this Thesis are discussed below in the context of data from studies investigating activation requirements for distinct NK cell events and the effect of interference from inhibitory signals.

6.3.1 Contact/Adhesion

The initial contact between an NK cell and a target cell may involve any of a number of receptors that induce LFA-1-dependent adhesion through ‘inside-out’ signals (Bryceson et al. 2009). Inside-out signals augment ligand affinity by inducing an open conformation in the LFA-1 receptor. In NK cells, LFA-1 can provide its own inside-out signal to promote a signal-dependent increase in binding of NK cells to ICAM-1 (Barber and Long 2003). This is in contrast to T cells, in which LFA-1 remains in a closed conformation unless the TCR or a chemokine receptor transmits inside-out signals that induce an extended conformation (Luo et al. 2007). Other NK cell receptors including CD2, 2B4, NKG2D, and DNAM-1 can also signal upstream of LFA-1, even in the absence of its ligands, to induce inside-out signals and promote NK cell adhesion to targets (Bryceson et al. 2009). Co-engagement of LFA-1 through its ligand ICAM-1, with some of these receptors results in a higher frequency of open LFA-1 relative to individual ligation, as shown in experiments using Drosophila S2 cells expressing individual and combined ligands for NK cell receptors (Bryceson et al. 2009). CTV-1 cells express ligands for at least three of these receptors: LFA-1, CD2 and NKG2D. Thus, it is likely that this ligand combination is responsible for synergistic NK cell adhesion and completion of the
first event in NK cell activation. LFA-1 is expressed at uniformly high levels on resting NK cells, as shown with conformation-independent mAbs in this Thesis (Chapter 3). Other studies using conformation-dependent mAbs 327C and mAb24 demonstrated heterogeneous staining of open-LFA-1 (Bryceson et al. 2009). The guanine exchange factor and actin regulator, VAV-1, has been postulated to be a common denominator of signalling pathways downstream of LFA-1 inside-out signals (Bryceson et al. 2011b). Interestingly, VAV-1 phosphorylation also provides a point at which interference by inhibitory receptor signals can oppose signals from activating receptors to abrogate target cell adhesion. Specifically, this is thought to occur through the recruitment of SHP-1, which binds phosphorylated VAV-1 (Stebbins et al. 2003).

6.3.2 Conjugate/Synapse formation

Another critical role of LFA-1 in NK cells is the organization of the cytotoxic immunological synapse (Long et al. 2013). Specifically ICAM-1 ligation has been shown to be crucial for the formation of stable NK cell-target cell conjugates (Barber et al. 2004; Bryceson et al. 2005; Bryceson et al. 2009). Studies by Liu et al. (Liu et al. 2009) imaging human primary NK cells on lipid bilayers carrying ligands of activation receptors have also shown that the formation of an organized natural cytotoxicity synapse to be dependent on the presence of ICAM-1. Additionally, ICAM-1 ligation is a requirement for the formation of a stable central zone at the synapse where active membrane internalization occurs and where exocytosed lysosome-associated protein (LAMP)-1 molecules are retrieved into an endocytic compartment, presumably for the recycling of lytic granule membranes (Liu et al. 2009). Again, the presence of ICAM-1 on CTV-1 cells is likely to play an important role in the formation of stable immunological synapses and the completion of the second event required in the progression of NK cells towards natural cytotoxicity. In the bead stimulation experiments (Chapter 5), beads coated with ICAM-1 formed more conjugates with NK cells than beads coated with ligands for NKG2D, CD2 or even anti-LFA-1, which supports the observation that ICAM-1 is important for the formation of stable conjugates with NK cells.
6.3.3 Granule Polarization

Finally, LFA-1 promotes the polarization of perforin-containing granules towards the target cell, facilitating efficient cytotoxicity (Barber et al. 2004; Bryceson et al. 2005). Granule polarization towards the immune synapse is the result of two different molecular processes. First, granules rapidly converge in a dynein-dependent manner to the microtubule-organizing centre (MTOC) (Mentlik et al. 2010) as initially described in T cells (Mentlik et al. 2010). In the NK cell line YTS, blockade of LFA-1 with antibodies results in an impairment of granule convergence at the MTOC upon target cell contact, suggesting that this process is LFA-1 dependent (Mentlik et al. 2010). Second, following granule convergence the MTOC and granules polarize towards the interaction site in a talin/paxillin-dependent manner (Bryceson et al. 2005; Mentlik et al. 2010). Blockade studies and bead stimulation experiments (Chapter 5) demonstrated the involvement of ICAM-1 as presented by CTV-1 cells in the tumour-priming of resting NK cells. Results in this Thesis suggest that ICAM-1 plays an important role in the tumour-priming signal. According to the literature discussed thus far ICAM-1 engagement should lead to the completion of NK cell activation events up to granule polarization. Future work should confirm that CTV-1 ligation by resting NK cells results in cytotoxic granule polarization.

6.3.4 Granule maturation and exocytosis

Vesicle exocytosis is the last requirement in NK cell activation events leading up to the triggering of natural cytotoxicity (Bryceson et al. 2006b). NK cells mediate target cell killing by polarised release of the contents of cytotoxic granules at the immunological synapse towards their target cells. In human NK cells, signals for granule polarization and for degranulation can be uncoupled, and the binding of LFA-1 to ICAM-1 is sufficient to induce polarization but not degranulation (Barber et al. 2004; Bryceson et al. 2005; Bryceson et al. 2009). This is in contrast to CD16, the engagement of which triggers degranulation, but not polarization (March and Long 2011). Recent studies in PLC-γ knockout mice have demonstrated an essential role for PLC-γ in granule exocytosis (Caraux et al. 2006; Tassi et al. 2005). NK cells
from these mice adhered to target cells and polarized their granules, but failed to mediate cytotoxicity. This is similar to the ability of CTV-1 cells to prime resting NK cells to activate, without triggering degranulation or NK cell-mediated killing of targets. Based on the ligand combination presented by CTV-1 tumour cells to resting NK cells, it is possible that CTV-1 stimulation overcomes the threshold for VAV-1 signalling, but not PLC-γ activation. NK cells from patients with mutations in either STIM1 or ORAI1 display defective degranulation, demonstrating a requirement for ORAI1-mediated store-operated Ca\textsuperscript{2+} entry (SOCE) for lytic granule exocytosis (Maul-Pavicic et al. 2011). This defect occurred at a late stage of the signalling process, because activation of LFA-1 and cytotoxic granule polarization were not impaired. How signals for PLC-γ activation by LFA-1 and CD16 selectively induce polarization or granule release, respectively, remains to be elucidated (March and Long 2011). The involvement of ICAM-1 in the delivery of the tumour-priming signal by CTV-1 as confirmed by blockade data (Chapter 5) suggests that the third event in NK cell activation (granule polarization) leading up to granule exocytosis should be complete. However, because CTV-1 cells are not susceptible to NK cell-mediated killing and resting NK cells do not degranulate in response to CTV-1 stimulation (Chapter 3), it is likely that tumour-priming by CTV-1 induces the progression of NK cell activation events up to granule polarization only. Upon additional stimulation, and the possible overcoming of the signalling threshold for PLC-γ, granule exocytosis and therefore NK cell killing is triggered. Figure 6.1 outlines the activation events discussed in this section coupled with their signalling requirements.
Resting NK cells progress through distinct activation events starting with contact/adhesion to target cells, followed by conjugate formation, and finally granule polarization and exocytosis. The requirement for receptor engagement for each activation event and the measurement assay for each distinct step are outlined.
6.3.5 Tumour-priming model for NK cell activation

In NK cells, the outcome of signalling by LFA-1 alone is sufficient to induce granule polarization (Barber et al. 2004; Bryceson et al. 2005; Bryceson et al. 2009). Individual engagement of the co-activation receptors CD2, 2B4 or NKG2D can induce LFA-1 inside-out signals for NK cell adhesion, independently of LFA-1 (Bryceson et al. 2009). However, co-engagement of LFA-1 by ICAM-1 on target cells, with any of those co-activation receptors results in enhanced activation signals. The final event in NK cell-mediated natural cytotoxicity seems distinct in its signalling requirements. Specifically, co-engagement of LFA-1+ 2B4 +NKG2D has been demonstrated as the minimal requirement for receptor cooperation in resting NK cells. Pre-activation with an exogenous cytokine such as IL-2 can reduce the signalling requirement for natural cytotoxicity to LFA-1 engagement only (Bryceson et al. 2009). This implies that priming by cytokines, which may be similar to the concept of ‘general priming’, decreases the threshold for full NK cell activation, but still requires the specificity of integrin co-engagement (Daniel Davis, verbal communication). Tumour-priming by CTV-1 circumvents this requirement, because it provides the activation signals needed for tumour cell killing specifically. Our group had previously hypothesized a two-stage hypothesis for tumour killing with distinct ‘priming’ and ‘triggering’ stages for NK cell activation by tumours (North et al. 2007). In this Thesis, I show through blockade and bead stimulation experiments that interactions between LFA-1-ICAM-1, CD2-CD15, and NKG2D-MICA/B are crucial for the delivery of the tumour-priming signal. The second ‘triggering’ signal can be provided upon subsequent stimulation with a target cell, as demonstrated by cytotoxicity assays with NK-resistant RAJI cells. Tumour-primed NK cells are then capable of killing previously-resistant targets as shown here, and in other studies by our group (North et al. 2007; Sabry et al. 2011). After NK cell-mediated killing of a tumour target cell is achieved, it can be envisaged that an NK cell is able to restart the activation cycle with the next target cell encounter, such as in the case of IL-2-activated NK cells, which have the capacity to serially hit up to 4 target cells (Bhat and Watzl 2007). Figure 6.2 demonstrates the proposed tumour-priming model.
Figure 6.2 NK cell activation stages and events.

Resting NK cells require an initial priming signal delivered by an activating cytokine or a target cell expressing the ligands necessary to induce adhesion, conjugate formation and granule polarization. Co-stimulation of additional NK cell activating receptors by the triggering ligands results in NK cell-mediated cytotoxicity against target cells through granule exocytosis.
6.4 Is it time to replace the missing-self hypothesis?

The ‘missing-self’ hypothesis was proposed in 1990 based on the observation that NK cells kill targets with reduced or absent self MHC class-I molecules, a phenomenon common to virally-infected and transformed cells (Karre et al. 1986; Ljunggren and Karre 1990). The subsequent characterization of NK cell inhibitory receptors supported this hypothesis by explaining the molecular mechanisms by which NK cells sensed the downregulation of MHC class I expression (Brooks et al. 1997; Carretero et al. 1997; Colonna and Samaridis 1995; D'Andrea et al. 1995; Karlhofer et al. 1992; Lazetic et al. 1996; Moretta et al. 1994; Phillips et al. 1996; Sivori et al. 1996; Wagtmann et al. 1995). However, when studies began to show that the absence of MHC class I molecules on tumour cells was insufficient to trigger NK cell lysis, it became clear that our understanding of NK cell target recognition was incomplete (Costello et al. 2002; Ruggeri 2002). As a wide array of activating receptors started to be discovered, the ‘dynamic equilibrium’ hypothesis was formulated, postulating that the integration of opposing signals from activating and inhibitory receptors determines the functional outcome of NK cell activity (Brumbaugh et al. 1998).

NK cells are negatively regulated by KIRs, which bind HLA-A, -B and -C, and C-type lectins, which form CD94/NKG2 receptor complexes recognizing HLA-E (Moretta et al. 2003). The role of HLA-mediated inhibition in regulating NK cell activity is evidenced by studies showing that transfection of appropriate HLA-C alleles into NK-susceptible target cells, such as K562, can render them resistant to NK-mediated lysis (Addison et al. 2005; Ciccone et al. 1992). Additionally, NK-resistant tumours such as the B lymphoma cell line RAJI are known to constitutively express type I and II HLA-C alleles. In the clinical setting, HSC transplantation across HLA barriers has been shown to trigger donor NK cell alloreactivity if the recipient lacks KIR ligands that are present in the donor, which is known as a ‘KIR-ligand mismatch’ (Farag et al. 2002; Parham and McQueen 2003). Studies involving acute myeloid leukaemia (AML) patients demonstrated that donor alloreactive NK
cells eliminate not only residual leukaemic cells but also host T cells and antigen-presenting cells, thereby preventing the development of GVHD (Ruggeri 2002). However, the impact of KIR-mismatch in other clinical settings remains very controversial with several studies showing no advantage of KIR-ligand incompatibility for survival or engraftment (Davies et al. 2002; Malmberg et al. 2005). Pre-incubation of NK cells with an activating cytokine such as IL-2, can result in NK cell killing of targets that were previously resistant. Tumour target cells can also be used to activate NK cells in a manner analogous to IL-2, as demonstrated in this Thesis and in other studies using the acute lymphoid leukaemia cell line CTV-1 (North et al. 2007; Sabry et al. 2011). Tumour-priming of NK cells has been shown here to generate NK cells that are able to lyse the NK-resistant tumour cell line RAJI, and other studies have extended these findings to primary leukaemias and solid tumours, in HLA-matched, allogeneic or autologous settings (North et al. 2007; Katodritou et al. 2011; Sabry et al. 2011). Moreover, transfection of resistant, HLA-expressing RAJI cells with specific ligands for NK cell-activating receptors renders them susceptible to NK cell lysis (Sabry et al. 2011). Similarly, blockade of certain tumour ligands for activating NK cell receptors on non-HLA expressing, sensitive K562 cells makes them resistant to NK cell-mediated killing.

Recent evidence has shown that when the minimal requirements for NK cell cytotoxicity are met, tumour killing can occur irrespective of the presence of inhibitory signals, which suggests that proponents of the missing-self theory might have been overstating their case. Upon contact with susceptible cells, a multiplicity of activating receptors can produce signals for NK cell-target cell adhesion. Engagement of the integrin LFA-1 mediates firm adhesion, provides signals for granule polarization and orchestrates the structure of an immunological synapse that facilitates efficient target cell killing. Other activating receptors signal for lytic granule exocytosis, a process that requires overcoming a threshold for activation of phospholipase C-\( \gamma \), which in turn induces SOCE entry as well as exocytosis mediated by SNARE-containing perforin syntaxin-11 and regulators thereof. Recent studies investigating the effect of licensing on different steps in NK cell cytotoxicity have
demonstrated that the involvement of inhibitory receptors in the determination of NK cell responsiveness is limited to the first event in NK cell activation. Only proximal signals, such as inside-out signalling by activating receptors, but not integrin outside-in signalling for granule polarization were affected by MHC class I engagement (Long et al. 2013). Unlicensed NK cells did not form as many stable conjugates with target cells, but for those unlicensed NK cells that did form conjugates, LFA-1-dependent granule polarization was similar to that in licensed NK cells. Thus, providing NK cells with the appropriate combination of activating stimuli unleashes full effector function, such that an NK cell can kill tumour targets even in the presence of strong inhibitory signalling.

6.5 Is NK cell priming different from NK cell memory?

A clear consensus on how the term ‘memory’ is defined in NK cell biology is currently lacking. Rolle et al. (Rolle et al. 2013) defined NK cell memory in the context of viral infections as the ability to respond more potently to a second challenge with the same antigen. Alternatively, the term ‘memory-like’ was proposed to describe long-lasting functional alterations that are induced by cytokines without clear evidence of antigen involvement. In the context of tumourigenesis, cytokine-priming induces long-term changes that can be considered memory-like traits in NK cells. For example, NK cells stimulated with the γ-chain cytokines used in this Thesis responded more potently to subsequent challenges, and secreted a plethora of cytokines that affect the tumour microenvironment and further regulate immune responses (Chapter 4). On the other hand, NK cell responses to tumour-priming are antigen-induced, and thus, according to this definition cannot be considered to have memory-like properties. Instead, tumour-primed NK cells fall somewhere in between memory and memory-like NK cells, because although they have undergone antigen-induced long-lasting functional changes, they do not necessarily respond more potently to a second challenge with the same tumour antigen initially encountered. In fact, it is likely, based on the evidence discussed in previous sections, that a primed NK cell requires further engagement of different, non-redundant receptors to overcome the signalling threshold for the triggering stage. This necessitates a new
definition that distinguishes priming from memory, and accommodates the involvement of antigens in the anti-tumoural processes of NK cells.

NK cells undergo clonal expansion after priming with viral targets or exogenous cytokines, as well as after sensitization with haptens (Min-Oo et al. 2013; Sun et al. 2011). Tumour-priming of NK cells was also demonstrated here to result in the expansion of NK cell subsets with downregulated expression of specific receptors such as CD16 and CD62L (Chapter 3). In humans, most reports suggesting the existence of NK cell memory highlight the increased proportion of NKG2C⁺ NK cells in viral infections (Della et al. 2012; Foley et al. 2012a; Foley et al. 2012c; Lopez-Verges et al. 2011). The broad range of viruses that are reported to trigger the expansion of the NKG2C⁺ NK cell subset suggests that this phenomenon relies on an induced or altered self-ligand rather than on a shared pathogen-derived structure. It is possible that NKG2C is functionally involved in the anti-viral immune response (Beziat et al. 2012; Bjorkstrom et al. 2011; Brunetta et al. 2010; Petitdemange et al. 2011). In the case of tumours, the increase in proportion of specific NK cell subsets after co-incubation with CTV-1 suggests that these receptors are functionally involved in anti-tumoural responses and can be used as markers for NK cell tumour-specific responses. Further studies investigating the phenotype of resting NK cells after co-incubation with different tumours can build the activation profiles of anti-tumoural NK cells and identify tumour-specific markers.

The expansion of certain human NK cell subsets observed in various viral or tumoural responses might also reflect a first step in the subsequent generation of memory NK cells. For example, in the case of CMV responses, the expanded NKG2C⁺ NK cells were shown to be more potent producers of IFN-γ, express higher levels of CD57, and respond faster to secondary stimulation (Foley et al. 2012; Lopez-Verges et al. 2011). NK cells primed with CTV-1 were previously shown by our group to maintain their primed status longer than IL-2-stimulated NK cells, even after cryopreservation, as measured by NK cell-mediated killing upon subsequent stimulation. The mechanisms involved in maintaining the tumour-primed status are
unknown. Some studies have shown IL-12 to be central to the generation of NK cells with memory-like features (Sun et al. 2012). Other studies with both mouse and human NK cells indicate that a short in vitro exposure to a combination of IL-12, IL-15 and IL-18 yields memory-like NK cells that display superior effector function and longevity in vitro and in vivo, and that these properties were also inherited by daughter cells (Cooper et al. 2009; Ni et al. 2012; Romee et al. 2012). It is possible that these cytokines, which were shown to be secreted by tumour-primed NK cells (Chapter 4) play a role in NK-NK cell priming maintenance of tumour-activation signals and the development of memory-like features. It is also possible that antigen exposure during the tumour-priming process drives the formation of memory-like NK cells that contribute to protection against re-challenge with tumour cells in a manner that resembles anti-viral NK cell responses. The specific antigens that drive the expansion of NK subsets and the formation of NK memory, as well as the involvement of cytokines for the generation of long-lived NK cell populations with superior effector function should be investigated further.

6.6 Conclusion: The remaining questions

Upon their discovery, NK cells were viewed as ‘null’ cells because they were thought to lack any defining cell surface markers and because their origins in relationship to other immune cells were not understood. In a recent study, Blish and colleagues (Horowitz et al. 2013) revealed the presence of 6,000–30,000 phenotypically distinct NK cell subsets in the blood of any single human. This provides an instant perspective on the extensive heterogeneity that exists within the PB NK cell population. Moreover, NK cells in the spleen, liver, bone marrow, lymph nodes, lung, and decidual tissues are known to be phenotypically different from PB NK cells. This diversity may have evolved to generate the various NK cell responses observed in different biological frameworks. Other than their role in tumour immunosurveillance, NK cells play important roles in the control of pathogen infections, maintenance of homeostasis in the lymphoid system as well as reproduction. The heterogeneity observed in NK cell responses to tumour-priming compared with cytokine-priming in this study, substantiates evidence for the
specificity of NK cell responses to varying stimuli and ligand combinations presented by target cells. This display of heterogeneity stresses the importance of re-evaluating certain concepts in NK cell biology. For example, the criteria used to describe NK cell impairments in cancer need to take into account the signalling requirements for different NK cell effector functions. The specificity of NK cell responses to varying stimuli should also be taken into consideration in the application of strategies involving \textit{ex vivo} culture of NK cells to enhance NK cell functional properties. In the case of cancer immunotherapy, studying tumour-specific responses of NK cells should be the focal point for better specificity and efficacy of treatments.

Another important aspect in the design of NK cell-based immunotherapeutic strategies is interference from inhibitory NK cell receptors. Inhibitory signalling by NK cells was traditionally viewed as the dominant regulatory mechanism for NK cell activity, but recent studies have demonstrated that inhibitory signalling can only exert influence on early events of NK cell activation. NK cells primed with CTV-1 were shown here to overcome inhibitory signalling by MHC class I molecules on RAJI cells and, thereby, kill target cells that were previously resistant to NK cell cytotoxicity. Thus, further work on defining NK cell activation is critical for the development of novel NK-based therapeutic strategies for the treatment of cancer.

NK cell activation relies on synergy between NK cell activating receptors, rather than a single dominant receptor for activation. This allows NK cells flexibility in sensing and responding to changes in the tumour environment. Further work should investigate the optimal pairing of NK cell receptors to achieve better synergy in the clinic, and shed light on the specific pathways instigated by different activating NK cell receptors. Other important questions in NK cell activation include what happens after triggering of NK cell killing? And how do NK cells detach from a target cell to restart activation events? This facet of NK cell biology is important to understand so that serial killing by NK cells is optimized in translational applications. The precise signals involved in the different priming mechanisms also remain to be elucidated and the relationship between priming and memory needs to be further defined. One
question that still remains is why NK cells manifest different responses upon target cell recognition or cytokine stimulation. Whether there is differential regulation of the signal transduction pathways downstream of the activating receptors involved in priming requires further study. Finally, it remains to be determined how these observations apply to the *in vivo* microenvironment, where NK cells, cytokines and target cells coexist with various activating and inhibitory factors. Progress in our understanding of how NK cells function *in vivo* is absolutely critical if NK cells are to be used successfully in cancer immunotherapy.
Chapter 7 References


177


179


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183


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201


Appendix 1: Publications

Appendix 2: Abstracts

- The unique profile of tumour-primed natural killer cells shows loss of activating receptor expression and pro-inflammatory cytokine secretion upon activation.
  British Society of Immunology, Liverpool, UK, 2013

- Natural killer cell responses to tumour-priming.
  European Association for Cancer Research, Barcelona, Spain, 2012

- What it takes to prime a resting natural killer cell.
  The Federation of European Biochemical Societies, Algarve, Portugal, 2011
Index I: Standard curves for cytokine concentration:

- **IL-1β**
- **IL-1Rα**
- **IL-6**
- **IL-8**
- **GM-CSF**
- **TNF-α**
- **IFN-γ**
- **IL-2**