

**Biology of Interleukin-6  
Production by Human Natural  
Killer Cells**

**Thesis submitted in accordance with the requirements  
of the University of Liverpool for the degree of Doctor  
of Philosophy**

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# Declaration

All the work presented in this thesis is my own and any other work is appropriately referenced.

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## Abstract

NK cells secrete a variety of immune-regulatory cytokines and chemokines such as interferon -  $\gamma$  (IFN- $\gamma$ ) and tumour necrosis factor-  $\alpha$  (TNF- $\alpha$ ). However, in some of infections where NK cells have an important role in the defence mechanism, IL-6 appears to play a significant anti-viral role. Considering the role of both IL-6 and NK cells in these infections raises the possibility that IL-6 secretion by NK cells could be a main defence mechanism against them. Moreover, NK cell-mediated IL-6 secretion may provide a critical link between the innate and adaptive immune response of the host. Furthermore, changes in this pathway in various autoimmune diseases, for example rheumatoid arthritis (RA) may be relevant for the pathogenesis of these disorders.

The results presented in this thesis demonstrated that peripheral blood NK cells from healthy individuals have the ability to secrete IL-6 after co-culture with target cells against which these cells are known to exhibit direct cytotoxicity (either K562 or HeLa cells). The described secretory response was rapid, with IL-6 being significantly higher as early as 1 hour in HeLa co-cultures compared to 6 hours in case of K562 co-cultures. These findings were further confirmed when NK cells were activated alone with high doses of IL-2 or with non specific chemical activators (PMA+ ionomycin). These experiments clearly showed that NK cells have the potential to secrete IL-6 following activation. To analyse whether IL-6 secretion in the co-culture experiments was the result of direct cell-cell interactions between NK cells and the target cells or was induced by the presence of soluble mediators, co-culture experiments were set up where the target cells were separated from NK cells using a 0.4  $\mu\text{m}$  pore size inserts. Separating NK cells and target cells abolished increases in cytokine production proving that direct interaction between NK cells and target cells is necessary for triggering IL-6 production by NK cells, as the semi-permeable membrane of Transwell chambers allows for the free passage of soluble factors but prevents direct cell-cell contact.

Investigating the activating pathway which triggers the secretion of IL-6 by NK cells was the next step. This aim was achieved by inducing NK cell activation with immobilized antibodies against NK cell activating receptors and assessing the effect of the engagement of these receptors on peripheral blood NK IL-6 gene expression and protein secretion by quantitative real time PCR and ELISA. The results demonstrated that NKG2D and NKP46 were the two main receptors involved in the IL-6 mRNA expression and secretion by NK cells.

The final aim of this thesis was to evaluate the biological significance of this secretion through an *in vitro* experimental model. We hypothesized that IL-6 secreted by NK cells could contribute to the migration of other inflammatory and immune cells to the site of inflammation. This hypothesis was based on the observations of others that IL-6 could induce direct CD4<sup>+</sup> T cell migration. To test this hypothesis, an *in vitro* transmigration assay using Transwell inserts with 8 and 3  $\mu\text{m}$  pore size were used. Our results demonstrated that CD4<sup>+</sup> T cell migration in response to NK cells was inhibited by about 30% in the presence of neutralizing antibody to IL-6. These results signify the relative biological importance of IL-6 induced secretion by NK cells.

In conclusion NK cells can contribute to IL-6 secretion. Given that NK cells appear at inflammation sites at the earliest stages of the process, where the number of other cells with a potential to secrete IL-6 is low, it is possible that NK cell-mediated IL-6 secretion is essential in orchestrating and potentiating the later stages of the adaptive immune response.

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# Abbreviations

<b>ADCC</b>	Antibody dependent cellular cytotoxicity
<b>AICL</b>	Activation induced C type lectin,
<b>AMV</b>	Avian Myeloblastosis Virus
<b>APCs</b>	Antigen presenting cells
<b>ATAC</b>	Activation-induced, T cell–derived, and chemokine-related
<b>BAT-3</b>	HLA-B associated transcript 3
<b>BCDF</b>	B-cell differentiation factor
<b>BCSF-2</b>	B cell stimulatory factor- 2
<b>BSA</b>	Bovine serum albumin
<b>Caco</b>	Colorectal adeno carcinoma
<b>CAI</b>	Codon Adaptation Index
<b>CD</b>	Cluster of differentiation
<b>CDF</b>	Cytotoxic T-cell differentiation factor
<b>cDNA</b>	Complementary DNA
<b>CHO</b>	Chinese hamster ovary
<b>CLC</b>	Cardiotrophin-like cytokine
<b>CMV</b>	Cytomegalovirus
<b>CNTF</b>	Ciliary neurotrophic factor
<b>CREB</b>	CRE-binding protein
<b>CRP</b>	C - reactive protein
<b>Ct</b>	Cycle threshold
<b>CT-1</b>	Cardiotrophin-1
<b>CTL</b>	Cytotoxic T lymphocytes
<b>CVB4</b>	Coxsackie B4 virus
<b>DCs</b>	Dendritic cells
<b>DEPC</b>	Diethylpyrocarbonate
<b>DMSO</b>	Dimethyl sulphoxide
<b>DNA</b>	Deoxyribonucleic acid
<b>DNAM-1</b>	DNAX accessory molecule-1
<b>dNK</b>	Decidual natural killer
<b>dNTPs</b>	Deoxyribonuclease triphosphates

<b>EAU</b>	Experimental autoimmune uveoretinitis
<b>EBV</b>	Epstein–Barr virus
<b>ECACC</b>	European Collection of Cell Cultures
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>EGF</b>	Epidermal growth factor
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>ER</b>	Endoplasmic reticulum
<b>ESR</b>	Erythrocyte sedimentation rate
<b>FCS</b>	Foetal calf serum
<b>FITC</b>	Fluorescein isothiocyanate
<b>FRET</b>	Fluorescence Resonance Energy Transfer
<b>GAPDH</b>	Glyceraldehyde-3-phosphate dehydrogenase
<b>GM-CSF</b>	Granulocytes monocytes-colony stimulating factor
<b>GPI</b>	Glycosylphosphatidylinositol
<b>HEK</b>	Human embryonic kidney
<b>HSF</b>	Hepatocyte stimulating factor
<b>HSPGs</b>	Heparan sulphate proteoglycans
<b>HSV</b>	Herpes simplex virus
<b>ICAM-1</b>	Intercellular Adhesion Molecule 1
<b>iDCs</b>	Immature dendritic cells
<b>IEL</b>	Intestinal intraepithelial lymphocytes
<b>IFN</b>	Interferon
<b>Ig</b>	Immunoglobulin
<b>IL-6</b>	Interleukin-6
<b>iNK</b>	Immature natural killer (cell)
<b>ITAM</b>	Immunoreceptor tyrosine-based activation motif
<b>ITIM</b>	Immunoreceptor tyrosine-based inhibition motif
<b>JAK</b>	Janus tyrosine kinases
<b>KIRs</b>	Killer-cell immunoglobulin-like receptors
<b>KLRF1</b>	Killer cell lectin-like receptor subfamily F member 1
<b>KSHV-IL6</b>	Kaposi's sarcoma associated herpes virus interleukin-6 like protein
<b>LB</b>	Luria-Bertani
<b>LCMV</b>	Lymphocytic choriomeningitis virus
<b>LFA-1</b>	Lymphocyte function-associated antigen 1

<b>LIF</b>	Leukemia inhibitory factor
<b>LPS</b>	Lipopolysaccharides
<b>mAb</b>	Monoclonal antibody
<b>MAP</b>	Mitogen-activated protein
<b>MAR-1</b>	Mouse-activating receptor 1
<b>MCMV</b>	Murine cytomegalovirus
<b>MCP-2</b>	Monocyte chemoattractant protein-2
<b>MCS</b>	Multiple cloning site
<b>MDC</b>	Macrophage-derived chemokine and regulated on activation
<b>MGB</b>	Minor groove binder
<b>MGI-2</b>	Monocyte-granulocyte inducer type 2
<b>MHC</b>	Major histocompatibility complex
<b>MICA/B</b>	MHC class I chain-related gene A/B
<b>MIP</b>	Macrophage inflammatory protein family members
<b>MMP-2</b>	Matrix metalloproteinase-2
<b>mRNA</b>	Messenger Ribonucleic acid
<b>NCAM</b>	Neural Cell Adhesion Molecule
<b>NCRs</b>	Natural cytotoxicity receptors
<b>NF-IL6</b>	Nuclear factor for IL-6 expression
<b>NK</b>	Natural killer (cell)
<b>NKATs</b>	Natural killer associated transcripts
<b>NKRs</b>	Natural killer receptors
<b>NKT</b>	Natural Killer T (cell)
<b>OSM</b>	Oncostatin M
<b>P/HGF</b>	Plasmacytoma / Hybridoma growth factor
<b>PBMCs</b>	Peripheral blood mononuclear cells
<b>PBS</b>	Phosphate Buffered Saline
<b>PCR</b>	Polymerase chain reaction
<b>PDGF</b>	Platelet derived growth factor
<b>PE</b>	Phycoerythrin
<b>PI3K</b>	Phosphatidylinositol 3-kinase
<b>PKC</b>	Protein kinase C
<b>PMA</b>	Phorbol 12-myristate 13-acetate



<b>PTMs</b>	Posttranslational modifications
<b>RA</b>	Rheumatoid arthritis
<b>RANTES</b>	Regulated upon activation normal T-cell expressed and secreted
<b>RNA</b>	Ribonucleic acid
<b>RT-PCR</b>	Reverse transcription–polymerase chain reaction
<b>SAA</b>	Serum amyloid A
<b>SAP</b>	SLAM-associated protein
<b>SD</b>	Standard deviation
<b>SDS-PAGE</b>	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
<b>SF</b>	Synovial fluid
<b>SHIP-1</b>	SH2-containing 5' inositol phosphatase 1
<b>SLAM</b>	Signaling lymphocytic activation molecule
<b>SLE</b>	Systemic lupus erythematosus
<b>SLTs</b>	Secondary lymphoid tissues
<b>SPR</b>	Surface plasmon resonance
<b>STAT3</b>	Signal transducer and activator of transcription 3
<b>SV40 pAn</b>	Simian Virus 40 late polyadenylation
<b>SYK</b>	Spleen tyrosine kinase
<b>TAE</b>	Tris-acetate-EDTA
<b>TBS</b>	Tris-buffered saline
<b>TCR</b>	T cell receptor
<b>TEMED</b>	Tetramethylethylenediamine
<b>TGF</b>	Transforming growth factor
<b>Th</b>	T helper cell
<b>TMB</b>	Tetramethylbenzidine
<b>TNF</b>	Tumour necrosis factor
<b>TRAIL</b>	Tumour necrosis factor-related apoptosis-inducing ligand
<b>TREM-1</b>	Triggering receptor expressed on myeloid cells
<b>TSS</b>	Transformation and storage solution
<b>ULBP</b>	UL-16 binding protein
<b>WT</b>	Wild type
<b>ZAP70</b>	ζ-chain-associated protein 70 kDa

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# Chapter 1

Introduction



2012

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## **1.1 Natural killer (NK) cells**

Human natural killer (NK) cells are large granular lymphocytes that function predominantly as cytotoxic effector cells of the innate immune system. They comprise about 5-15 % of all circulating lymphocytes and play a major role in the early host defence against tumours and viruses prior to initiation of the adaptive immune response (Biron and Brossay, 2001; Zamai et al, 2007; Fietta and Delsante, 2009). They were named “natural killers” because of the initial notion that they did not require any previous immunisation or prior sensitisation in order to exert their cytotoxic functions. In addition NK cells also function as regulators of immune responses, via the secretion of immune-regulatory cytokines and chemokines and / or interaction with other immune cells to trigger an adaptive, or antigen-specific, immune response (Lünemann et al, 2009). In contrast to T- lymphocytes, natural killer cells do not express T-cell antigen receptor (TCR) or the pan T cell marker (CD3) (Ritz et al, 1988 ; Murphy et al, 1992). They also fail to express surface immunoglobulins (Ig) which characterise B cells (Murphy et al, 1992). Instead, human natural killer cells express the surface marker (CD56), a neural cell adhesion molecule (NCAM) isoform found on NK cells and a minority of T cells, and CD16 (FcγRIII). Based on the surface expression of CD56 and CD16, peripheral blood NK cells can be divided into at least two functional subsets. CD56<sup>dim</sup> CD16<sup>bright</sup> NK cells constitute about 90% of total blood NK cells and CD56<sup>bright</sup> CD16<sup>dim/-</sup> cells make up <10% of NK cells in the blood, but they are enriched in secondary lymphoid tissues (Cooper et al, 2001*a*).

### **1.1.1 Origin and development of NK cells**

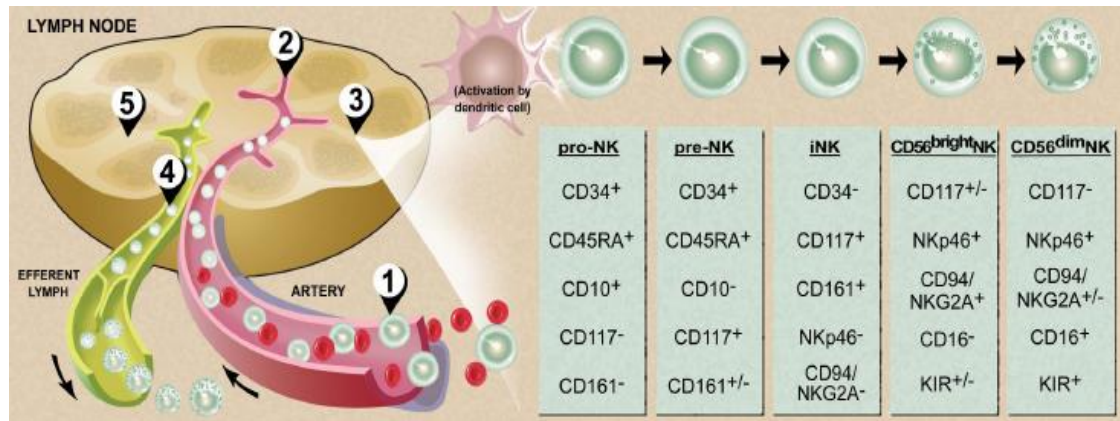
It has been generally accepted that NK cell development primarily occurs within the bone marrow (BM) from the same hematopoietic CD34<sup>+</sup> progenitor cells

(HPCs) as B and T cells but then they diverge from other lymphocyte lineages fairly early in their development (Fietta and Delsante, 2009). In the bone marrow NK cells develop from CD34<sup>+</sup> progenitor cells and express the phenotype CD34<sup>+</sup> CD45RA<sup>+</sup> (Figure 1.1). In mice, CD122 co-expression in the absence of other mature NK cell markers identifies these pre-NK cells (Freud and Caligiuri, 2006). In humans, pre-NK cells do not express CD122 and therefore identification of these pre-NK cells is more difficult (Freud and Caligiuri, 2006). Some studies suggested that co-expression of CD7, CD10, CD117 and CD45RA on CD34<sup>+</sup> cells are associated with a significant restriction toward NK cell development (Freud and Caligiuri, 2006; Caligiuri, 2008). Nevertheless, this phenotype is not specific for the NK cell lineage as B cells, T cells, and dendritic cells (DCs) can all be derived from this cell population (Galy et al, 1995). Functional NK cells can be derived *in vitro* following culture of CD34<sup>+</sup> cells in the presence of either interleukin-2 (IL-2) or IL-15, which signal through the shared IL-2 / IL-15 receptor  $\beta$  chain and the common  $\gamma$  chain (Mingari et al, 1997). These functional NK cells phenotypically and functionally resemble CD56<sup>bright</sup> NK cells (Carson et al, 1997). The expansion and differentiation of CD34<sup>+</sup> CD45RA<sup>+</sup> pre-NK cells is sustained by growth factors within the BM micro-environment including c-kit (KL) ligand, flt-3 (FL) ligand and cytokines such as IL-2, IL-7, IL-15 and IL-21 (Fietta and Delsante, 2009). c-kit and flt-3 ligands are crucial in the early commitment of CD34<sup>+</sup> progenitor cells (HPCs) towards the NK cell lineage. They also up-regulate the expression of IL-15 receptor complex on NK cell precursors (Yu et al, 1998).

Current evidence indicates that only the very early stages of NK cells development occur exclusively within the BM and the thymus and secondary lymphoid tissues (SLTs) are the fundamental sites for NK lymphocyte maturation (Di Santo and Vosshenrich, 2006; Fietta and Delsante, 2009). CD34<sup>+</sup> CD45RA<sup>+</sup> NK cells

are not only found in the BM, but also in thymus (Sánchez et al, 1994), cord blood (Canque et al, 2000) and SLTs (Freud et al, 2005) in concentrations even higher than those found in the BM. This enrichment of CD34<sup>+</sup> CD45RA<sup>+</sup> pre-NK cells together with immature NK (iNK) cells within SLT relative to BM or blood, along with an abundance of dendritic cells (DCs) and other antigen presenting cells (APCs) that express membrane bound IL-15, which is required for NK-cell maturation, suggested that SLTs may be a site for NK-cell development *in vivo* (Caligiuri, 2008).

Since the discovery of the CD56<sup>bright / dim</sup> NK cell subsets it has been postulated that CD56<sup>dim</sup> NK cells may represent a subsequent stage of CD56<sup>bright</sup> NK cell maturation (de Toledo et al, 2010). This hypothesis is supported by some data as only CD56<sup>bright</sup> (but not CD56<sup>dim</sup>) cells have convincingly been generated *in vitro* from CD34<sup>+</sup> CD45<sup>+</sup> pre-NK cells following treatment with IL-2 and/or IL-15 (Mrózek et al, 1996; Carson et al, 1997) , and only CD56<sup>bright</sup> NK cells express the c-kit immature cell marker (Cooper et al, 2001a). It is important to note that CD56<sup>bright</sup> NK cells appear to be the only lymphocytes with constitutive expression of the high-affinity IL-2R, while CD56<sup>dim</sup> NK cells express only the intermediate affinity IL-2R and do not express c-kit marker (Cooper et al, 2001a). This could explain, in part, why CD34<sup>+</sup> HPCs in LN differentiate preferentially into CD56<sup>bright</sup> NK cells, and why CD56<sup>dim</sup> NK cells proliferate weakly in response to high doses of IL-2 or IL-15 (de Toledo et al, 2010). CD56<sup>bright</sup> NK cells have been found to be predominantly present within SLTs (Freud and Caligiuri, 2006). Since CD56<sup>dim</sup> NK cells are more abundant in peripheral blood, it had been postulated that the CD56<sup>bright</sup> NK population is less mature than the CD56<sup>dim</sup> subset and activation of CD56<sup>bright</sup> NK cells within SLTs increased the expression of NK receptors that are characteristic of the CD56<sup>dim</sup> subset.



**Figure 1.1: Different stages of human NK cell development.** CD34<sup>+</sup>CD45RA<sup>+</sup> HPCs pro- NK cells leave BM circulate in the blood and then enter secondary lymphoid tissues (SLTs) through extravasation. Here, pro-NK cells are activated through contact with dendritic cells (DCs) and progress through distinct stages of maturation, namely immature NK (iNK), CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells. Mature CD56<sup>dim</sup> NK cells return to the circulation via the efferent lymph whereas most CD56<sup>bright</sup> NK cells remain within the SLTs. Illustration by Debra T. Darte. Reproduced from Caligiuri MA (2008).

## **1.1.2 NK cell subsets:**

### **1.1.2.1 Phenotypic properties**

NK cells are large granular lymphocytes possess a prominent kidney shaped nucleus and numerous granules, many of which are secretory granules containing the lytic effector molecules involved in NK cell-mediated cytotoxic activity. In addition, they have extensive and well developed endoplasmic reticulum and Golgi networks suggesting a significant level of constitutive protein synthesis.

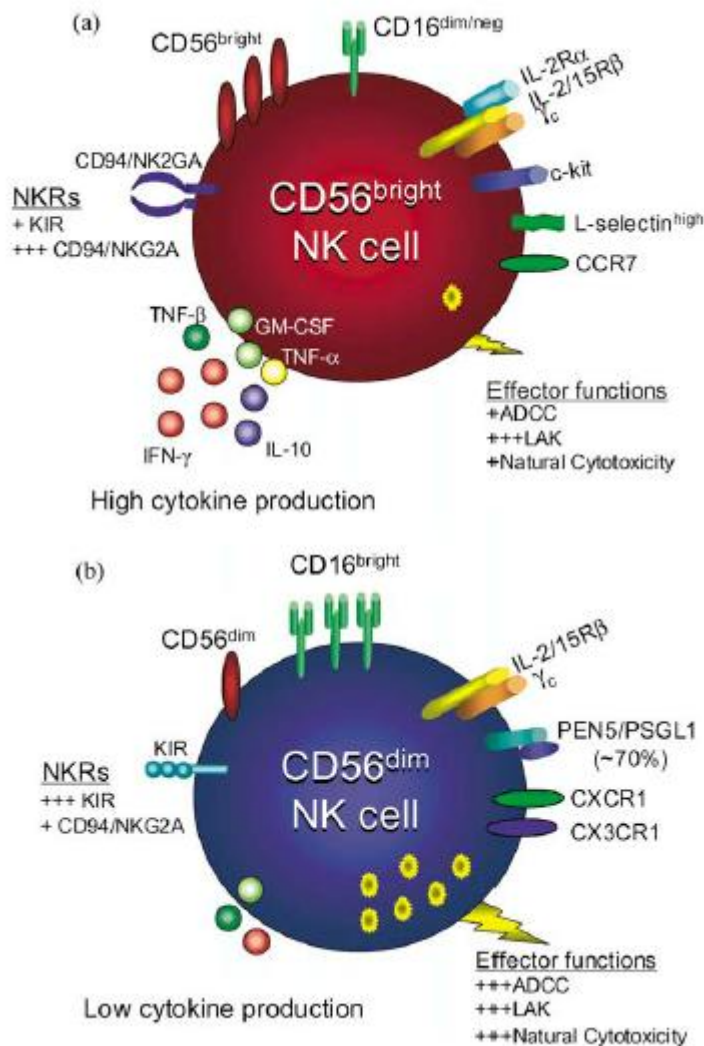
Human NK cells selectively express CD56, an isoform of the human neural cell adhesion molecule (NCAM) that is generally not expressed by other haematopoietic cells or lymphocytes (Lanier et al, 1989). The function of CD56 on human NK cells is controversial. Early studies suggested that this molecule might mediate interactions between human NK cells and target cells (Nitta et al, 1989; Suzuki et al, 1991). However, mouse NK cells do not express CD56 suggesting that this functional role on NK cells is not conserved (Hayakawa et al, 2006). Recent studies suggested a role for CD56 in promoting the terminal maturational step of NK cells (Caligiuri, 2008). Using an *in vitro* co-culture system consisting of purified CD56<sup>bright</sup> NK cells and human fibroblasts, Chan et al (2007) demonstrated that antibody blockade of the interaction between CD56 and fibroblast growth factor receptor-1 significantly inhibited the generation of CD56<sup>dim</sup> NK cells. Nevertheless, CD56 is particularly useful as a pan NK cell marker in humans.

The other widely used marker for the characterisation of NK cells is CD16, a low-affinity receptor for the Fc portion of IgG isotypes (Schumann et al, 1994). It is also found on monocytes / macrophages and neutrophils (Selvaraj et al, 1988; Wirthmueller et al, 1992). NK cell and macrophage isoforms of CD16 (FcγRIIIa) are integral membrane proteins in contrast to the GPI-linked isoform of neutrophils



(FcγRIIIb) (Fossati et al, 2001). CD16 is a potent activator of NK cell cytotoxicity, allowing NK cells to recognize and bind Ab-coated (opsonized) targets (Mandelboim et al, 1999). This mechanism is termed antibody-dependent cellular cytotoxicity (ADCC). Differences in the level of expression of CD16 have functional consequences for ADCC by NK-cell subsets, as will be discussed later.

Based on the cell surface density of CD56 and CD16, human NK cells have been phenotypically divided into at least two subsets with different functional patterns and tissue locations (Figure 1.2). In the peripheral blood of healthy individuals the majority (90%) of NK cells belong to the CD56<sup>dim</sup> CD16<sup>bright</sup> subset while a minority (10%) are CD56<sup>bright</sup> CD16<sup>dim/-</sup> (Cooper et al, 2001a). However, CD56<sup>bright</sup> CD16<sup>dim/-</sup> cells prevail in SLTs representing more than 75% of NK cells in these tissues (Moretta, 2010). It is also noteworthy that the infiltrating NK cells in a number of human inflammatory lesions were found to be mainly CD56<sup>bright</sup> CD16<sup>dim/-</sup> NK cells (Dalbeth et al, 2007) suggesting a prominent role for these cells both in secondary lymphoid tissues as well as at sites of infection and inflammation. In contrast to the CD56<sup>dim</sup> CD16<sup>bright</sup> NK cell subset, which efficiently kill target cells and secrete only low levels of cytokines, activated CD56<sup>bright</sup> CD16<sup>dim/-</sup> cells produce large amounts of cytokines including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and granulocyte monocyte-colony stimulating factor (GM-CSF), but acquire cytotoxicity after prolonged activation (Strowig et al, 2008). Recent studies revealed that CD56<sup>dim</sup> CD16<sup>bright</sup> cells can also be a potent source of cytokines (Moretta, 2010). CD56<sup>bright</sup> CD16<sup>dim/-</sup> and CD56<sup>dim</sup> CD16<sup>bright</sup> NK cells also differ from each other in the expression of inhibitory and activating NK cell receptors as well in the expression of adhesion molecules and chemokine receptors which facilitate homing to lymphoid tissues and the sites of inflammation. These will be discussed later on in this chapter.



**Figure 1.2: NK cell subsets.** NK cells are not a homogenous group of cells. Instead they are generally divided into two subsets based on the surface expression of CD56 and CD16 markers. **A)** CD56<sup>bright</sup> CD16<sup>dim/-</sup> NK cells account for ~ 10% of peripheral blood NK cells but they are enriched in SLTs. **B)** CD56<sup>dim</sup> CD16<sup>bright</sup> cells account for ~ 90% of peripheral blood NK cells. In addition to the differences in their localization, the two groups also differ in their surface expression of activating and inhibitory receptors, and adhesion molecules. Reproduced from Cooper et al (2001c).

### 1.1.2.2 Cytokine and chemokine receptors

All NK cells express the low-intermediate affinity interleukin-2 receptor (IL-2R $\beta\gamma$ ), whereas the high affinity heterodimeric receptor, IL-2R $\alpha\beta\gamma$  (CD25), is exclusively expressed on the CD56<sup>bright</sup> cells (Caligiuri, 1990). This subset has a high proliferative response to low doses of IL-2 alone and can be expanded *in vitro* and *in vivo* in response to picomolar concentrations of IL-2 in contrast to resting CD56<sup>dim</sup> NK cells which show almost no proliferation in response to high doses of IL-2 *in vitro* (Cooper et al, 2001a). High level of expression of some of monokine receptors including IL-1RI and IL-18R has also been noted in the CD56<sup>bright</sup> subset (Cooper et al, 2001a; Poli et al, 2009) suggesting that these cells probably receive some of their earliest activation signals from monocytes during the innate immune response.

The two subsets of NK cells also differ in terms of chemokine receptors and adhesion molecule expression, suggesting that they have different homing properties. CD56<sup>dim</sup> cells express high level of the chemokine receptors CXCR1, CXCR3, CXCR4 and CX3C-chemokine receptor 1 (CX3CR1) but lack the expression of CCR7 (Robertson, 2002). In contrast, CD56<sup>bright</sup> cells show little expression of CXC receptors but they do express high levels of CCR7 (Robertson, 2002). None of the NK cell subsets express CXCR2, CXCR5, or CCR5. These different chemokine profiles suggest the existence of differential trafficking for both CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cell subsets, influencing their functions in innate and adaptive immunity.

CD56<sup>bright</sup> CD16<sup>dim/-</sup> NK cells also constitutively express the adhesion molecule CD62L (L-selectin) (Frey et al, 1998). This molecule, together with CCR7, allows the recruitment of CD56<sup>bright</sup> subset from the blood into lymphoid tissues and site of inflammation. CD56<sup>bright</sup> NK cells also display a stronger expression of CD2, CD11c, CD44, CD49e and CD54 adhesion molecules, whereas CD56<sup>dim</sup> NK cells

express more CD11a (Poli et al, 2009). At sites of inflammation, CD56<sup>bright</sup> CD16<sup>dim/-</sup> cells display an activated phenotype (CD69), and undergo reciprocal stimulatory cell-to-cell interactions with monocytes/macrophages and DCs (Fietta and Delsante, 2009). Such a NK/DC “stimulatory synapse” promotes the DC secretion of IL-12, crucial not only for inducing the release of IFN- $\gamma$  by NK cells, but also for increased cytotoxicity (Fietta and Delsante, 2009).

### **1.1.2.3 Functional properties**

#### **1.1.2.3.1 Response to and production of cytokines and chemokines**

Although interleukin 15 (IL-15) has no sequence homology with IL-2, it interacts with components of the IL-2 receptor (IL-2R) (Carson et al, 1994; Freud and Caligiuri, 2006). High concentrations of IL-15 (nanomolar) can induce proliferation of CD56<sup>bright</sup> NK cells via signalling through the IL-2 receptor  $\beta$  and  $\gamma$  subunits that are common to both the IL-2 and IL-15 receptors (Carson et al, 1994). The proliferative effects of IL-15 on CD56<sup>bright</sup> NK could be abrogated by anti-IL-2R  $\beta$  and anti-IL-2R  $\gamma$  but not by anti-IL-2R  $\alpha$  antibodies. IL-15 is produced by activated monocytes/macrophages thus these cells may regulate NK cell function (Ferlazzo et al, 2004). Resting human NK cells constitutively express the IL-10 receptor (Carson et al, 1995). Although IL-10 does not induce proliferation of CD56<sup>bright</sup> or CD56<sup>dim</sup> NK cells, low concentrations of IL-10 significantly augment IL-2-induced proliferation of CD56<sup>bright</sup> NK cells (Carson et al, 1995). IL-10 is also able to induce significant NK cytotoxic activity against NK-resistant tumour cell targets in both subsets of NK cells in a dose-dependent fashion. Furthermore, the combination of IL-10 and IL-2 had an additive effect on NK cytotoxic activity (Carson et al, 1995). Production of IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF by IL-2-activated NK cells was also significantly enhanced by IL-10

(Carson et al, 1995). It is noteworthy that CD56<sup>bright</sup> cells require no co-stimulus to initiate their proliferative response to IL-2 or IL-15. This is in sharp contrast to both T-cells and B-cells which require additional signals from CD28 and CD40 respectively (Langley, 2005). This observation is consistent with an early effector role of NK cells in the innate immune response.

Freshly isolated CD56<sup>bright</sup> human NK cells are the primary source of NK-cell-derived immune-regulatory cytokines. These include IFN- $\gamma$ , TNF- $\alpha$ , and GM-CSF (Diefenbach and Raulet, 2001). During the innate immune response, when macrophages encounter pathogens, they produce a variety of monocyte-derived cytokines (monokines) which include IL-2, IL-12, IL-15 and IL-18. These monokines can then activate the production of cytokines by CD56<sup>bright</sup> NK cells. Studies have shown that IL-12 combined with IL-18 is the optimal stimulus for the production of IFN- $\gamma$  while IL-15 together with IL-18 results in optimal production of GM-CSF (Cooper et al, 2001a). These data suggest that the qualitative and quantitative production of monokines following infection of the host is probably important in determining the cytokine response of CD56<sup>bright</sup> NK cells. The production of these cytokines is also differentially regulated by IL-4 and IL-12. Whereas production of IFN- $\gamma$  in response to IL-2 is significantly inhibited or induced by IL-4 and IL-12 respectively, the production of other cytokines remains relatively unaffected (Perussia, 1996). Secretion of IFN- $\gamma$  by CD56<sup>dim</sup> NK cells is negligible when co-cultured with recombinant monokines. However, these cells seem to be a potent source for IFN- $\gamma$  and TNF- $\alpha$  when they come in contact with target cells (Fauriat et al, 2010).

Resting NK cells have been shown to express mRNA for different chemokines. These include macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ; CCL3), monocyte chemoattractant protein-2 (MCP-2; CCL8) and activation-induced, T cell-

derived, and chemokine-related (ATAC; XCL1) (Roda et al, 2006). Following activation, however, NK cells have been shown to dramatically up-regulate their expression of chemokine transcripts and to secrete chemokines with the capacity to recruit T cells, B cells, neutrophils, and other activated NK cells. These include MIP-1 $\alpha$ , MIP-1 $\beta$ , IL-8, macrophage-derived chemokine (MDC; CCL22), and regulated on activation, normal T-cell expressed and secreted (RANTES; CCL5) (Fehniger et al, 1999; Diefenbach and Raullet, 2001; Roda et al, 2006). NK cells produce these chemokines after activation with IL-2 or IL-12, or following activation of their receptors during interactions with target cells (Roda et al, 2006; Fauriat et al, 2010). However, differential secretion of chemokines by specific NK cell subsets has not been fully evaluated.

#### **1.1.2.3.2 NK cell cytotoxicity**

Unlike cytotoxic T lymphocytes (CTLs), NK cells do not require antigen-specific recognition to kill target cells, and as such are capable of limiting infection prior to the induction of adaptive immune response. Consistent with their more granular appearance, the cytotoxic activity of CD56<sup>dim</sup> NK cells is significantly higher than that of CD56<sup>bright</sup> cells (Poli et al, 2009). However, after activation with IL-2 CD56<sup>bright</sup> and CD56<sup>dim</sup> cells exhibit similar levels of cytotoxicity. The high expression level of CD16 on CD56<sup>dim</sup> cells makes them efficient mediators of antibody-dependent cellular cytotoxicity (ADCC), whereas CD56<sup>bright</sup> CD16<sup>dim/-</sup> NK cells perform ADCC only weakly or not at all (Poli et al, 2009). NK cell cytotoxicity is initiated via two main pathways, the *granule - exocytosis pathway* and the *receptor mediated (ligation of death receptors) pathway* (Cullen et al, 2008). In the granule - exocytosis pathway, several cytotoxic secretory granules are released from the

cytoplasm of NK cell into the immunological synapse between it and the target cell. These cytotoxic granules are formed during development and therefore, NK cells are equipped to respond rapidly to the threat of tumourigenicity or infection. The granule components are numerous powerful proteases together with other lytic molecules which, upon delivery to the target, cause rapid cell death in less than 20 min (Cullen et al, 2008). Of the proteases, perforin and granzymes are the most abundant. Upon release by NK cells, perforin integrates into the membrane of the target cell and disrupts its integrity allowing entry of granzymes to the inside of target cell. When delivered to the target cell, granzymes promote programmed target cell death (apoptosis) either indirectly through the activation of a family of death-inducing proteases called caspases or directly by fragmentation of the target cell DNA (Trapani and Smyth, 2002). Therefore, even if caspases are inhibited by viral proteins or are inactivated through mutation in an attempt from the pathogens to prolong the life of the infected cell, the granzyme-mediated proteolysis of other cellular substrates remains rapid, powerful and multi-faceted, and cell death follows in minutes, thereby limiting pathogen replication and spread.

Natural killer (NK) cell cytotoxicity was initially thought to be only dependent on perforin and granzymes. However, granzyme - deficient NK cells were also shown to retain potent *in vivo* antitumour activity despite the fact that these cells can not cause cell death by DNA fragmentation (Cullen et al, 2008). This suggests that the NK cells can employ another killing mechanism(s). Although many studies, including studies in perforin - deficient animals, have led to the conclusion that perforin / granzyme-mediated induction of cell death is still a principal pathway used by NK cells to eliminate virus-infected or transformed cells (Van den Broek et al, 1995; Kägi et al, 1996; Smyth et al, 1999; Pardo et al, 2002), it is now clear that NK

cells are also capable of using death ligands like Fas ligand (FasL) or tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) to induce cytotoxicity (Oshimi et al,1996; Eischen and Leibson, 1997; Kayagaki et al,1999; Wallin et al, 2003). However, still relatively little is known about the control of the "perforin and granzyme-independent" cytotoxicity, for example, the regulation of FasL expression on NK cells.

### **1.1.3 Regulation of NK cell functions**

#### **1.1.3.1 Missing self hypothesis (role of MHC-class I molecules)**

The first important clue as to the regulation of NK cell function came in 1986 from the observation that major histocompatibility complex (MHC) - class I deficient tumour cells were attacked by NK cells *in vivo* (Kärre et al, 1986). Since then, a "missing self" hypothesis was introduced proposing that NK cells discriminate target cells according to the level of self MHC-class I expression (Kärre et al, 1986; Ljunggren and Kärre, 1990). Thus, the absence or altered expression of MHC class I molecules would render target cells susceptible to NK cell attack while normal levels of MHC class I molecules would inhibit NK cells. Further support for the "missing self" hypothesis was shown in allogeneic BM grafts and T-cells lymphoblasts from  $\beta$ 2-microglobulin deficient mice that were attacked by NK cells from syngeneic wild-type (WT) mice and this rejection could be prevented by expressing a H-2 transgene (MHC-class I antigen) in the donor mice (Ohlén et al, 1989). In addition, the phenomenon of hybrid resistance, where a (A x B) F1 host rejects A or B grafts (A and B refers to MHC genotype), was known to be dependent on NK cells (Davenport et al, 1995; Kumar et al, 1997). Moreover, the notion that the rejection of class I MHC deficient bone marrow cells by NK cells from MHC-matched mice was a strong



suggestion that susceptibility to NK cell lysis of the parental graft was, at least in part, linked to the absence of specific MHC class I products in the donor (Ohlén et al, 1989; Yu et al, 1992; Borrego, 2006). Based on the "missing self" hypothesis NK cells survey the surface of host cells for the expression of autologous MHC class I molecules via surface receptors that deliver signals inhibiting NK cell functions. The failure to express one or more MHC-class I alleles by target cells represents a major threat to host integrity rendering them more susceptible to NK cell lysis. Certain viruses including herpesviruses and adenoviruses induce MHC-class I down regulation as strategies to evade HLA-class I- restricted CTLs (Petersen et al, 2003). However, this down regulation of MHC- class I molecules makes them more susceptible to NK cells lysis. This is particularly important in the early stages of viral infection when CTLs are unable to mediate protection and may represent a general mechanism by which NK cells limit virus spreading during the early phases of infection. Regarding MHC-class I expression in tumour cells, these often express low levels of these molecules and over 85% of human metastatic carcinomas display deficient HLA-class I expression (Garrido and Ruiz-Cabello, 1999; Moretta et al, 2004). This could reflect a loss of a single allele, or a general down regulation of all class I alleles. This regulatory mechanism has been demonstrated experimentally where NK cells lysed MHC-class I deficient EBV-transformed B-lymphoblastoid cell lines, whereas transfection of MHC-class I alleles into target cells inhibited lysis (Storkus et al, 1989; Shimizu and DeMars, 1989; Farag et al, 2002).

### **1.1.3.2 MHC - class I independent regulation of NK cell function**

Some studies have demonstrated that NK cells are still able to efficiently attack some target cells that express normal levels of MHC - class I molecules. For

example, some virus-infected cells that maintain expression of MHC class I at the cell surface can still be killed by autologous NK cells (Routes, 1992; Malnati et al, 1993; Farag et al, 2002). On other hand, some other cells are not sensitive to NK cell lysis despite low or absent class I MHC expression. For example, NK cells are unable to reject MHC class I-deficient non hematopoietic tissues, such as skin grafts, and *in vitro* they fail to lyse fibroblasts, even from  $\beta$ 2-microglobulin null mice that lack class I expression (Zijlstra et al, 1992; Farag et al, 2002). NK cells are also unable to attack human erythrocytes despite low or absent MHC class I expression by these cells (Lanier, 2003). Furthermore, ligation of NK activating receptors with membrane-bound molecules of target cells or monoclonal antibodies results in NK cell cytotoxicity and cytokine production (André et al, 2004). These observations point to the additional importance of activating receptors in regulating NK cell effector function. Therefore the regulation of NK functions appears to be more complicated than it was thought, and it is now largely accepted NK cell cytotoxicity and cytokine secretion are ultimately regulated by a balance of signals from activating and inhibitory receptors that interact with MHC class I and non class I-like molecules on target cells.

#### **1.1.4 NK cell receptors (NKR)**

Three major superfamilies of NKRs have been described in humans (Lanier, 1998; Cooper et al, 2001a): the killer cell immunoglobulin - like receptor (KIR) superfamily, which primarily recognizes classical MHC-class I molecules (HLA-A, -B and -C); the C-type lectin superfamily, which includes CD94 and NKG2 receptors recognizing non classical MHC class I molecules (HLA-E); and natural cytotoxicity receptors (NCRs) whose ligands are unknown. In general, triggering KIRs as well as

the CD94/NKG2A receptor on NK cells by classical and non-classical MHC class I molecules respectively delivers negative signals that prevent target cell killing (Lanier, 2005), although some activating KIRs are also available. In contrast, engagement of activating receptors such as NCRs by naturally occurring ligands provides an NK cell activating signal, which can also be mimicked by stimulating these receptors with appropriate antibodies (Moretta et al, 2001).

#### **1.1.4.1 MHC class I receptors**

##### **1.1.4.1.1 Killer-cell immunoglobulin-like receptors (KIRs)**

KIRs, originally known as natural killer associated transcripts (NKATs), are a family of protein molecules found on the surface of the NK and T cells that specifically recognize HLA-A, HLA-B and HLA-C alleles. They are encoded by a diverse and rapidly evolving family of genes located on chromosome 19p13.4 and different individuals differ in the number and type of inherited KIR genes (Trowsdale, 2001; Vilches and Parham, 2002). The KIR family of proteins has also been designated CD158 a-k (André et al, 2001).

KIRs are named according to whether they have two extracellular domains (2D) or three extracellular domains (3D) and whether they possess a short (S) or long (L) cytoplasmic tail (Colonna and Samaridis, 1995; Long et al, 1996). KIRs with long cytoplasmic tails contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) and have inhibitory functions (Colonna and Samaridis, 1995; Long et al, 1996; Trowsdale, 2001; Vilches and Parham, 2002). Therefore, NK cells expressing KIR with ITIM sequences in their cytoplasmic domains (KIR2DL and KIR3DL) are inhibited from lysing target cells that express MHC class I molecules. This interaction usually also inhibits cytokine production by the effector cells (D'Andrea et al, 1996).

In contrast, expression of KIRs with short cytoplasmic tails without an ITIM (e.g. KIR2DS) have a potentially activating function through interactions with the adaptor molecule DAP12 and NK cells expressing such receptors appear to promote cytotoxicity against target cells expressing an appropriate MHC class I molecules (Moretta et al, 1995; Vilches and Parham, 2002). Despite the functional differences, activating and inhibitory variants of KIR molecules share the same MHC class I ligand due to the almost identical extracellular domain (Borrego et al, 2002). CD56<sup>bright</sup> CD16<sup>dim/-</sup> cells have low to absent expression of KIRs which are, in contrast, expressed by CD56<sup>dim</sup> CD16<sup>bright</sup> NK cells (Cooper et al, 2001a). The differential expression of the more complex inhibitory and activating KIR receptor repertoire by the latter subset of human NK cells probably enables the unique regulation of cytotoxic properties by these cells (Lünemann et al, 2009).

#### **1.1.4.1.2 CD94/NKG2 receptors**

In humans CD94/NKG2 receptors are a group of molecules structurally characterized by c-type lectin extracellular domain that require Ca<sup>2+</sup> for carbohydrate recognition (Drickamer, 1988). These receptors are type II integral proteins expressed as disulphide-linked heterodimers composed of an invariant common sub-unit, CD94, linked to a distinct glycoprotein member of NKG2 family (Lazetic et al, 1996; Brooks et al, 1997; Cantoni et al, 1998). It appears that the NKG2 glycoproteins are usually unable to be expressed on the cell membrane unless they are disulphide-linked to CD94. CD94 has very short cytoplasmic tail and by itself is unable to transduce signals, but is necessary for expression of NKG2 on the cell surface (Lanier, 1998).

CD94 and NKG2 genes are closely linked on human chromosome 12 in the NK gene complex (Houchins et al, 1991; Renedo et al, 1997). Whereas CD94 is

encoded by a single gene with limited or no allelic polymorphism, the NKG2 family is encoded by five genes designated NKG2A, NKG2C, NKG2E (Houchins et al, 1991), NKG2F (Plougaste and Trowsdale, 1997) and NKG2H which is an alternative splice variant of NKG2E (Bellón et al, 1999). Although NKG2D is encoded within the NK gene complex on human chromosome 12 but it is distantly related to NKG2 and shows only 20% sequence homology with other NKG2 family members (Houchins et al, 1991) and does not interact with CD94 (Wu et al, 1999). Apart from the NKG2F, NKG2 genes show some degree of sequence homology with each other within same species for example; NKG2C and NKG2E genes in humans are 92% identical indicating that, like the KIRs, they probably arose from recent duplication events (Middleton et al, 2002). Similar to KIRs, the CD94/NKG2 family of receptors has individual members with activating and inhibitory features that correlate with ITAMs or ITIMs subunits in their transmembrane and intracytoplasmic tails respectively.

The inhibitory members of this family are NKG2A and an isoform of NKG2A generated by alternative spliced pre-mRNA referred to as NKG2B (Houchins et al, 1991). NKG2A and NKG2B express two cytoplasmic ITIMs capable of interacting with SH2 containing phosphatase (SHP)-1 and SHP-2. CD94/NKG2A (B) heterodimers bind HLA-E, a non-classical MHC class I molecule in humans (Braud et al, 1998; Lee et al, 1998*b*; Navarro et al, 1999; López-Botet et al, 2000). The interaction of CD94/NKG2A (B) with HLA-E is a central mechanism by which NK cells indirectly monitor the expression of other classical MHC class I molecules within a target cell. To be expressed, HLA-E needs to bind conserved sequences derived from the leader sequences of classical MHC-class I molecules (Braud et al, 1998; Lee et al, 1998*a*). In other words, optimum expression of HLA-E molecules on target cells is dependent on the expression of other classical MHC-class I molecules. Thus NK cells

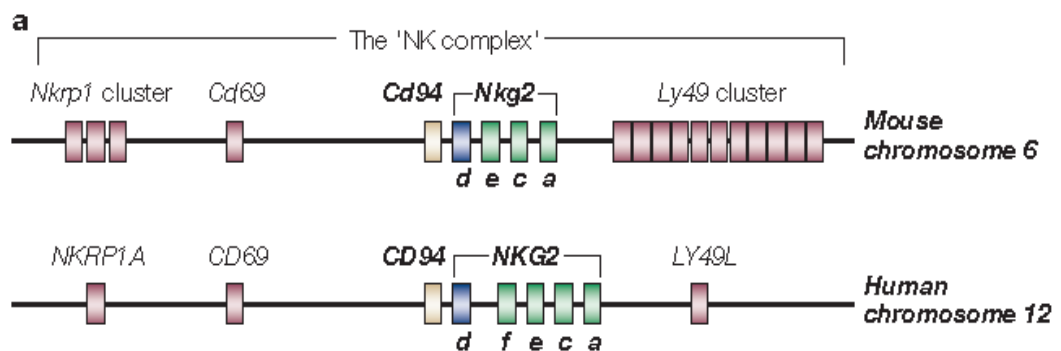
expressing CD94/NKG2A (B) are able to monitor indirectly the integrity of the expression of other MHC class I molecules.

The activating forms of NKG2 receptors are NKG2C, NKG2E and NKG2H with NKG2E and NKG2H being generated by alternative splicing of the same pre-mRNA transcript (Bellón et al, 1999). These molecules have intracellular tails without ITIM motifs and a charged amino acid in the transmembrane region necessary for the association with DAP-12, an immuno-receptor tyrosine-based activation motif (ITAM) - bearing adaptor molecule (Lanier et al, 1998; Braud et al, 1998; Vance et al, 1999; López-Botet et al, 2000). The ligand for these receptors is HLA-E (Braud et al, 1998; López-Botet et al, 2000). An interesting member of the NKG2 family is NKG2F. It differs from other NKG2 genes in that it has a shortened extracellular domain because of two additional nucleotides that lead to a premature stop codon but the transcripts of this gene have been detected in most NK clones (Plougaste and Trowsdale, 1997; Middleton et al, 2002). The putative NKG2F protein has a charged residue in the transmembrane region, a mock ITIM-like motif in the cytoplasmic tail and does not contain any C-type lectin domain (Kim et al, 2004). However, it can be associated with DAP12 in its transmembrane region thereby providing activation signaling potential (Kim et al, 2004). A conserved 24-amino acid sequence, present in all members of the NKG2 family, suggests that NKG2-F could form heterodimers with CD94 (Borrego et al, 2002). Regarding the expression of CD94/NKG2 receptors on different subsets of NK cells, CD56<sup>bright</sup> NK cells preferentially express the inhibitory CD94/NKG2A complex (de Matos et al, 2007; Lünemann et al, 2009).

## 1.1.4.2 Non - MHC class I specific receptors

### 1.1.4.2.1 NKG2D

The stimulatory natural killer group 2 D (NKG2D) receptor, also known CD314, is a type II trans-membrane protein (Houchins et al, 1991) with an extracellular C-type lectin-like domain (Li et al, 2001; Wolan et al, 2001). It was originally identified in 1991 as an orphan activating receptor expressed on human NK cells (Houchins et al, 1991). Subsequently, mouse, rat, and porcine homologues have also been identified. NKG2D is also expressed on CD8<sup>+</sup> T cells and subpopulations of  $\gamma\delta$  T cells (Bauer et al, 1999). The NKG2D gene is encoded within the "NK complex" (Figure 1.3) on human chromosome 12 (Glienne et al, 1998) and mouse chromosome 6 (Ho et al, 1998). Despite its name and its genomic location, NKG2D differs dramatically in sequence from the other NKG2 proteins (only 21% identity in amino acid sequence) (Houchins et al, 1991), does not associate with CD94 and does not recognize Qa-1/HLA-E (Wu et al, 1999; Diefenbach and Raulet, 2001).



**Figure 1.3: The human and mouse NKG2D gene.** The NKG2D gene is located within the NK cell complex on chromosome 6 in mouse and chromosome 12 in human. Other genes found in the human “NK complex” include CD69, CD94, NKR-P1A, and the NKG2 family while the mouse “NK complex” includes the NKG2 family, the Ly49 family, the NKR-P1 family, CD94 and CD69. Reproduced from Raulet DH (2003).

#### 1.1.4.2.1.1 NKG2D structure and distribution

Crystal structures of human and mouse NKG2D revealed that NKG2D retains an overall structure that is surprisingly homologous to other members of the C-type lectin family including CD94, Ly49A, and CD69, despite the relatively low sequence identity (Diefenbach and Raulet, 2001). NKG2D itself is more closely related to CD94 than other members of the C-type lectin family and it does not contain a putative carbohydrate binding site or any of the features that are characteristic of the Ca<sup>2+</sup> binding sites of C-type lectins (Middleton et al, 2002). Kinetic analysis indicated that the NKG2D interaction with its ligands such as MICA might be more stable than TCR - ligand and other NK receptor - ligand complexes (Li et al, 2001; Middleton et al, 2002). Although there is little obvious sequence similarity between human and mouse NKG2D ligands, there appears to be considerable structural similarity as shown by the ability of mouse NKG2D to react with human ligands, MICB, ULBP1, and ULBP2 (Kubin et al, 2001). Structural conservation is also indicated by the fact that, although the MICA and MICB proteins of various primate species are highly diverse, they are readily recognized by receptors on human intestinal epithelial  $\gamma\delta$  cells (Groh et al, 1998).

There are significant differences in the expression pattern of NKG2D receptors between mice and humans. In mice, NKG2D is expressed on the cell surface of almost all NK cells (Raulet, 2003). Increased expression is observed when NK cells are treated *in vitro* with IL-2 or IL-15 (Kubin et al, 2001). Resting peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells do not express significant levels of NKG2D, but the expression of the receptor is up-regulated by T-cell receptor (TCR) cross-linking in all CD8<sup>+</sup> but not in CD4<sup>+</sup> T cells (Diefenbach et al, 2000). Co-stimulation with anti-CD28 does not increase further NKG2D expression by CD8<sup>+</sup> T cells (Diefenbach et al, 2000;



Diefenbach and Raulet, 2001; Raulet, 2003). Approximately 25% of mouse splenic  $\gamma\delta$  T cells, all of which also express CD44, express NKG2D (Raulet, 2003). *In vivo* treatment of severe combined immunodeficiency syndrome (SCID) mice with IL-15 leads to up-regulation of NKG2D on splenocytes (Kubin et al, 2001). Almost all of the  $\gamma\delta$  T cells in the mouse epidermis (dendritic epidermal T cells) also express NKG2D, whereas most mouse intestinal intraepithelial  $\gamma\delta$  T cells do not (Raulet, 2003). In humans, virtually all NK cells also express NKG2D, and the levels are increased by exposure to IL-15 (Sutherland et al, 2002). In contrast to resting mouse CD8<sup>+</sup> T cells that do not express NKG2D, almost all human peripheral blood CD8<sup>+</sup> T cells express this receptor constitutively without activation, including those that lack expression of the co-stimulatory molecule CD28 (Diefenbach and Raulet, 2001; Raulet, 2003). Expression of NKG2D receptors by these cells is maintained after activation. As in mice, human CD4<sup>+</sup> T cells do not express NKG2D (Bauer et al, 1999; Diefenbach and Raulet, 2001). All human  $\gamma\delta$  T cells express NKG2D in contrast to mouse  $\gamma\delta$  T cells whose expression appears to be primarily restricted to the subset of cells that expresses CD44 (Diefenbach and Raulet, 2001; Raulet, 2003). In addition, although intestinal intraepithelial  $\gamma\delta$  T cells lack NKG2D expression in mice, almost all human intestinal intraepithelial  $\gamma\delta$  T cells express NKG2D at low levels, which can be increased by incubation with IL-15 (Raulet, 2003).

#### **1.1.4.2.1.2 NKG2D ligands**

Several families of ligands have been described for NKG2D in humans, all of which are distantly related to MHC class I molecules.

## 1. The MIC family of proteins (MICA and MICB)

MICA (MHC class I chain-related gene A) is a heavily glycosylated transmembrane glycoprotein that functions as a ligand for human NKG2D (Bauer et al, 1999). A closely related protein, MICB, shares 85% amino acid identity with MICA (Bahram and Spies, 1996, Bahram et al, 1996*b*). Although they share some structural similarity with MHC-class I molecules in that they possess three extracellular Ig-like domains ( $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  domains) there is only 27% amino acid identity with human MHC class I, they do not associate with  $\beta 2$ -microglobulin and have no capacity to bind peptide (Li et al, 1999). The genes encoding these proteins are found within the MHC locus in most but not all mammalian species (Groh et al, 1996; Steinle et al, 1998). However, they are absent in mice where no MIC homologues have been identified. Instead, in this species NKG2D binds to H60 (Cerwenka et al, 2000; O'Callaghan et al, 2001, Cerwenka et al, 2002) and the members of the Rae-1 family (O'Callaghan et al, 2001). Both MICA and MICB loci are highly polymorphic with more than 50 and 12 recognized human alleles respectively (Petersdorf et al, 1999; Stephens, 2001). The functional significance of this polymorphism remains unclear. Because of MICA's proximity to HLA-B, which is associated to a number of autoimmune diseases, most efforts to study the role of MICA in human diseases are mainly focused on the potential associations with autoimmune diseases (Choy and Phipps, 2010). For example, Behçet's disease was among the first to be associated with a MICA polymorphism (Choy and Phipps, 2010). However, several later studies concluded that the disease is instead associated with HLA-B\*51 and the perceived association with MICA alleles was due to linkage disequilibrium (Choy and Phipps, 2010). Ankylosing spondylitis is another disease known to be primarily associated with HLA-B\*27, whereas MICA association appears

secondary. Therefore, more refined disease studies on MICA polymorphism, independent of HLA association, need to be undertaken to confirm its pathological role.

Under normal circumstances, the expression of MIC proteins is highly restricted to the human intestinal epithelium, endothelial cells, and fibroblasts (Groh et al, 1996; Groh et al, 1998; Zwirner et al, 1999). Additionally, in the sub-capsular cortex of infant thymi a population of stellate MIC expressing epithelial cells could be identified (Groh et al, 1996; Diefenbach and Raulet, 2001). Expression is up-regulated in various transformed cells, particularly those of epithelial origin such as tumours of lung, breast, kidney, ovary, prostate and colon (Groh et al, 1999; Dunn et al, 2004). In thymomas, it is over-expressed in cortical and medullar epithelial cells (Hüe et al, 2003). Expression of the MIC proteins can also be up-regulated under conditions of “stress” such as heat shock (Groh et al, 1996; Stern-Ginossar and Mandelboim, 2009), viral infections such as CMV, Sendai and influenza A viruses (Groh et al, 2001; Das et al, 2001; Siren et al, 2004) and some bacterial infections such as Mycobacterium tuberculosis (Das et al, 2001) and diarrhoeagenic E.coli (Tieng et al, 2002). Expression was also observed in kidney and pancreatic allografts undergoing rejection episodes (Hankey et al, 2002; Racca et al, 2009) and after oxidative stress (Yamamoto et al, 2001). Exposure to DNA damaging conditions such as high doses of ionizing radiation or genotoxic stress (Gasser et al, 2005) and proteasome inhibitors (Valés-Gómez et al, 2008) can also increase MIC expression by target cells. Therefore MIC expression can thus be viewed as an “identification system” that cells use to provoke attack by the immune system.

## **2. The ULBP family of NKG2D ligands**

UL16 binding proteins (ULBPs) are another group of human NKG2D ligands first identified for their ability to bind UL16, a type I glycoprotein expressed by CMV infected cells (Sutherland et al, 2001). UL16 also binds to a member of the MIC family of proteins, MICB but not to MICA (Sutherland et al, 2001; Cosman et al, 2001). ULBP1 was the prototype of this family while the ULBP2 and ULBP3 were subsequently identified as proteins encoded by ULBP1-related expressed sequence (Cosman et al, 2001). All three ULBPs are distantly related to members of the extended MHC class I family with amino acid sequences that are 55– 60% identical (Cosman et al, 2001; Sutherland et al, 2001; Sutherland et al, 2006). The extracellular domain structure shows similarity to the  $\alpha 1$  and  $\alpha 2$  domains of MHC class I molecules but they lack the  $\alpha 3$  domain (Cosman et al, 2001). They comprise an overall amino acid identity of < 25% to human HLA molecules and only 23-26% identity with the MIC proteins (Diefenbach and Raulet, 2001). ULBPs also do not associate with  $\beta 2$ -microglobulin and they lack many of the conserved amino acids that are known to be important for peptide binding by classical MHC class I and are therefore unlikely to present peptide antigens (Cosman et al, 2001; Sutherland et al, 2002). Unlike the MIC family, which are true transmembrane proteins, ULBPs are anchored to the membrane via a GPI- linkage (Cosman et al, 2001). However, two family members, ULBP4 (RAET1E) and ULBP5 (RAET1G), have transmembrane domains (Chalupny et al, 2003; Eagle et al, 2009).

Unlike most members of the extended MHC class I family and MIC family, which map to chromosome 6p21 the ULBP genes map outside the MHC region to chromosome 6q25 (Sutherland et al, 2001). The ULBP gene family is homologous to the mouse Rae1/H60/Mult1 gene family (Raulet, 2003). This is why ULBP proteins

are sometimes referred to as RAET1 proteins in recognition of their relatedness to the mouse proteins. This also might explain why human ULBP1 and ULBP2 can interact with mouse NKG2D (Kubin et al, 2001). Polymorphism in ULBP genes and its impact on NK cell functions is still under analysis. Cao et al (2007) demonstrated that a soluble form of ULBP4 (RAET1E) protein produced in COS-7 tumour cell lines from selective splicing of the ULBP4 gene resulted in inhibition of NKG2D - mediated NK cytotoxicity.

The ULBPs are broadly expressed in a number of tissues and high levels of expression were observed on normal human epithelial and endothelial cells of various origin. These include the heart, brain, lung, liver, testis, lymph node, thymus, tonsil and bone marrow (Diefenbach and Raulet, 2001). ULBP transcripts were also abundant in foetal heart, brain, lung and liver (Diefenbach and Raulet, 2001). However, in some tissues / cells where high ULBP mRNA was detected, such as the Raji cell line, no cell surface ULBP expression was detected with a monoclonal antibody. ULBPs are also widely expressed on transformed cells including neuroblastomas (Raffaghello et al, 2004), malignant gliomas (Friese et al, 2003), leukemias (Poggi et al, 2004) and ovarian carcinomas (Conejo-Garcia et al, 2004). Correlations have been made between ULBP expression and good tumour prognosis suggesting that ULBPs may have a role in mediating antitumour immune responses *in vivo* (Poggi et al, 2004; Conejo-Garcia et al, 2004).

#### **1.1.4.2.1.3 NKG2D signalling pathway**

Many NK cell activating receptors consist of ligand recognition subunits that are non-covalently associated with transmembrane signalling 'adaptor' subunits. Most of these adaptor subunits, including CD3 $\zeta$ , Fc $\epsilon$ RI $\gamma$  and DAP12, possess

immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails (Mistry and O'Callaghan, 2007). Receptor engagement by their ligands induces tyrosine phosphorylation of the ITAMs, recruitment and activation of Syk or  $\zeta$ -chain-associated protein 70 kDa (ZAP70) tyrosine kinases, and phosphorylation of downstream effectors that trigger cell activation (Raulet, 2003). Human NKG2D has two charged residues in the transmembrane domain leading to the prediction that NKG2D is a stimulatory receptor that uses these residues to associate with an adapter molecule (Diefenbach and Raulet, 2001). However, none of the known adapter molecules involved in NK-cell signalling (CD3 $\zeta$ , Fc $\epsilon$ RI $\gamma$  and DAP12) was known to be associated with human NKG2D. Instead, the receptor was shown to associate with a new adaptor molecule known as DAP10, which is unusual as it lacks an ITAM in its cytoplasmic domain but contains a different tyrosine-based motif (YxxM) that is similar to those found in co-stimulatory receptors such as CD28, inducible co-stimulatory molecule (ICOS) and CD19 (Raulet, 2003). Engagement of NKG2D in human NK cells results in tyrosine phosphorylation of DAP10 and recruitment and activation of the p85 subunit of phosphatidylinositol 3- kinase (PI3K) and the anti-apoptotic kinase AKT (Wu et al, 1999; Raulet, 2003). DAP10 is expressed by several human NK-cell lines, a myeloid cell line (U937) and in CD8<sup>+</sup> T cells (Chang et al, 1999). Despite their gene similarities, DAP10 and DAP12 form distinct receptor complexes with their ligand-binding partners in NK cells and transfectants, activate different kinase pathways and contain different tyrosine based motifs (Chang et al, 1999; Wu et al, 2000).

Depending on the length of their intracellular cytoplasmic tail, two isoforms of NKG2D receptor are present in the mouse. These are NKG2D short (NKG2DS) which can be associated with both DAP 10 and DAP 12 and NKG2D long (NKG2DL)

which can only associate with the DAP10 molecule (Rosen et al, 2004). Cross-linking of either DAP10- or DAP12- associated receptors has been shown to be sufficient to trigger NK cell-mediated cytotoxicity in both mouse and humans (Wu et al, 2000). Regarding the cytokine secretion cross-linking of mouse NKG2D on the surface of NK cells by monoclonal antibodies (mAbs) is known to trigger IFN- $\gamma$  secretion by NK cells and TNF- $\alpha$  and nitric oxide production by macrophages (Diefenbach et al, 2002; Raulet, 2003). In humans, NKG2D cross-linking by mAbs failed to induce IFN- $\gamma$  and GM-CSF secretion by NK cells in some studies (Billadeau et al, 2003; Castriconi et al, 2003; Andre et al, 2004). This secretion was only induced when soluble recombinant ligands to NKG2D were used (Sutherland et al, 2001; Sutherland et al, 2002; Andre et al, 2004). This leads to the assumption that NKG2D in humans might function as a co-stimulatory receptor rather than as a primary recognition receptor. Studies of CD8<sup>+</sup> T-cell activation stimulated by NKG2D-ligand-transfected target cells or by antibody cross-linking supported the conclusion that NKG2D functions as a co-stimulatory receptor that amplifies TCR-mediated activation of CD8<sup>+</sup> T cells (Raulet, 2003). However, further clarification of the role of NKG2D in humans is needed before this conclusion can be made.

#### **1.1.4.2.1.4 Functions of NKG2D**

Engagement of human NKG2D on the surface of NK cells and  $\gamma\delta$  T cells by its ligands can trigger cytolytic activity as demonstrated in transfectants that express MIC and the ULBP proteins (Bauer et al, 1999; Cosman et al, 2001). This lytic activity is strongly inhibited by antibodies against NKG2D receptors (Bauer et al, 1999). Similar results were observed in mouse transfectants expressing the mouse NKG2D ligands (Rae-1 proteins, and H-60) (Cerwenka et al, 2000). Moreover,

expression of human MIC or murine RAE-1 on epithelial tumour cells causes NKG2D dependent rejection of tumour cells by NK cells and primed cytotoxic T cells (Groh et al, 1999; Cerwenka et al, 2000). Overall, these findings signify the potential role of NKG2D in innate and adaptive immune responses against tumours.

NKG2D appears to function in some cells as a co-stimulatory molecule. When CD8<sup>+</sup> CD28<sup>-</sup> αβ-T cell clones, specific for HCMV antigens, were tested for their capacity to kill HCMV-infected fibroblasts the interaction between NKG2D and MIC strongly augmented T cell responses under conditions of suboptimal TCR stimulation (Groh et al, 2001). Thus, induced expression of MIC may overcome the interference of viral gene products with antigen processing and presentation and the down modulation of MHC class I that is frequently associated with tumours. NKG2D can also trigger T cells in a TCR- independent manner (González et al, 2006). Normal freshly isolated intestinal intraepithelial lymphocytes (IEL) exhibit markedly diminished expression of NKG2D, which may be down modulated to prevent chronic T cell stimulation and autoreactive bystander T cell activation. High levels of NKG2D can be induced by IL-15 (Roberts et al, 2001), which is produced by intestinal epithelial cells on external stimuli and infection. In patients with active celiac disease, however, NKG2D is strongly expressed because of high local levels of IL-15 and MIC is up-regulated in intestinal epithelial cells (Hüe et al, 2004). Under these conditions, freshly isolated intraepithelial CD8<sup>+</sup> αβ T cells lyse intestinal epithelial cell lines independent of TCR engagement.

NKG2D signalling can also elicit cytokine secretion. Cross-linking of mouse NKG2D receptors by monoclonal antibody (mAb) on the surface of NK cells can induce IFN-γ secretion by these cells (Diefenbach et al, 2002). In human, NK cell stimulation with soluble recombinant NKG2D ligands such as MICA or ULBP1 (but



not with mAbs) induces IFN- $\gamma$  and GM-CSF secretion by these cells (Kubin et al, 2001; Sutherland et al, 2001; Sutherland et al, 2002; Andre et al, 2004). This effect is accompanied by increased expression of IL-2 receptor (CD25) and proliferation of NK cells (Kubin et al, 2001; Andre et al, 2004). Similarly, secretion of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-4 secreted by HCMV-specific  $\alpha\beta$ -T cells is strongly enhanced when exposed to target antigen presented by MICA-transfected C1R cells (Groh et al, 2001). Infection by Mycobacterium tuberculosis induces expression of MIC on dendritic and epithelial cells, resulting in NKG2D-mediated co-stimulation of V $\gamma$ 2/V $\delta$ 2 T cell cytotoxicity, proliferation, and release of IFN- $\gamma$  and IL-2 (Das et al, 2001).

#### **1.1.4.2.2 Natural cytotoxicity receptors (NCRs)**

The search for receptors responsible for NK-cell triggering in non-MHC-restricted natural cytotoxicity identified three novel NK-cell-specific triggering surface molecules. These molecules are termed natural cytotoxicity receptors (NCRs) and appeared to play a critical role in the induction of NK-cell-mediated cytotoxicity. All NCRs (except NKp80) are type I transmembrane proteins with one or two extracellular immunoglobulin domains, a transmembrane domain and a short cytoplasmic tail (Moretta et al, 2000). The transmembrane region of NCRs contains a positively charged amino acid residue that is crucial for their association with distinct signal-transducng adaptor molecules bearing ITAM sequences (DAP12, CD3  $\zeta$  and Fc $\epsilon$ RI $\gamma$ ). A putative NCR should satisfy the following requirements (Moretta et al, 2000): 1) its expression should be restricted to NK cells. 2) its mAb in a redirected killing assay should trigger NK-cell cytotoxicity. 3) mAb-mediated masking of the NCR should inhibit the NK-cell-mediated cytotoxicity. Three NCRs were discovered: NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3) (Moretta et al, 2000). NKp80 is

type II transmembrane protein that may act as a co-receptor rather than a primary recognition receptor (Vitale et al, 2001). Little is known about the expression of NCRs by NK-cell subsets. Although some reports suggest that NCRs are not expressed differentially on NK-cell subsets (Moretta et al, 2000; Moretta and Moretta, 2004), other studies demonstrated that their expression is significantly up-regulated in CD56<sup>bright</sup> compared to CD56<sup>dim</sup> in some diseases such as in women with recurrent spontaneous abortions and implantation failures (Fukui et al, 2006). Both NKp46 and NKp30 are expressed by all NK cells irrespective of their activation status (Sivori et al, 1997; Sivori et al, 1999; Moretta et al, 2000; Moretta and Moretta, 2004). However, NKp44 is absent in freshly isolated peripheral blood NK cells but is progressively expressed by NK cells *in vitro* upon culture in the presence of IL-2 (Vitale et al, 1998; Moretta et al, 2000; Moretta and Moretta, 2004). Therefore, NKp44 appears to be a marker specific for activated human NK cells.

#### **1. NKp46 (NCR1 or CD335)**

NKp46 is a 46 kDa type I transmembrane glycoprotein, characterised by two C2 - type immunoglobulin-like domains in the extracellular portion (Sivori et al, 1997; Pessino et al, 1998; Biassoni et al, 1999). NKp46 lack the intrinsic signalling motifs and instead it associates with the ITAM-bearing CD3 $\zeta$  or FC $\epsilon$ R1 $\gamma$  adaptor subunits (Pessino et al, 1998). The NKp46 gene is expressed in human on chromosome 19 (Pessino et al, 1998). The mouse homolog has been identified independently as MAR-1 (mouse-activating receptor 1) and is expressed on chromosome 7 (Biassoni et al, 1999). Since NKp46 expression is confined only to NK cells (both resting and activated) therefore it is a true NK-specific molecule, and probably the most reliable surface marker to identify NK cells. The engagement of this

receptor with activating mAbs induces cytotoxicity, calcium mobilization and synthesis of cytokines (Sivori et al, 1999; Moretta et al, 2000; Moretta et al, 2005). This receptor appears to have a central role in the lysis of different targets including normal and tumour-transformed cells (Sivori et al, 1999). NKp46 masking by specific blocking mAbs inhibits lysis of the majority of human tumours belonging to different histotypes, including lung, liver and breast carcinomas, melanoma, and Epstein Barr virus (EBV)-transformed cell lines (Moretta et al, 2000). In particular, the use of murine-tumour target cells (including Bw1502 and YAC cells) provided direct evidence that NKp46 is sufficient to mediate NK cytolytic anti-tumour activity that does not necessarily require co-engagement of other triggering receptors (Moretta et al, 2000; Moretta et al, 2005). In addition, Vankayalapati et al (2002) have reported that NKp46 can also contribute to NK-cell lysis of mononuclear phagocytes infected with an intracellular bacterium. The surface density of NKp46 seems to be important in determining the magnitude of the cytotoxic response from NK cells (Moretta et al, 2005). In a control population, < 20% donors display the NCR<sup>dull</sup> phenotype while most donors express a high density of NCRs on NK cells, NCR<sup>bright</sup> phenotype (Sivori et al, 1999). In general, NCR<sup>dull</sup> individuals displayed low cytolytic activity against a variety of NK-susceptible tumour targets. De Maria et al (2003) have also reported that the reduced cell surface expression of NKp46 and other NK-cell receptors is associated with the impaired NK-cell cytolytic function in viraemic HIV-1 infection. Decreased NKp46 expression on NK cells has also been reported in the elderly, potentially impacting on susceptibility to infectious, inflammatory, and neoplastic diseases (Almeida-Oliveira et al, 2011).

## 2. **NKp44 (NCR2 or CD336)**

NKp44 is a 44 kDa protein first identified by Vitale et al (1998) and its gene has then been cloned by Cantoni et al in 1999. It is type I transmembrane glycoprotein with one immunoglobulin-like domains in the extracellular portion. The NKp44 gene is only found in humans and shows three different splice forms varying in their stalk and intracellular domains (Hollyoake et al, 2005). The crystal structure of NKp44 shows structural homology with a number of other immunoglobulin-like structures including TREM-1 (triggering receptor expressed on myeloid cells); TREM-like transcript-1, and sialoadhesin (Joyce and Sun, 2011). NKp44 also lacks the intrinsic signalling motifs. However, unlike NKp46 (associates with CD3 $\zeta$  or FC $\epsilon$ RI $\gamma$  adaptor subunits) and NKp30 (associates with CD3 $\zeta$  adaptor subunit), NKp44 is associated with another adaptor molecule (DAP12) and is expressed only on the surface of activated NK cells (Vitale et al, 1998; Cantoni et al, 1999). Furthermore, in contrast to other markers of lymphocyte activation, NKp44 is absent from activated T cells or T-cell clones and therefore, NKp44 appears to be the first marker specific for activated human NK cells (Bottino et al, 2000). Cross-linking of NKp44 by mAbs strongly activates tumour target cell lysis by NK cells in a redirected killing assay indicating that NKp44 can mediate triggering of NK cell non-MHC restricted cytotoxicity (Vitale et al, 1998). mAb-mediated masking of NKp44 resulted in partial inhibition of cytolytic activity against certain MHC - class I Fc $\gamma$ R negative NK-susceptible target cells. Remarkably, the degree of inhibition was greatly increased by the simultaneous masking of NKp46 (Vitale et al, 1998). In addition to tumour lysis, NKp44 is also reported to be involved in triggering NK-cells' cytolytic activity against virus infected cells. These include influenza virus (Arnon et al, 2001; Arnon et al, 2006; Ho et al, 2008), Sendai virus (Arnon et al, 2001; Arnon et al, 2006) and flavivirus (Hershkovitz

et al, 2009). Therefore, NKp44 appears to function as an NCR that is selectively expressed by activated NK cells and that might cooperate with other NCRs in the process of non-MHC-restricted lysis of tumour and virus-infected cells.

### **3. NKp30 (NCR3 or CD337)**

NKp30, the third NCR molecule, was first discovered by Pende et al in 1999. It is a 30 kDa glycoprotein that contains only one immunoglobulin-like extracellular domain, (Moretta et al, 2000; Joyce et al, 2011). Its transmembrane portion contains an arginine residue which is involved in the association with the CD3 $\zeta$  chain for transduction of downstream activating signals (Pende et al, 1999). With the exception of NK cells in lymph nodes and in the endometrium during the menstrual cycle (Manaster et al, 2008) NKp30 is selectively expressed by all human NK cells, both freshly isolated and cultured in IL-2. However, it is present as a non-expressed pseudogene in mouse (Hollyoake et al, 2005). The crystal structure of NKp30 displays strong structural similarity to the CD28 family of receptors (Joyce and Sun, 2011). CD59 is physically and functionally associated with NKp30 and NKp46 and therefore may act as a co-receptor to trigger NCRs -mediated cytotoxicity by NK cells (Marcenaro et al, 2003). NKp30 was found to co-operate with NKp46 and NKp44 in the induction of cytotoxicity against a variety of normal and transformed cells (Pende et al, 1999). Moreover, NKp30 is the major receptor responsible for the killing of some tumour target cells that are relatively resistant to NKp46/NKp44 mediating killing such as MEL-15 melanoma cells suggesting that NKp30 probably recognizes distinct ligands (Pende et al, 1999). Depending on the type of target cells, mAb masking of NKp30 results in partial to complete inhibition of NK-cell mediated cytotoxicity. NKp30 is also shown to recognize an unknown ligand expressed on the

surface of immature dendritic cells (iDCs) suggesting that NKp30 may be involved in the NK cell mediated killing of iDCs (Fauriat et al, 2005; Farag, 2005). Furthermore, NK-mediated induction of DC maturation is also dependent on NKp30. Vitale et al (2005) showed that upon NK/DC interaction NK cells produced TNF- $\alpha$  and IFN- $\gamma$  that, in turn, promoted DC maturation. Masking of NKp30 with specific monoclonal antibodies (mAbs) strongly reduced maturation of DCs co-cultured with NK cells. The surface expression of NKp30 parallels that of NKp46, as NK cells displaying an NKp46<sup>dull</sup> or an NKp46<sup>bright</sup> phenotype were also characterized by dull or bright NKp30 fluorescence (Pende et al, 1999; Moretta et al, 2000). The demonstration that NK cells express parallel densities of different triggering receptors might explain the existence of NK-cell subsets displaying different natural cytolytic activity.

#### **4. NKp80**

NKp80, also known as killer cell lectin-like receptor subfamily F member 1 (KLRF1), is the last discovered member of NCR receptors. It is a type II transmembrane protein with a C-type lectin domain in its extracellular region and encoded by the human NK cell gene complex region (Vitale et al, 2001; Biassoni et al, 2001). No mouse homologue to human NKp80 has been yet identified. NKp80 is shown to be expressed on the cell surface of virtually all fresh or activated NK cells, a minor subset of T cells that express CD56 (Vitale et al, 2001) and a small fraction of CD8<sup>+</sup> effector memory cells (Kuttruff et al, 2009) as a dimer of 80 KDa. NKp80 surface expression was also detected in all CD3<sup>-</sup> and in a proportion of CD3<sup>+</sup> lymphoproliferative diseases of large granular lymphocytes (Vitale et al, 2001). The transmembrane region is characterized by non-polar amino acids and therefore disfavours association with classical adaptor molecules such as DAP12, CD3  $\zeta$  and

FcεRIγ (Biassoni et al, 2001). The signal transduction pathway of NKp80 is still largely unknown although some recent reports demonstrated that NKp80 uses an atypical hemi-ITAM and Syk kinase to trigger cellular cytotoxicity (Dennehy et al, 2011). There was some controversy regarding the role of NKp80 as activating or inhibitory receptor. One early study suggested that NKp80 functions as an inhibitory receptor for NK cells based on the presence of two immunoreceptor tyrosine-based inhibitory-like motifs (ITIMs) within the cytoplasmic tail of NKp80 (Roda-Navarro et al, 2000). However, subsequent studies clearly indicated that NKp80 displays an activating function. mAb- mediated cross-linking of NKp80 in NK cells resulted in induction of cytolytic activity and Ca<sup>2+</sup> mobilization (Vitale et al, 2001; Welte et al, 2006). In fact, Vitale et al (2001) suggested that NKp80 functions as a co-receptor that cooperates with other NCRs rather than acting as a primary recognition receptor. Moreover, Welte et al (2006) demonstrated that when NKp80 comes in contact with its proposed ligand, activation induced C type lectin (AICL), pro-inflammatory cytokine secretion is stimulated.

#### **1.1.4.2.2.1 NCR ligands**

Very little is known regarding the cellular ligands of NKp46, NKp44 and NKp30. However, haemagglutinin molecules of different influenza and Sendai viruses and neuraminidase of Sendai viruses were suggested as the first specific NKp46 and NKp44 ligands (Mandelboim, 2001; Arnon et al, 2001; Arnon et al, 2004). In these studies, recognition of the haemagglutinin of influenza and haemagglutinin / neuraminidase of Sendai viruses by NKp46 and NKp44 was needed to kill the infected cells. Vimentin, a surface marker expressed by Mycobacterium tuberculosis-infected human monocytes, was also proposed as a ligand for NKp46. NKp30 is suggested to

interact with heparan sulphate proteoglycans (HSPGs) (Bloushtain et al, 2004). This binding may be critical for tumour cell killing but its relevance to virally infected cell killing is unclear. However, both NKp46 and NKp30 bind to this molecule (Bloushtain et al, 2004). Keeping in mind the differential susceptibility of targets to the killing mediated by NKp46 and NKp30, and the likelihood that these receptors recognize different cellular ligands HSPG is probably a co-ligand for both these receptors rather than a specific ligand for NKp30. The HCMV tegument protein (pp65) was found to antagonize NKp30 activation by causing a dissociation of the adaptor molecule CD3  $\zeta$  from NKp30. pp65 is an intracellular protein and it is therefore suggested that soluble pp65 derived from direct lysis of virally infected cells or from apoptotic cells may bind to NKp30 and hamper its activity (Arnon et al, 2005). NKp30 is also suggested to recognize intracellular ligands. This is supported by the finding that the nuclear factor HLA-B associated transcript 3 (BAT3) released from tumour cells can directly bind to NKp30 and initiate NKp30 cytotoxicity. BAT3 is thus the first cellular ligand identified for NKp30 (Pogge von Strandmann et al, 2007). Most recently, NKp30 was shown to recognize a B7 family homolog (B7-H6) as its ligand (Brandt et al, 2009). Unlike other proposed NKp30 ligands, B7-H6 expression is confined to tumour cells and is not expressed by normal cells (Brandt et al, 2009). B7-H6 is expressed on a number of tumour cell lines, such as K562 and Raji, as well as on primary cancer cells (Joyce and Sun, 2011). However, the role of NKp30 in cancer immunity against these cell lines is still unclear. The activation-induced C-type lectin (AICL) (which is also encoded in the NK-gene complex) was identified as a ligand of NKp80 (Welte et al, 2006). AICL is a myeloid-specific receptor expressed by monocytes, macrophages and granulocytes. Interaction between NK cells and monocytes resulted in a cytokine release that was partially dependent on NKp80 engagement. Hence, it seems that



NKp80-AICL interaction is involved in the crosstalk between NK cells and myeloid cells and thus may influence the initiation and maintenance of immune responses in humans (Welte et al, 2006).

#### **1.1.4.2.3 CD16**

CD16 or Fc-gamma receptor III (Fc- $\gamma$  RIII) is another activating receptor of NK cells that recognizes non-MHC class I molecules (Diefenbach and Raulet, 2001). It is one of the three subtypes of Fc-IgG receptors that bind to the Fc region of IgG antibody (Kimberly et al, 1995). All these receptors belong to the immunoglobulin superfamily and are distributed widely on many cells of the immune system (Ivan and Colovai, 2006). They are **1)** Fc $\gamma$ RI (CD64), a high affinity receptor capable of binding monomeric IgG and expressed on monocytes and activated neutrophils. **2)** FcR $\gamma$ II (CD32), a low-intermediate affinity receptor that binds multimeric IgG and is expressed on the surface of B cells, monocytes, neutrophils, and non-hematopoietic cells such as epithelial cells. **3)** Fc $\gamma$ RIII (CD16), also a low affinity receptor capable of binding multimeric IgG. They can also contribute to the pathogenesis of immune complex and auto-antibody mediated disorders such as vasculitis, rheumatoid arthritis, idiopathic thrombocytopenic purpura, or autoimmune neutropenia (Takai T, 2005; Ivan and Colovai, 2006). Human CD16 is expressed in two distinct (CD16a and CD16b) isoforms, which are products of two different highly homologous genes (Schumann et al, 1994). CD16a (Fc- $\gamma$ RIIIa) is a 50-65 kDa polypeptide-anchored transmembrane protein that is expressed on the surface of NK cells (Lanier et al, 1989; Anderson et al, 1990), activated monocytes or macrophages (Wirthmueller et al, 1992) and mast cells (Kurosaki et al, 1992). CD16b (Fc- $\gamma$ RIIIb) or HNA (human neutrophil antigen) is a GPI-linked monomeric receptor that is over 95% homologous to CD16a

in its extracellular domain and is expressed specifically on neutrophils (Selvaraj et al, 1988; Huizinga et al, 1988; Huizinga et al, 1989). CD16a requires association of the gamma subunit of Fc epsilon (FcεRIγ) or TCR-CD3ζ for cell surface expression (Nagarajan et al, 1995). Engagement of CD16 induces phosphorylation of ITAMs on the adaptor chains CD3ζ and FcεRγ, which in turn recruit and activate Syk and ZAP70 tyrosine kinases for downstream signalling (Ting et al, 1995; Nagarajan et al, 1995). Activation of CD16 on the surface of NK cells by IgG aggregates or IgG-antigen complexes or mAb induces the release of cytokines such as IFN-γ (Márquez et al, 2010) and cytotoxic mediators like perforin and granzymes (Werfel et al, 1989; Mandelboim et al, 1999). The latter is known as antibody-dependent cell-mediated cytotoxicity (ADCC). Ligation of CD16 can also induce highly activated NK cells to undergo Fas ligand induced apoptosis (Eischen et al, 1996).

#### **1.1.4.3 Other NK cell receptors**

Several other receptors and co-receptors have been identified to be expressed on the surface of NK cells such as DNAX accessory molecule-1 (DNAM-1), CD69, and 2B4. DNAM-1 is a transmembrane glycoprotein with two immunoglobulin-like domains expressed on the surface NK and T cells in association with lymphocyte function antigen-1 (LFA-1) (Shibuya et al, 1996). It is a signal transducing adhesion molecule that is involved in the adhesion of certain tumour cells to CTLs and NK cells and mediates their cytotoxicity (Shibuya et al, 1996; Lakshmikanth et al, 2009). Anti-DNAM-1 mAbs inhibits T and NK cell-mediated cytotoxicity against a variety of tumour cell targets and blocks cytokine production by alloantigen-specific T cells (Shibuya et al, 1996). Recent studies also demonstrated

that DNAM-1 can synergize with other activating NK cell receptors in the pro-inflammatory cytokine secretion by these cells (Fauriat et al, 2010).

CD69, also known as activation inducer molecule or early T- cell activation antigen (P60), is a type II transmembrane glycoprotein with a C-type lectin-binding domain encoded by the CD69 gene located on chromosome 12 (Borrego et al, 1999). NK cells can express CD69 after activation by different stimuli such as phorbol 12-myristate 13-acetate (PMA), IL-2, IL-12, IFN- $\gamma$  or anti-CD16 mAbs (Borrego et al, 1999). It is commonly used as quantitative measure of NK cell or T cell activation. Ligation of this molecule with activating anti-CD69 mAbs results in triggering of NK-cell-mediated cytolytic activity (Borrego et al, 1999). However, this activity can be abrogated by simultaneous stimulation of CD94/NKG2A inhibitory receptors.

2B4 is a glycoprotein that belongs to the signalling lymphocyte activation molecule (SLAM) family of receptors (Velikovskiy et al, 2007). It is expressed on NK cells, monocytes, basophils, TCR $\gamma\delta^+$  T cells, and a subset of CD8 $^+$  T cells (Rodríguez-Navarro et al, 2004). Engagement of 2B4 by mAb induces its phosphorylation and recruitment of SLAM-associated protein (SAP) through cytoplasmic tyrosine-based motifs (Chen et al, 2004). The ligand of 2B4 is CD48, which is expressed on hematopoietic cells (Bryceson et al, 2006). 2B4 may function as a co-receptor in human NK-cell mediated cytotoxicity, leading to induction of cytokine (IFN- $\gamma$ ) and matrix metalloproteinase (MMP-2) production (Chuang et al, 2001).

## **1.2 Interleukin-6 (IL-6)**

Interleukin-6 (IL-6) together with other interleukins such as IL-1 and IL-10 are pleiotropic in their effect (Kishimoto, 2006; Apte and Voronov, 2002; Commins et al, 2008). However, the IL-6 is considered the prototypic pleiotropic cytokine among them. This is reflected in the variety of names originally assigned to IL-6 based on its various functions. These include interferon beta 2 (IFN-B2) (Zilberstein et al, 1986), B cell stimulatory factor- 2 (BCSF-2) (Hirano et al, 1985; Hirano et al, 1986), B-cell differentiation factor (BCDF) (Kishimoto, 1989), plasmacytoma / hybridoma growth factor (P/HGF) (Van Damme et al, 1987; Van Snick et al, 1988), 26 kDa protein (Haegeman et al, 1986), hepatocyte stimulating factor (HSF) (Baumann et al, 1987), monocyte-granulocyte inducer type 2 (MGI-2) (Shabo et al, 1988), and cytotoxic T-cell differentiation factor (CDF) (Uyttenhove et al, 1988; Ming et al, 1989). Sequencing the cDNA clones have shown that all these molecules were identical and are encoded by a single gene (Poupart et al, 1987). The name IL-6 was proposed for this molecule in December 1988 (Shegal et al, 1989; Sehgal et al, 1995). This protein has been proven to play important roles in acute phase reactions (Heinrich et al, 1990), inflammation (Hodge et al, 2005), haematopoiesis (Rodríguez et al, 2004), bone metabolism (Ishimi et al, 1990; de la Mata et al, 1995) , and cancer progression (Hodge et al, 2005; Bomberg and Wang, 2009).

### **1.2.1 Structure of IL-6**

IL-6 is a member of a family of cytokines called the IL-6 cytokine family. This family also includes interleukin 11 (IL-11), interleukin 27 (IL-27), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), leukemia inhibitory factor (LIF), oncostatin M (OSM) and Kaposi's sarcoma

associated herpes virus interleukin-6 like protein (KSHV-IL6) (Kishimoto et al, 1995; Taga and Kishimoto, 1997). All known members of the IL-6 cytokine family have similar helical structure (Somers et al, 1997), induce hepatic expression of acute phase proteins (Somers et al, 1997) and signal through transducing receptors containing the gp 130 subunit (Boulanger et al, 2004).

Human IL-6 cDNA encodes a polypeptide of 212 amino acids with 28 amino acids as signal sequence and a 184 amino acids as mature segment (Hirano et al, 1986; Simpson et al, 1997; Li et al, 2004). The mature protein is N-glycosylated at positions 73 and 172 respectively. Due to different degrees of glycosylation and phosphorylation, IL-6 has a molecular weight ranging between 21-28 kDa (Park and Philinger, 2007). An alternative splice variant of IL-6 was identified in monocytes and lymphocytes (Kestler et al, 1995). This form is 17 kDa (148 amino acids long) and appears to lack a binding site for the IL-6 signal transducing molecule gp 130. The human IL-6 molecule contains four conserved cysteine residues involved in the formation of two disulphide bonds (Boulanger et al, 2003). A region containing nine amino acids between two of these cysteine residues are identical which led to the proposition that these cysteines were essential for the IL-6 activity. However, the biological activity of recombinant IL-6 lacking the cysteine residues suggests that these cysteines may be not necessary (Park and Philinger, 2007). Mouse and rat IL-6 also have been cloned and are approximately 65% identical to human IL-6 at cDNA level and about 40% identical at the protein level (Chiu et al, 1988; Van Snick, 1990). Unlike human IL-6, mouse and rat IL-6 lack potential N-linked glycosylation sites but may be O-glycosylated (Van Snick, 1990). Although human IL-6 is active on mouse and rat cells, mouse IL-6 is not active on human cells (Chiu et al, 1988; Northemann et al, 1989; Hammacher et al, 1994).

### **1.2.2 Gene structure**

The human IL-6 genomic DNA segment has a length of approximately 5 kb and contains five exons and 4 introns (Li et al, 2004; Park and Philinger, 2007). It maps to human chromosome 7p14 and contains a c-fos serum responsive enhancer element and sequences for cAMP induction, activator protein-1 binding and glucocorticoid –responsive elements (Park and Philinger, 2007). The wide range of regulatory pathways involved in controlling IL-6 transcription confirms the central role of IL-6 in inflammatory responses. The murine gene also consists of five exons and 4 introns, but it maps to chromosome 5 (Tanabe et al, 1988). The comparison between human and mouse IL-6 genomic segments reveals highly homologous regions extending approximately 350 bp upstream of the cap site (Tanabe et al, 1988). The nucleotide sequences of IL-6 and GM-CSF genes also resemble each other in a way suggesting a possible evolutionary relationship. Four polymorphisms in the IL-6 gene have been described, with possible functional consequences (Brull et al, 2001). For example the common G→C polymorphism at position -174 results in lower stimulated IL-6 promoter activity *in vitro* and lower circulating IL-6 concentrations in healthy individuals compared with the G allele (Fishman et al, 1998). Ferrari et al (2001) demonstrated that postmenopausal women homozygous for the -174 C allele have low serum levels of C-terminal cross-linking of type I collagen (sCTX), a marker of bone resorption.

### **1.2.3 Expression and secretion of IL-6**

Various cell types are capable of IL-6 expression / or synthesis including T-lymphocytes (Sad et al, 1995), monocytes (Kestler et al, 1995), macrophages (Stowet et al, 2009), eosinophils (Melani et al, 1993), mast cells (Gagari et al, 1997) fibroblasts

(Cichy et al, 1996), synoviocytes (Miyazawa et al, 1998) , hepatocytes (Panesar et al, 1999), endothelial cells (Xin et al, 1995) , skeletal and smooth muscle cells (Keller et al, 2003; Bouchelouche et al, 2006), chondrocytes (Guerne et al, 1990), keratinocytes (Jiang et al, 1994) glial cells (Sun et al, 1997), astrocytes (Lafortune et al, 1996) and adipocytes (Fried et al, 1998; Lyngsø et al, 2002). Production and expression of IL-6 is also up-regulated by numerous signals including viral infections (Sharma et al, 2005; Jiang et al, 2008; Cai et al, 2008), lipopolysaccharide (Grimaldi et al, 1998; Zhang et al, 2009), calcium ionophore (Holmes et al, 2004), interleukins (IL-1, IFN, TNF) (Sancéau et al, 1995; Flower et al, 2003), platelet derived growth factor (PDGF) (Franchimont et al, 1999), bacterial toxins (Onogawa et al, 2002), and hormones (Bell et al, 2003). In contrast, IL-4 inhibits IL-6 expression in monocytes (Donnelly et al, 1993). In fibroblasts the synthesis of IL-6 is enhanced by treatment with TNF- $\alpha$ , IL-1i, prostaglandin E2, and various activators of the intracellular signalling pathways acting through proteinkinase A (PKA) or protein kinase C (Baumann and Kushner, 1998). In epithelial and endothelial cells the secretion of IL-6 is also induced by IL-17 (Laan et al, 2001). IL-6 is not constitutively expressed. Some studies suggest that IL-6 is secreted in circadian pattern with two peaks at about 19.00 and 05.00 hrs and its circadian secretion correlates with sleep (Vgontzas et al, 2005; Burgos et al, 2006). Vgontzas et al concluded that IL-6 is a mediator of sleepiness and its circadian pattern reflects the homeostatic drive for sleep. Finally, IL-6 can also stimulate or inhibit its own synthesis, depending upon the cell type.

#### **1.2.4 Normal IL-6 concentration**

Circulating IL-6 can be found in the blood of normal individuals in the 1 pg/ml range (D'Auria et al, 1997) with slight elevations during the menstrual cycle

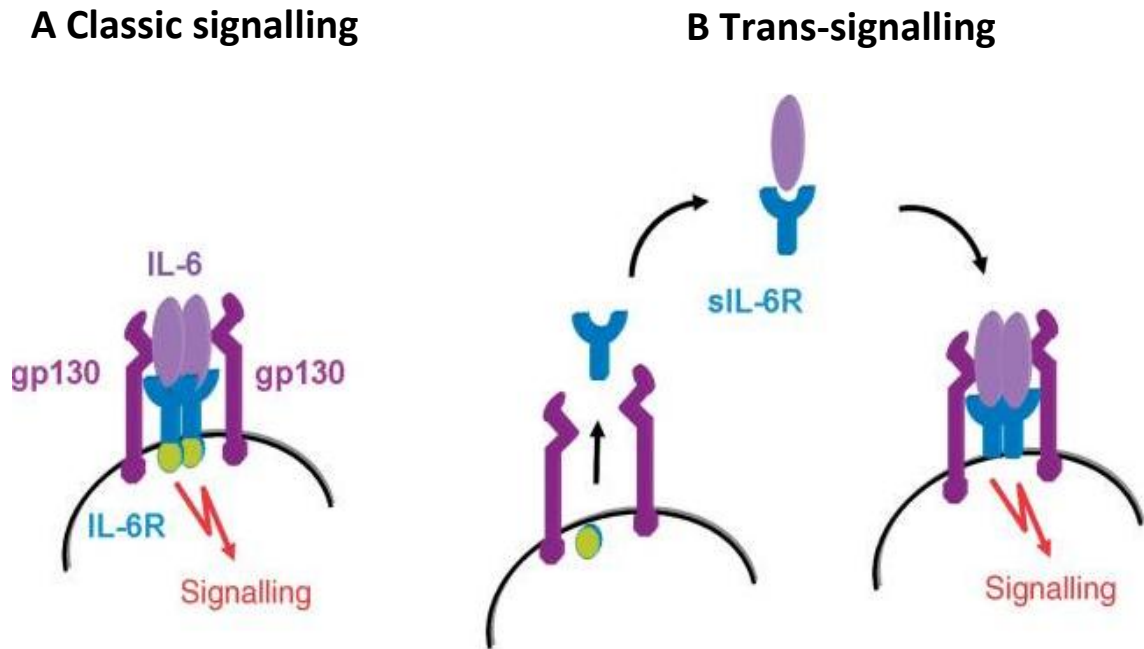
(Angstwurm et al, 1997), and large elevations (up to 430 pg/ml) after surgery (Sakamoto et al, 1994). There is a controversy regarding the major source of circulating IL-6 in healthy individuals. While Keller et al (2003) demonstrated that skeletal muscle is a major source of blood-borne IL-6, Mohamed-Ali et al (1997) demonstrated that one third of total circulating concentrations of IL-6 originate from adipose tissue. Plasma IL-6 concentrations may be influenced by a person's age with higher concentrations detected in the elderly. Weight, disease conditions, medications/supplements, alcohol consumption, composition of the last meal eaten, and activity level can all influence IL-6 levels (Picotte et al, 2009). Despite these sources of variation, a positive association between circulating IL-6 levels and increased mortality rate has been described (Fernandez-Real et al, 2001). Yudkin et al (2000) speculated that IL-6 is the link among obesity, inflammation, stress, and coronary heart disease.

### **1.2.5 IL-6 receptors and signalling pathway**

IL-6 induces signalling through a cell surface heterodimeric receptor complex composed of a ligand binding subunit (IL-6 R) and a signal transducing subunit, gp130 (Kamimura et al, 2003). Both are members of the class I cytokine receptor superfamily. Although IL-6 binding to gp130 has been described, it is generally accepted that the IL-6 R is the actual IL-6 binding protein (Hibi et al, 2003). IL-6 can signal via two different pathways (Figure 1.4): *classical signalling* via the membrane bound IL-6R and *IL-6 trans-signalling* via a naturally occurring soluble IL-6R (sIL-6R). In classical signalling, IL-6 engagement of membrane bound IL-6R results in association of the intracellular segment of IL-6R with the gp130. The resulting complex is a hexamer comprised of two IL-6 molecules, two IL-6R and two



gp130. Dimerized gp130 activates intracytoplasmic Janus tyrosine kinases (JAK) which in turn induce tyrosine phosphorylation of STAT3. STAT-3 is a member of STAT family of cytoplasmic latent transcription factors which consists of seven members: STAT-1-4, STAT-5a, STAT-5b, and STAT-6. Once STAT 3 is activated, it translocates to the nucleus. The translocation of STAT3 to the nucleus then induces IL-6 regulated changes in gene expression (Park and Philinger, 2007). These changes include up-regulation of expression of genes that control cell proliferation, survival, differentiation, and development, and new gene expression of acute phase proteins, which subsequently are secreted (Heinrich et al, 2003; Brantley and Benveniste, 2008). The membrane bound IL-6R is only expressed on hepatocytes, neutrophils, macrophages and some lymphocytes (Drucker et al, 2010). Accordingly, the effect of IL-6 through the classical signalling pathway is restricted to these cells. However, this pathway does not explain the wide biological effect of IL-6 on a broad spectrum of cells that do not express IL-6R. Therefore, the alternative IL-6 pathway is trans-signalling pathway in which IL-6 binds to a naturally occurring soluble IL-6R (sIL-6R) and this IL-6/sIL-6R complex activates gp130 (Rose-John et al, 2006). Since gp130 is expressed on all cells of the body, therefore most cells can respond to IL-6 by this way. However, IL-6 trans-signalling affects also cells that express membrane bound IL-6R, e.g. hepatocytes. In this setting an activation of the IL-6 trans-signalling can enhance stimulatory effects of IL-6 classical pathway (Drucker et al, 2010). It is also noteworthy to mention that two soluble splice forms of gp130, lacking the transmembrane segment, also exist (Sharkey et al, 1995; Diamant et al, 1997). When bound to the IL-6/IL-6R complex, these soluble gp130 block trans-signalling from the IL-6/IL-6 R but not from other cytokines that utilize gp130 as a co-receptor (Narazaki et al, 1993).



**Figure 1.4: IL-6 receptors and signalling pathways. A) Classic IL-6 signalling pathway:** IL-6 binds to membrane bound IL-6R resulting in dimerization of gp130. The actual complex is comprised of two IL-6 molecules, two IL-6Rs and two gp130. Dimerized gp130 activates intracytoplasmic Janus tyrosine kinases (JAK) which in turn induce tyrosine phosphorylation of STAT3 which translocates into the nucleus and initiates IL-6 induced changes in gene expression. **B) IL-6 trans-signalling pathway:** sIL-6R is generated either by limited proteolysis of the membrane-bound IL-6R or by alternative mRNA splicing. IL-6 binds to soluble IL-6R (sIL-6R) and this IL-6/sIL-6R complex activates gp 130. Reproduced from Dayer and Choy (2010)

### **1.2.6 Functions of IL-6**

As mentioned before, IL-6 is a pleiotropic cytokine with diverse biological activities. These include:

#### **1. Acute phase response**

IL-6 is one of the most important mediators of acute phase responses (Heinrich et al, 1990). These include induction of hepatic acute phase proteins, fever, increased erythrocyte sedimentation rate (ESR), increased secretion of glucocorticoids and the activation of the complement and clotting cascades. Similarly to IL-1, IL-11 and LIF, IL-6 can induce the synthesis of hepatic acute phase proteins both *in vivo* and *in vitro* by regulating changes in the gene transcription rate of these proteins (Moshage, 1997; Akira and Kishimoto, 2004). These are C - reactive protein (CRP), serum amyloid A (SAA), alpha-1-antichymotrypsin and haptoglobin (Moshage, 1997; Akira and Kishimoto, 2004). It also increases the synthesis of fibrinogen, an important clotting agent but decreases the synthesis of albumin and transferrin (Park and Philinger, 2007). When secreted by T-cells and macrophages, it stimulates the immune response to trauma, especially against burns or other tissue damage leading to inflammation (Vindenes et al, 1998).

#### **2. T-cells**

Interleukin-6 has a prominent regulatory role in T-cell proliferation, differentiation and survival (Dienz and Rincon, 2009). In the early stages of T-cell differentiation, it activates resting T-cells by inducing both IL-2 production and IL-2 receptor expression and thus with IL-2, drives the differentiation of naïve T-cells cells into cytotoxic T cells (Garman et al, 1987; Noma et al, 1987). IL-6 also functions as

the required second signal in both antigen- or mitogen-activated T-cells and induces the proliferation of mitogen-activated T cells and thymocytes (Park and Philinger, 2007). Details of the effect of IL-6 on T cells will be discussed in the following chapters.

### **3. B-cells**

Interleukin-6 is very important in the stimulation of differentiation of B cells into plasma cells and it was first described as a B-cell activation or differentiation factor (Schwartz et al, 1985; Hirano et al, 1986; Muraguchi et al, 1988). It is involved in the induction of B-cell activation and enhances the production of antibodies by activated B-cells, but not their proliferation (Hirano, 1998). Here this cytokine stimulates mostly the release of IgG and IgA antibodies from these cells.

### **4. Haematopoiesis**

The IL-6 is a promoter of haematopoiesis. It has blast cell growth factor activity and can synergize with IL-3 to shorten the G0 period of early haematopoietic progenitors (Park and Philinger, 2007). In addition, IL-6 has been found to synergize with IL-3 in increasing the number, size and average ploidy value of megakaryocyte colonies formed from mouse or human bone marrow cells *in vitro* (Ishibashi et al, 1989; Kimura et al, 1990). IL-6 was also found to increase platelet numbers (Gutierrez et al, 1995; Clarke et al, 1996).

## **5. Bones**

IL-6 is a modulator of bone resorption (Ishimi et al, 1990; Palmqvist et al, 2002). Osteoclast formation is increased in the presence of IL-6, apparently through the activation of gp130 (Heymann and Rousselle, 2000). Given the recent interest in osteoclasts as mediators of bony erosion in RA, these observations suggest a possible direct role for IL-6 in RA joint destruction (Lipsky, 2008).

## **6. Muscles and fatty tissues**

IL-6 stimulates energy mobilization from muscles and fatty tissues, which leads to increased body temperature (Petersen et al, 2005). It is also a 'Myokine' a cytokine produced from the muscle, and it is elevated in response to muscle contraction (Febbraio et al, 2005; Pedersen et al, 2007).

## **7. Hybridomas and malignancy**

IL-6 has growth factor activities and stimulates the growth of hybridomas (Van Damme et al, 1987), plasmacytomas (Pattengale, 1997; Schirmacher et al, 1998) and multiple myelomas (Hitzler et al, 1991; Lokhorst et al, 1994; Pattengale, 1997). Multiple studies have documented high IL-6 levels in the serum of patients with certain carcinomas such as breast (Sansone et al, 2007; Knupfer and Preiss, 2007; Schafer and Brugge, 2007), lung (Schafer and Brugge, 2007; Pine et al, 2011), colon and rectum (Becker et al, 2005) and lymphoma (Kurzrock et al, 1993; Schafer and Brugge, 2007). In these patients high IL-6 levels correlated with a poor clinical prognosis. IL-6 also appears to be especially important in some sarcomas such as AIDS-related Kaposi's sarcoma (Miles et al, 1990; Yang et al, 1994) glioblastomas (Van Meir et al, 1990) and later stages of melanomas (Francis et al, 1996). However,

in contrast to its growth stimulatory activities, it is interesting to know that IL-6 is also a growth inhibitor for a number of carcinomas such as hepatocellular carcinoma (Moran et al, 2008); and early stages of melanoma (Lu et al, 1992).

## **8. Chronic inflammation and autoimmune diseases**

IL-6 has shown to be an important key player in several chronic and autoimmune diseases. It not only maintains inflammation, but also modifies the immune responses. It has been suggested that IL-6 can change the direction of immune responses to self or non-self, or activation or tolerance, by modifying the quality of peptide presentation by dendritic cells (Ishihara and Hirano, 2002). An overproduction of this protein has been reported to be involved in the pathology of rheumatoid arthritis (Lipsky, 2006; Nishimoto, 2006 ; Park and Philinger, 2007), Castleman's disease (Yoshizaki et al, 1989), juvenile idiopathic arthritis (Lepore et al, 1994), ankylosing spondylitis (Gratacós et al, 1994), systemic lupus erythematosus (Gröndal et al, 2000), psoriasis (Arican et al, 2005) and Crohn's disease (Ito, 2003). On the other hand, defective production of this protein has been proposed in the pathogenesis of other autoimmune disorders, such as liver autoimmune diseases (Tovey, 1989).

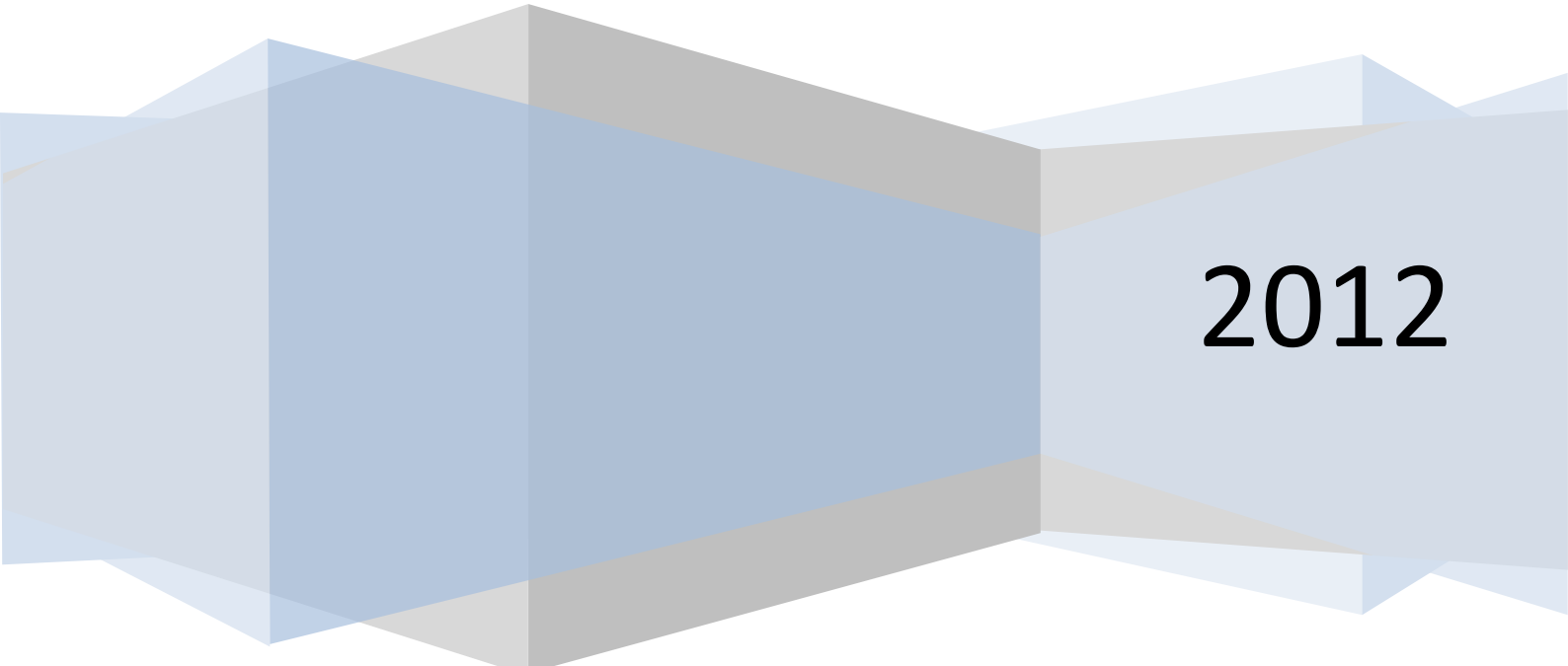
### **1.3 Aims and objectives of this study**

The aims of this study were:

1. To investigate the secretion and expression of interleukin-6 (IL-6) by human peripheral blood natural killer (PBNK) cells from healthy individuals by triggering NK cells with different stimuli, including co-cultures with K562 and HeLa cells, incubation with cytokines and non-specific chemical activators (PMA+ ionomycin).
2. To study which NK receptors are involved in the induction of IL-6 expression and secretion.
3. To evaluate the biological significance of IL-6 secretion by PBNK cells through an *in vitro* model.

# Chapter 2

## Materials and Methods



2012



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## **2.1 Cells**

### **2.1.1 Healthy donors**

Ethical approval from the South Sefton Ethics Committee was obtained for this study. Blood samples from healthy volunteers without previous history of autoimmune diseases or current infections were obtained at the University of Liverpool. All donors signed forms of consent to participate in the research project. Their age varied from 30-65 years and the M/F ratio was 2/5.

### **2.1.2 Mononuclear cell separation**

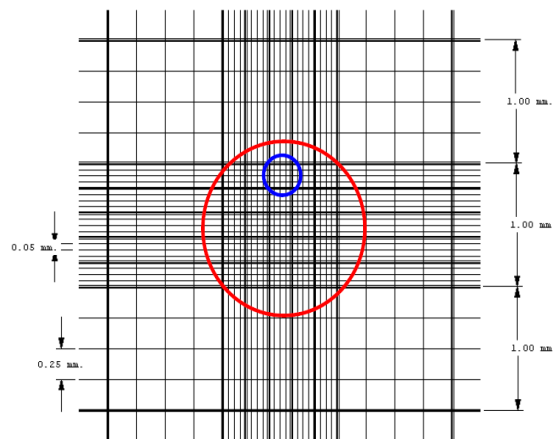
Heparinized venous blood of healthy volunteers was collected in sterile universal tubes. The amount of blood varied between 15 – 20 ml per donor. The blood was then diluted with equal volume of 1X Phosphate Buffered Saline (PBS) and the mixture was then slowly layered on top of 10 ml Ficoll Paque<sup>TM PLUS</sup> (Amersham Bioscience, Sweden). The samples were centrifuged at 400g (1800 rpm) for 20-25 minutes at 22 °C without braking during deceleration. Thus, the blood cells are distributed in the solution in layers based on the differences in their density/size. Resultant layers from top to bottom are: plasma – platelets – peripheral blood mononuclear cells (PBMCs) – Ficoll – red blood cells (with granulocytes). The PBMCs become visible as buffy coat between the plasma and the Ficoll, which was carefully removed with a pastette and transferred into new universal tube and washed twice with 1X PBS. After washing, the PBMCs were counted using a haemocytometer and the viability of the cells were assessed by staining with 0.4% trypan blue.

### 2.1.3 Cell Counting using a haemocytometer

For the cell counting procedure, a haemocytometer of 0.1 mm depth was used. The mononuclear cells were suspended in 10 ml culture medium. Ten  $\mu\text{l}$  aliquots of the cell suspension were then placed in a suitably-sized conical centrifuge tube (Eppendorf tube) and mixed with equal volume of 0.4 % trypan blue in PBS. A Gilson pipette was used to transfer the mixture of cells and trypan blue to the chambers of the haemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowing each chamber to fill by capillary action. The cells were then viewed under a microscope at 100 X magnification. Under the microscope, a grid of nine 1mm squares should be seen (Figure 2.1). The central 1mm square (big red circle) is divided into 25 groups of 0.25 mm small squares (small blue circle). All the viable cells in the central 1mm square were counted. The non-viable cells stained blue by trypan blue and were excluded from the total. Each large square of the haemocytometer, with cover-slip in place, represents a total volume of  $0.1 \text{ mm}^3$  ( $1.0 \text{ mm} \times 1.0 \text{ mm} \times 0.1 \text{ mm}$ ) or  $10^{-4} \text{ cm}^3$ . Since  $1 \text{ cm}^3$  is equivalent to approximately 1 ml, the total number of cells per ml was then determined using the following formula:

**Number of cells/ml = Total number of viable cells in 1mm central square x (dilution factor for trypan blue) x  $10^4$**

**Total cell number = cells per ml x the original volume of fluid from which cell sample was removed.**



**Figure 2.1: Cell counting using a haemocytometer.**

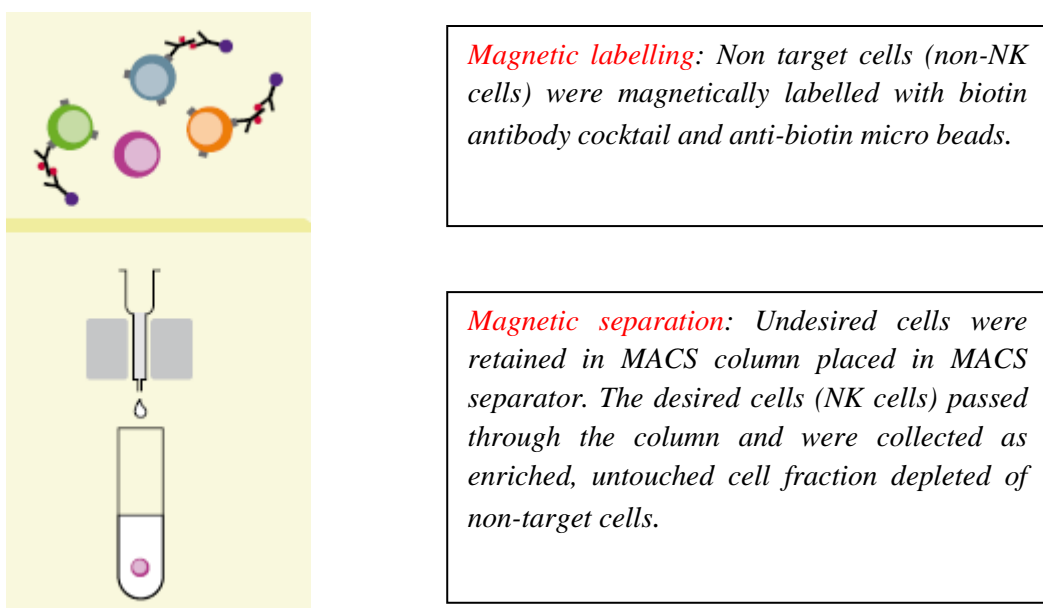
#### **2.1.4 Freezing cells**

After cell counting, the PBMCs were re-suspended in freezing medium that consisted of 90% foetal calf serum (FCS) and 10% dimethyl sulphoxide (DMSO). The cells were then transferred into small cryogenic vials at a concentration of  $1 \times 10^7$  viable cells/ 1ml freezing medium / vial and frozen at - 80 °C overnight. On the next day, they were transferred into liquid nitrogen and stored for further use.

#### **2.1.5 Effector cells (peripheral blood natural killer cells)**

The frozen cells (PBMCs) were taken out from the liquid nitrogen on the day of the experiment, warmed to 37 °C in a water bath and then transferred into a universal tube and warm culture medium (complete RPMI 1640 or DMEM medium) was added to fill the universal tube. Complete RPMI 1640 medium and Dulbecco's modified medium (DMEM) from (Sigma-Aldrich, UK) were supplemented with 10% foetal calf serum (FCS), 2 mL-glutamine (GIBCO, Invitrogen), 100 U of penicillin and 100 mg of streptomycin (GIBCO, Invitrogen) per ml. The cells were then spun at 400g (1800 rpm) for 10 minutes at 22 °C and the pellet re-suspended with culture medium (CM) in a plastic petri dish and incubated at 37 °C in atmosphere of 5% CO<sub>2</sub> for 2 hours. After incubation, the adherent cells containing the monocytes were discarded while the non-adherent cells containing the lymphocytes were collected and their cell number was determined. The peripheral blood natural killer (PBNK) cells were isolated from the other lymphocytes by negative magnetic separation using a commercial NK cell isolation kit (Miltenyi Biotec, Germany). Using this kit, the non NK cells, i.e. T cells, B-cells, stem cells, dendritic cells, monocytes, granulocytes and erythrocytes were magnetically labelled with biotin conjugated antibodies and a cocktail of micro beads. Upon passing the cells over a MACS® column (Miltenyi

Biotech, Germany) that is placed in a magnetic field, the magnetically labelled non-NK cells were retained on the column while the unlabeled NK cells passed through (negative selection). The procedure was carried out according to the manufacturer's instructions. Briefly, PBMCs cells were centrifuged at 400g (1800 rpm) for 10 minutes and the supernatant was removed completely. The cell pellet was re-suspended in 40  $\mu$ l of MACS buffer per  $10^7$  total cells and 10  $\mu$ l of NK cell selection biotin antibody cocktail was added. The cells were incubated for 10 minutes on ice. After this incubation, 30  $\mu$ l of MACS buffer and 20  $\mu$ l of micro bead cocktail were added to  $10^7$  total cells. The cells were incubated again for 15 minutes on ice. Following the second incubation, the cells were washed with 2 ml of MACS buffer / $10^7$  cells and centrifuged at 400g (1800 rpm) for 10 minutes. The supernatant was then removed completely and the cells were re-suspended in 500  $\mu$ l of MACS buffer and passed through an LS MACS column placed in a MACS separator. After three washings with 3 ml of MACS buffer, the total effluent was collected. This fraction represents the enriched NK cells. For higher purity, the cell suspension resulting from this selection process was applied to a second LS column.



**Figure 2.2: Diagram to illustrate purification of NK cells by immunomagnetic depletion.**

## **2.2 Cell lines**

### **2.2.1 Target cell lines**

For the purposes of activating resting peripheral blood natural killer cells (PBNK) cells, two different NK cell sensitive tumour cell lines were used as target cells:

#### **2.2.1.1 K562**

This is an erythroleukemia cell line derived from a 53-year-old female with chronic myeloid leukaemia in blast crisis (Lozio and Lozio, 1975). The cells are non-adherent and rounded with some morphology similar to undifferentiated granulocytes and erythrocytes (Gahmberg and Andersson, 1981). These cells do not express major histocompatibility complex (MHC) class I molecules and therefore can be easily killed by NK cells as they lack the MHC class I complexes required to inhibit NK activity (Zamai et al, 1998).

#### **2.2.1.2 HeLa**

HeLa cells are large adherent immortal cervical cancer cells derived from Henrietta Lacks, who died from her cancer in October, 1951 (Lucey et al, 1991). They are larger than K562s and divide in unlimited number of times in culture. These cells do express MHC class I molecules. However, they are known to be susceptible to NK cell lysis through a mechanism mediated by NKG2D, a dominant NK cell stimulatory receptor.

Both K562 and HeLa tumour cell lines were gifts from the Inflammation Research Unit/ Teaching Hospital Aintree, University of Liverpool. The K562 cells were maintained in 75 cm<sup>2</sup> tissue culture flask (Corning) in complete RPMI 1640

medium (Sigma-Aldrich, UK) supplemented with 10% foetal calf serum (FCS), 2 mM-glutamine (GIBCO, Invitrogen), 100 U of penicillin and 100 mg of streptomycin (GIBCO, Invitrogen) per ml. HeLa cells were maintained in 75 cm<sup>2</sup> tissue culture flask (Corning) in Dulbecco's modified medium (DMEM) from Sigma-Aldrich supplemented with 10% foetal calf serum (FCS), 2 mM-glutamine, 100 U of penicillin, and 100 mg of streptomycin per ml. The flasks were kept in a tissue culture incubator at 37 °C, 5% CO<sub>2</sub> and a humid atmosphere. Every 2-3 days, the non-adherent cells were centrifuged, washed once with 1 X PBS and the pellet re-suspended in a fresh CM. The adherent cells were trypsinized with trypsin (Sigma-Aldrich, UK) to detach them from the wall of culture flask, centrifuged and washed once with 1 X PBS. The pellet was then re-suspended in fresh CM. Aliquots of both K562 and HeLa cells were cryo-preserved in liquid nitrogen at a concentration of 1 x 10<sup>7</sup> cells / vial for future use.

### **2.2.2 Other cell lines**

Several other cell lines were used for the purposes of transfection and for studying the signalling pathways involved in the secretion of IL-6 by human NK cells. These included:

#### **2.2.2.1 CHO cells**

Chinese hamster ovary (CHO) cells are the most widely used mammalian cells for transfection, protein expression, and large-scale recombinant protein production. CHO cells were purchased from European Collection of Cell Cultures (ECACC). Upon receipt these cells were maintained in Ham's F12 medium (Sigma-Aldrich, UK) supplemented with 10% foetal calf serum (FCS), 2 mM-glutamine



(GIBCO, Invitrogen), 100 U of penicillin and 100 mg of streptomycin (GIBCO, Invitrogen) per ml. Since these cells were prepared from low passage cell bank cultures, they were frozen in multiple cryo vials to ensure having an adequate supply of early-passage cells. Culturing CHO cells was also attempted in serum free EX-CELL™ 302 medium (SAFC Biosciences, UK) supplemented with 2 mM L-glutamine (GIBCO, Invitrogen), 100 U of penicillin and 100 mg of streptomycin (GIBCO, Invitrogen) per ml. The latter is a serum free medium specifically developed for long term growth and maintenance of transfected and untransfected CHO cells.

#### **2.2.2.2 HEK - 293 cells**

HEK - 293 is an adherent cell line derived from human embryonic kidney cells (Graham et al, 1977). The source of these cells was a healthy, aborted foetus. Like CHO cells, HEK cells are easy to grow and transfect and have been widely used for cell biology research and also used by the biotechnology industry to produce therapeutic proteins and viruses for gene therapy. HEK - 293 cells were a gift from the Inflammation Research Unit/ Teaching Hospital Aintree, University of Liverpool.

#### **2.2.2.3 Caco-2 cells**

The Caco-2 cell line is a continuous line of heterogeneous human epithelial colorectal adeno carcinoma cells (Trainer et al, 1988). They are adherent and known to express ligands for NKG2D receptors, namely MICB and ULPBs (Butler et al, 2009) and therefore their RNA was used to synthesize the cDNA necessary for amplification of these ligands. Both HEK- 293 and Caco-2 cells were grown and maintained in 75 cm<sup>2</sup> tissue culture flasks containing complete DMEM medium.

## **2.3 Activation of resting PBNK cells**

Different methods were used for activating the resting PBNK cells after isolation. These included co-culturing the NK cells with target cells (K562 or HeLa cells), stimulation with cytokines (IL-2) at different concentrations and using phorbol diesters (e.g. Phorbol Myristate Acetate [PMA]) together with ionomycin).

### **2.3.1 Co-cultures of PBNK cell with target cells**

#### **2.3.1.1 NK cells and K562**

After isolation,  $1 \times 10^5$  NK cells from each healthy donor were co-cultured with  $10^5$  K562 cells in a 96-well round bottom tissue culture plate. Cell concentrations were adjusted to obtain effector: target (E: T) ratios of 1:1. Additionally, two sets of controls were prepared. The first control was containing only target cells (same concentration as in the co-cultures), and the other containing only  $1 \times 10^5$  NK cells in culture medium. All wells were prepared in duplicates whenever sufficient numbers of effector cells were available. The plate was then placed in a humidified incubator with 5% CO<sub>2</sub> atmosphere at 37 °C for up to 24 hrs. Supernatants from this co-culture as well as from the controls were collected at 1, 3, 6, 18 and 24 hrs. The supernatants were then stored at - 80 °C for further analysis.

#### **2.3.1.2 NK cells and HeLa**

Co-culturing of PBNK cells with HeLa cells was carried out in a similar way to co-culturing NK cells with K562 cells. The only difference was that the cell concentrations were adjusted to obtain effector: target (E: T) ratios of 5: 1 due to the fact that HeLa cells are significantly larger than K562s.

### **2.3.2 Stimulation of resting PBNK cells with PMA and ionomycin**

Isolated PBNK cells, at the same concentrations as those in the co-cultures, were washed twice with 1X PBS and incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> in the presence of 10 ng/ml of PMA (Sigma-Aldrich, UK) and ionomycin (1µg/ml). Supernatants were collected at 1, 3, 6 and 18 hrs (depending on the numbers of the effector cells) and stored at - 80 °C.

### **2.3.3 Activation of resting PBNK cells with IL-2**

Resting NK cells collected from healthy donors were washed twice with 1X PBS and then activated with 500 U / ml of recombinant human IL-2 (R & D Systems, UK). Another sample was activated with only 100 U / ml of IL-2. The cell concentration was the same in these co-cultures. Cells were then incubated at 37 °C with 5% CO<sub>2</sub>. Aliquots of tissue culture supernatants were collected at different time points i.e. 1, 3, 6 and 18 hrs and stored at - 80 °C until further analysis.

## **2.4 Analysis of isolated PBNK cells for CD56 surface antigen expression**

The purity of the enriched PBNK cells after negative magnetic selection was assessed by flow cytometry. Four aliquots of 100 µl of the enriched PBNK cell fraction were taken from each sample in Falcon tubes and washed twice with FACS buffer. The first aliquot was stained with PE-conjugated antibody against the NK cell marker (CD56). The second one was stained with FITC - conjugated antibody against a T - cell marker (CD3) and the third one was dual labelled with both CD56 PE and CD3 FITC. A fourth unstained sample was used as a control. The aliquots were then incubated on ice in the dark for 45-60 minutes. Following the incubation, the cells were washed twice and re-suspended in 500 µl of FACS buffer and run on a Cyan

ADP flow cytometer (DakoCytomation Coulter, USA). Analysis was carried out using the Summit software package (DAKO). The samples were acquired until 20,000 target cells had been counted. Gates on the NK cell populations were set manually whereby the cell debris and dead cells were excluded to assess NK cell purity (CD3<sup>-</sup> CD56<sup>+</sup>). All flow cytometry reagents and antibodies were from BD bioscience.

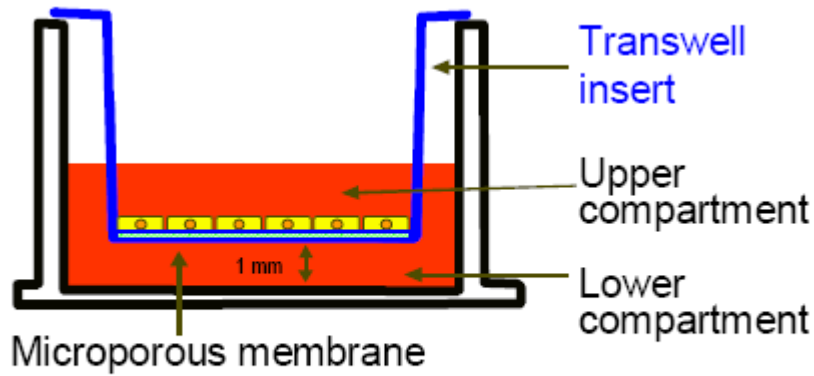
## **2.5 Measurement of IL-6 secretion by ELISA**

The IL-6 concentration in the co-cultures, IL-2 and PMA - activated supernatants was assessed using the 'Human IL-6 ELISA Ready-SET-Go' ELISA Kit (eBioscience, USA). This kit has a detection limit as low as 2 pg/ml; the procedure was done according to the manufacturer's instructions. 100 µl of monoclonal antibody specific for IL-6 (at a dilution of 48 µl/12 ml coating buffer) has been coated onto a 96-well ELISA micro plate (Corning) and incubated overnight at 4 °C. The wells were then washed with washing buffer. 100 µl of the standards in serial dilutions (from 200 to 0 pg / ml) and the samples were pipetted into the wells and incubated for 2 hrs. IL-6, if present in a sample, was bound to the wells by the immobilized antibody. The wells were washed and 100 µl of biotinylated anti-human IL-6 antibody (concentration of 48 µl/12 ml assay buffer) was added for 1 hr. After washing to remove any unbound biotinylated antibody, 100 µl of HRP-conjugated streptavidin was pipetted into the wells and the plate was incubated further for 30 minutes. After a further washing step 100 µl of a 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate solution was added to the wells. A blue colour developed in proportion to the amount of IL-6 bound. The colour development was stopped by adding Stop solution which changed the colour from blue to yellow. Finally, the intensity of the colour was measured at 450 nm and a calibration curves is plotted on semi log paper and values of optical density (OD) of

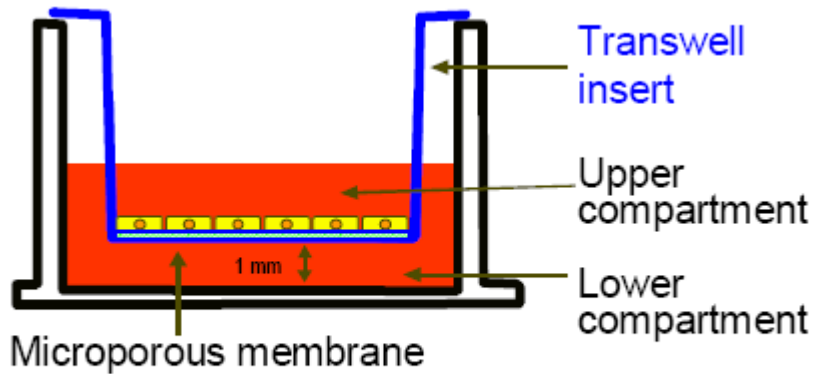
samples calculated from the standard. A TECAN plate reader was used to measure the absorbance (optical density) at 450 nm. The generation of standard curve and measurement the concentration of unknowns were done by using Microsoft Excel.

## **2.6 Assessment of IL-6 secretion using Transwell plates**

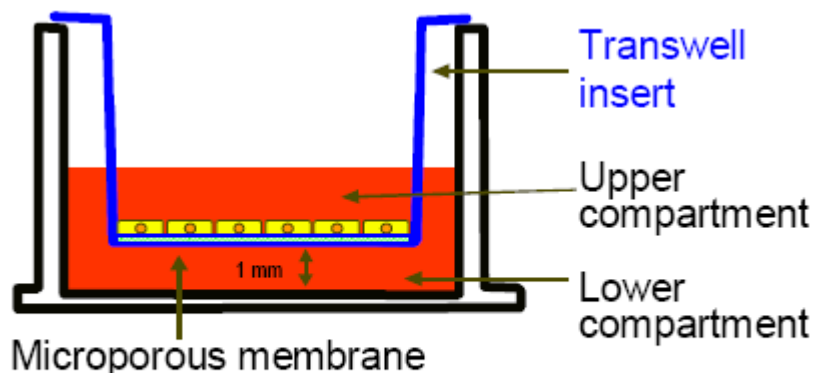
In this assay, a 24-well polycarbonate Transwell plates (Corning) with 0.4  $\mu\text{m}$  pore size inserts were used. In the first well, resting PBNK cells from healthy donors were added at a concentration of  $1 \times 10^5$  cells / 100  $\mu\text{l}$  of culture medium in the upper chamber of the Transwell plate.  $1 \times 10^5$  K562 (or  $2 \times 10^4$ ) HeLa cells suspended in 600  $\mu\text{l}$  of culture medium were added to the lower chamber to act as target cells (Figure 2.3A). In the second well,  $1 \times 10^5$  NK cells and an identical number of K562 cells (or  $2 \times 10^4$  HeLa cells) were placed in the lower compartment of the Transwell plate and adjusted to a total volume of 600  $\mu\text{l}$  of culture medium. 100  $\mu\text{l}$  of culture medium, containing no cells was added to the upper compartment (Figure 2.3B). A third well was used as a negative control in which 100  $\mu\text{l}$  of culture medium was added to the upper compartment while the lower compartment received  $1 \times 10^5$  K562 (or  $2 \times 10^4$  HeLa cells) (Figure 2.3C). The wells were incubated at 37  $^\circ\text{C}$  with 5%  $\text{CO}_2$  for 24 hrs and the supernatants were collected from the lower compartments. IL-6 was then measured in the supernatants by ELISA.



**Figure 2.3A:**  $1 \times 10^5$  resting PBNK cells from healthy donors in  $100 \mu\text{l}$  of culture medium were added to the upper chamber of the Transwell well. The K562 with same concentration as the PBNK cells (or  $2 \times 10^4$  HeLa cells) suspended in  $600 \mu\text{l}$  of culture medium were added to the lower chamber to act as target cells.



**Figure 2.3B:** The NK cells in a concentration of  $1 \times 10^5$  cells were co-cultured with same concentration of K562 (or  $2 \times 10^4$  HeLa cells) in the lower compartment of the Transwell plate and adjusted to a total volume of  $600 \mu\text{l}$  of culture medium.  $100 \mu\text{l}$  of culture medium without cells was added to the upper compartment.



**Figure 2.3 C:** Control sample in which  $100 \mu\text{l}$  of culture medium was added to the upper compartment while the lower compartment received  $1 \times 10^5$  K562 (or  $2 \times 10^4$  HeLa) cells. Pictures were reproduced from [www.corning.com](http://www.corning.com)

## **2.7 Reverse transcription–polymerase chain reaction (RT-PCR)**

### **2.7.1 RNA handling**

Obtaining high quality, intact RNA is the first and often the most critical step in performing many molecular biology experiments, such as Northern analysis, RT-PCR, and quantitative PCR (qPCR). As RNA is very unstable all reagents and materials for its handling were treated to remove possible Ribonucleases (RNase) activity. RNAses are very stable and are difficult to inactivate. Therefore, throughout the extraction, measures were taken to minimize RNase activity. These included working in a dedicated RNA room, changing gloves regularly, and using previously un-opened RNase-free tips and dedicated pipettes.

### **2.7.2 Total RNA extraction from NK cells and cell lines**

Total RNA was extracted from NK cells and cell lines (HeLa and Caco-2 cells) using the QIAamp RNA Blood Mini Kit (Qiagen, UK) according to the manufacturer's instructions. The purified NK cells and cell lines were disrupted or lysed using Buffer RLT to which  $\beta$ -Mercaptoethanol was added. The lysate was then added into a QIAshredder<sup>TM</sup> spin column in a 2 ml collection tube and centrifuged for 2 minutes at maximum speed (14,000 rpm) to homogenize. The QIAshredder<sup>TM</sup> spin column was then discarded and the homogenized lysate was saved. One volume of 70% ethanol was then added to the homogenized lysate to maximize the binding capacity and mixed by pipetting. The sample was then carefully pipeted into QIAamp spin column in a 2 ml collection tube without moistening the rim, and centrifuged for 15 seconds at 10,000 rpm. This step is necessary to bind the RNA to silica membrane. The QIAamp spin column was then transferred into a new 2 ml collection tube and Buffer RW1 was added into the QIAamp spin column and centrifuged for 15 seconds

at 10,000 rpm to wash .The QIAamp spin column was then transferred into a new 2 ml collection tube and washed with 500 µl buffer RPE (with ethanol) and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded. This washing step was repeated again with the same buffer at full speed (14,000 rpm) for a longer period (3 minutes) to dry the membrane. The QIAamp spin column was placed in a new 2 ml collection tube and the old collection tube was discarded. The column then centrifuged at full speed for 1 minute to remove any excess of ethanol. The total RNA was then eluted from the membrane by adding 30 - 50 µl of RNase-free water directly onto the QIAamp spin column followed by centrifugation for 1 minute at 10,000 rpm. The eluted RNA was then either used immediately or stored in a - 80°C freezer for further use.

### **2.7.3 Measuring RNA concentration**

The following formula was used for measuring RNA concentration:

$$\text{RNA conc.} = \text{Optical Density at 260 nm} \times \text{Dilution Factor} \times 40\mu\text{g/ml}$$

$$\text{Total RNA yield} = \text{Concentration} \times \text{Volume of stock in ml}$$

### **2.7.4 Determining RNA purity**

The ratio between the readings at 260 nm and 280 nm (A260/A280 ratio) provides estimates of the purity of RNA.

Ratios more than 1.8 were taken to indicate good RNA purity (Manchester, 1996).



### **2.7.5 Determining the RNA integrity**

The integrity of RNA is a major concern for gene expression studies and traditionally has been evaluated by comparing the intensity of the 28S and 18S rRNA bands on agarose gel. A denaturing gel system is suggested because most RNA forms extensive secondary structure via intra-molecular base pairing, and this prevents it from migrating strictly according to its size. Formaldehyde is usually used for analysis of RNA on gels and to keep the RNA denatured.

### **2.7.6 Formaldehyde - Denaturing Agarose Gel Electrophoresis**

The electrophoresis tank and comb were cleaned with a detergent solution, thoroughly rinsed with 70% ethanol and left overnight in DEPC (diethylpyrocarbonate) treated RNase-free water. On the next day, 1 g agarose was melted in 82 ml of 1X TAE buffer prepared using DEPC treated water. The mixture was heated in microwave until dissolved and then allowed to cool to 50°C. After that, 18 ml of 37% formaldehyde and 1 µl of ethidium bromide were added to the gel mixture. The gel was mixed gently and then poured carefully onto the casting tray with the combs already inserted and left to set at room temperature for 30 - 45 minutes. When fully set, the gel was assembled in the electrophoresis tank and the comb was carefully removed. 1X TAE buffer was then poured to cover the gel surface and used as running buffer. 4 volumes of RNA sample was mixed with 1 volume of 5X RNA sample buffer. The mixture was then heated at 65°C for 15 minutes and then immediately chilled on ice for 1 minute to denature the RNA. The RNA sample was then loaded onto the formaldehyde agarose gel. The gel was run at 1-5 V/ cm until the dye front has migrated the appropriate distance. The gel was visualized under UV light and digitally recorded.

### 2.7.7 Reverse transcription (cDNA synthesis)

The AMV (Avian Myeloblastosis Virus) Reverse Transcription System (Promega, UK) was used to synthesize single-stranded complementary DNA (cDNA) from the isolated total RNA. Since this system uses the AMV polymerase enzyme, a short double-stranded sequence is needed at the 3' end of the mRNA which acts as a start point for the polymerase. This is provided by poly (A) tail found at the 3' end of most eukaryotic mRNAs to which a short complementary synthetic oligonucleotide (oligo dT primer) is hybridized (polyT-polyA hybrid). The total RNA template and all other reaction components were thawed and stored on ice. The optimal amount of starting RNA varied from 100 ng up to 1µg depending on each experimental design and the concentration of target cells. The RNA was heated at 70 °C for 10 minutes, centrifuged briefly and then placed on ice. A 20 µl reaction master mix was then prepared as follows:

Reagent	Volume	Final Concentration
10 X AMV RT Buffer	2	1X
MgCl <sub>2</sub> (25mM)	4	5mM
Deoxyribonucleose triphosphates (dNTPs),10mM	2	1Mm
Oligo dt(15) Primer	0.5	0.5 µg
RNase inhibitor	0.5 µl	1u/µl
AMV reverse Transcriptase	1 µl	15 u
RNase –free water	variable	
Template RNA	variable	100 ng up to 1 µg
Total	20 µl	

The reverse transcription reaction was incubated in a thermal cycler (Eppendorf Mastercycler, Germany) at 42°C for 15 minutes. During this step the oligo dT primer anneals to poly-A tail of mRNA and the AMV reverse transcriptase synthesizes a complementary DNA strand on the mRNA template (RNA-DNA hybrid). At the same time the AMV reverse transcriptase enzyme has a strong ribonuclease activity (RNase H) which recognises the RNA component of the RNA:DNA hybrid. This results in the cleavage of the RNA at a number of non-specific sites leaving a single stranded cDNA copy of the RNA template. After the completion of the reverse transcription the sample was heated at 95°C for 5 minutes, and then incubated at 0 - 5°C for 5 minutes. This inactivates the AMV reverse transcriptase and prevents it from binding to the cDNA. The first-strand cDNA was then stored at -20°C until use.

## **2.8 PCR based DNA cloning**

### **2.8.1 General primer design considerations**

The most vital step in the development of a PCR reaction is the design of suitable primers. A PCR primer pair consists of two oligonucleotides that hybridize to complementary stands of the DNA template, and thus identify the region to be copied. The following considerations were taken into account when designing the primers for PCR reactions:

1. Primer length 18-30 bp
2. Primers should be in 5' to 3' orientation
3. Primer melting temperature (T<sub>m</sub>) 55 °C -65 °C.
4. T<sub>m</sub> difference between forward and reverse primers ≤ 5°C
5. Guanine-Cytosine (GC) content 40 - 60 %
6. No secondary structures such as self dimers or hairpin.

## 2.8.2 Fc-Fusion protein primers

The human NKG2D (hNKG2D) ligands were planned to be expressed as Fc-fusion proteins to investigate the role of NKG2D signalling in the initiation of IL6 production by NK cells. This can be achieved by the use of overlap extension PCR. In the first round (PCR1) the genes encoding for the extracellular portions of hNKG2D ligands (MICA, MICB, ULBP1, ULBP2 and ULBP3) were amplified. In PCR2, the genes encoding the hinge region, CH2 and CH3 domains of heavy chain of mouse IgG (m IgG) were amplified. The primers were specifically designed so that the 3' ends of PCR1 and PCR2 were overlapped. This was followed by third round of PCR (PCR3) in which these two intermediate products were hybridized together and were extended to generate the full length recombinant gene using the forward primer of NKG2D ligand and the reverse primer of the Fc fragment respectively. These primers also included restriction enzyme sites for inserting the product into an expression vector for cloning purposes. The following primers were used to generate hNKG2D - mFc fusion proteins throughout this study (Table 2.1). All of them were purchased from Invitrogen and their designs are discussed in more details in Chapter 5:

**Table 2.1: Primers used to generate hNKG2D ligands - mFc fusion proteins**

Primer name	Primer sequence	Primer length
EcoRI – MICA forward primer1- sense	5' TCTGAATTCATGGGGCTGGGCCCGGTC 3'	27 bp
EcoRI – MICB forward primer1- sense	5' TCTGAATTCATGGGGCTGGGCCCGGTC 3'	27 bp
EcoRI – ULBP1 forward primer1- sense	5'TCTGAATTCATGGCAGCGGCCCGCCAGC 3'	27 bp
EcoRI – ULBP2 forward primer1- sense	5' TCTGAATTCATGGCAGCAGCCCGCCGCT 3'	27 bp

EcoRI – ULBP3 forward primer1 - sense	5' TCTGAATTCATGGCAGCGGCCAGCCCC 3'	27 bp
EcoRI – MICA forward primer2 - sense	5' TCTGAATTCGGAGCCCCACAGTCTTCGTTA3'	30 bp
EcoRI – MICB forward primer2 - sense	5'TCTGAATTCGGAGCCCCACAGTCTTCGTTA3'	30 bp
EcoRI – ULBP1 forward primer2 - sense	5'TCTGAATTCGTGTGAAGTTCAAGGCCTGGT 3'	30 bp
EcoRI – ULBP2 forward primer2- sense	5' TCTGAATTCGTGTGCGGTTCAAGGCCAGGT 3'	30 bp
EcoRI – ULBP3 forward primer2- sense	5'TCTGAATTCGTGTGAGGTCCAGAGCCAGGT 3'	30 bp
MICA reverse (pFUSE overlap) - antisense	5' TGTGGGCCCTCTGGGAGAGGGCACAGGGTG 3'	30 bp
MICB reverse (pFUSE overlap) - antisense	5' TGTGGGCCCTCTGGGAGGGTGAGTGCCGTG 3'	30 bp
ULBP1 reverse (pFUSE overlap)-antisense	5' TGTGGGCCCTCTGGGTTGTTCCAGTACAT 3'	30 bp
ULBP2 reverse (pFUSE overlap) - antisense	5' TGTGGGCCCTCTGGGGCTGTCCATGCCCAT 3'	30 bp
ULBP3 reverse (pFUSE overlap)-antisense	5' TGTGGGCCCTCTGGGCTTCTTCTGTGCAT 3'	30 bp
pFUSE forward (MICA overlap) - sense	5' CACCCTGTGCCCTCTCCCAGAGGGCCCCACA 3'	30 bp
pFUSE forward (MICB overlap) - sense	5' CACGGCACTCACCTCCCAGAGGGCCCCACA 3'	30 bp
pFUSE forward (ULBP1 overlap) -sense	5' ATGTA CTGGGAACAACCCAGAGGGCCCCACA 3'	30bp
pFUSE forward (ULBP2 overlap) - sense	5' ATGGGCATGGACAGCCCCAGAGGGCCCCACA 3'	30bp
pFUSE forward (ULBP3 overlap) - sense	5'ATGCACAGGAAGAAGCCCAGAGGGCCCCACA3'	30bp
pFUSE reverse (anti-sense)	5' CTGGGTCATCCTCGCTCACATCCACCA 3'	27 bp
BglIII - MICA reverse-antisense	5'GTTAGATCTAGAGGGCACAGGGTGAGTGCT 3'	30 bp

### 2.8.3 Setting up the overlap extension PCR reaction

#### 2.8.3.1 PCR1 for the amplification of genes encoding the extracellular portion of hNKG2D ligands (MICA, MICB, ULBP1, ULBP2 and ULBP3)

Whenever these PCR1 reactions were conducted, two 50  $\mu$ l PCR reactions (one for the sample and other for negative control) were set up. All components were thawed and kept on ice and all the work was carried out in a clean, aseptic environment using gloves and clean RNase free disposable pipettes. Phusion® High Fidelity polymerase (Finnzymes) was used for this step to decrease error rate. The master mix was prepared as follows:

Reagent	Volume	Final concentration
<b>5X Phusion HF buffer</b> (Finnzymes)	10 $\mu$ l	1X
<b>dNTPS, 10mM</b> (Promega)	1 $\mu$ l	200 $\mu$ M each
<b>Sense primer*</b>	1 $\mu$ l	0.5 $\mu$ m
<b>Anti-sense primer*</b>	1 $\mu$ l	0.5 $\mu$ m
<b>cDNA**</b>	1 $\mu$ l	
<b>Phusion® High-Fidelity DNA polymerase</b> (Finnzymes)	0.5 $\mu$ l	0.02 U/ $\mu$ l
<b>H2O</b>	Up to 50 $\mu$ l	
<b>Total</b>	50 $\mu$ l	

\* H2O was used instead of the sense and anti-sense primers for the negative control.

\*\* HeLa cDNA was used as a template to amplify the genes encoding for the extracellular portion of MICA.

Caco-2 cDNA was used as a template to amplify the genes encoding for the extracellular portion of MICB and ULBPs.

After setting up the reaction mix, the tubes were spun down briefly to bring all the components of PCR reactions to the bottom and then incubated in a Thermalcycler (Eppendorf, Germany). The cyclic parameters were as follows:

Cycle step	Temperature	Time	Cycles
<b>Initial denaturation</b>	98°C	30 seconds	1
<b>Denaturation</b>	98°C	5-10 seconds	30 cycles
<b>Annealing</b>	*	10-30 seconds	
<b>Extension</b>	72°C	1 minute	
<b>Final extension</b>	72°C	5 minutes	1
	4°C	hold	

\*MICA/B = 62°C

\*ULBP1 = 60°C

\*ULBP2 = 62°C

\*ULBP3 = 60 °C

### 2.8.3.2 PCR 2 for the amplification of genes encoding for the Fc portion of mouse IgG

The pFUSE mIgG2A-Fc plasmid (Invivogen) was used as a template to amplify the Fc portion of the mouse IgG2A. pFUSE-Fc plasmids are family of plasmids specifically developed to facilitate the construction of Fc-Fusion proteins by fusing a sequence encoding a given protein to the Fc region of an immunoglobulin.

Two set of reaction mixes (sample and negative control) were prepared as follows:

Reagent	Volume	Final concentration
5X Phusion HF buffer (Finnzymes)	10 $\mu$ l	1X
dNTPS, 10mM (Promega)	1 $\mu$ l	200 $\mu$ M each
Sense primer*	1 $\mu$ l	0.5 $\mu$ M
Anti-sense primer*	1 $\mu$ l	0.5 $\mu$ M
cDNA**	1 $\mu$ l	
Phusion®High-Fidelity DNAPolymerase (Finnzymes)	0.5 $\mu$ l	0.02 U/ $\mu$ l
H2O	Up to 50 $\mu$ l	
<b>Total</b>	50 $\mu$ l	

\* H2O was used instead of the sense and anti-sense primers for the negative control.

\*\* pFUSE-mIgG2A-Fc plasmid was used as a template to amplify the Fc portion of mIgG2A.

The thermocyclic parameters were as follows:

Cycle step	Temperature	Time	Cycles
<b>Initial denaturation</b>	98°C	30 seconds	1
<b>Denaturation</b>	98°C	5-10 seconds	30 cycles
<b>Annealing</b>	*	10-30 seconds	
<b>Extension</b>	72°C	1 minute	
<b>Final extension</b>	72°C 4°C	5 minutes hold	1

\* pFUSE (MICA overlap) = 62

\* pFUSE (ULBP1 overlap) = 60

\* pFUSE (ULBP2 overlap) = 62

\* pFUSE (ULBP3 overlap) = 60



After amplification, the PCR1 and PCR2 were purified using Qiagen MinElute PCR Purification Kit (Qiagen, UK). In brief, one volume of PCR1 or PCR2 products was mixed with 5 volumes of Qiagen PB buffer. The mixture was added to Qiagen MinElute column and centrifuged for 1 minute at maximum speed (14,000 rpm). Following centrifugation, the column was washed with 750  $\mu$ l of PE buffer (with ethanol) and centrifuged again for 1 minute at maximum speed. After discarding the flow-through, the column was centrifuged for additional 1 minute to get rid of any residual ethanol from PE buffer. Finally, the column was placed in a clean 1.5 ml microcentrifuge tube and 50  $\mu$ l of elution buffer (EB buffer) was added to the centre of the column membrane. The DNA was then eluted from the column membrane by centrifugation for 1 minute at maximum speed.

A 1% agarose gel in 1X TAE buffer was used to analyze and visualise the DNA fragments generated during PCR1 and PCR2 reactions. 1.5 g of agarose powder (Web Scientific, UK) was mixed with 150 ml of 1X TAE buffer and heated in a microwave oven until completely melted. After melting, the gel was cooled to about 50 °C and 1  $\mu$ l of ethidium bromide was added to the gel at this point to facilitate the visualization of DNA after electrophoresis. The gel was then poured into a casting tray containing a comb and allowed to solidify at room temperature. After the gel set the comb was removed, and the gel (still in its plastic tray), was inserted horizontally into the electrophoresis chamber and just covered with 1X TAE buffer. 5 $\mu$ l DNA samples from the PCR1 and PCR2 products were mixed with 6X loading buffer (NEB, UK) and pipetted into the sample wells along with 10000 bp DNA ladder (Web Scientific, UK ; NBS Biologicals, UK). After electrophoresis, the amplified fragments were checked for their size and approximate concentration and a picture was taken by digital camera.

### 2.8.3.3 PCR3 for fusing hNKG2D ligand - mFc recombinant genes

In this PCR, a recombinant DNA construct for hNKG2D ligands was generated by fusing the sequence encoding for the extracellular portion of NKG2D ligand proteins (MICA/B, ULBP1, ULBP2 and ULBP3) to the Fc region of mouse IgG2A. The PCR3 reaction was set up in 2 steps:

In the first step 15µl of PCR 1 were mixed with 15 µl of PCR 2 in 0.5 ml PCR tube and the reaction was allowed to run on Thermocycler (Eppendorf, Germany) for 10 cycles without primers. During this step the annealing temperature of the overlap area was determined and the extension time increased to allow the annealing of PCR1 with PCR2. The reaction was set as follows:

Reagent	Volume	Final concentration
<b>5X Phusion HF buffer</b> (Finnzymes)	10 µl	1X
<b>dNTPS, 10mM</b> (Promega)	1 µl	200 µM each
<b>Sense primer</b>	-	-
<b>Anti-sense primer</b>	-	-
<b>cDNA</b>	15µl of PCR1+15 µl of PCR 2	
<b>Phusion®High-Fidelity DNAPolymerase</b> (Finnzymes)	0.5 µl	0.02 U/µl
<b>H2O</b>	Up to 50 µl	
<b>Total</b>	50 µl	

The cycling parameters were as follows:

Cycle step	Temperature	Time	Cycles
<b>Initial denaturation</b>	98°C	30 seconds	1
<b>Denaturation</b> <b>Annealing</b> <b>Extension</b>	98°C * 72°C	5-10 seconds 10-30 seconds 1.5 minutes	10 cycles
<b>Final extension</b>	72°C 4°C	5 minutes hold	1

\*MICA/B fusion = 66

\*ULBP1 fusion = 61

\*ULBP2 fusion = 65

\*ULBP3 fusion = 62

Following this step, 1 µl of the forward primer (either MICA/B or ULBP1 or ULBP2 or ULBP3 forward primer) and 1 µl of the pFUSE-mIgG2A-Fc reverse primer were added to the reaction. More Phusion® Polymerase enzyme (0.3 µl) was also added to the PCR mix at this step and the reaction was run for another 25 cycles using the following cycling conditions:

Cycle step	Temperature	Time	Cycles
<b>Initial denaturation</b>	98°C	30 seconds	1
<b>Denaturation</b> <b>Annealing</b> <b>Extension</b>	98°C * 72°C	5-10 seconds 10-30 seconds 1.5 minutes	25 cycles
<b>Final extension</b>	72°C 4°C	5 minutes hold	1

\*MICA/B fusion = 66

\*ULBP1 fusion = 61

\*ULBP2 fusion = 65

\*ULBP3 fusion = 62

#### **2.8.4 Visualization and extraction of the fused PCR products**

After analysing on 1% agarose gel/1X TAE buffer, the PCR3 (recombinant DNA products) were then gel purified using PureLink™ Quick Gel Extraction Kit (Invitrogen) according to the manufacturer's instructions. In brief, the gel area containing the correct size recombinant DNA fragment was carefully excised using a clean, sharp razor blade. The excised gel was then placed in a 1.5 ml polypropylene micro-centrifuge tube and weighed. Three volumes of Quick Gel Solubilisation Buffer were then added to the gel and allowed to melt at 50 °C for at least 10 minutes. After complete dissolution of the gel, 1 volume of 100% isopropanol was added to the DNA-gel mixture and the mixture was then loaded onto Quick Gel Extraction column and centrifuged at 10,000 rpm for 1 minute. This step is necessary to bind the DNA to silica membrane. Following centrifugation, the flow-through was discarded and the membrane was washed with 500 µl of Quick gel Wash Buffer (with ethanol) and centrifuged at 10,000 rpm for another 1 minutes. To remove any residual ethanol and Wash Buffer, the Quick Gel Extraction column was centrifuged again for 2-3 minutes. The wash tube was then discarded together with the flow-through and the Quick Gel column was placed in a new 1.5 ml eppendorf tube. Finally, the DNA was eluted from the membrane by adding 30 - 50 µl of Quick Gel Elution Buffer directly onto the column followed by centrifugation for 1 minute at 13,000 rpm. The eluted DNA was either used immediately or stored in a - 80°C freezer until further use.

## 2.9 Cloning and sequencing of the recombinant gene products

### 2.9.1 Restriction enzyme digestion and T4 ligation

The recombinant DNA products (hNKG2D ligand- mFc recombinant genes) were cloned into pFUSE-mIgG2A - Fc1 and pFUSE-mIgG2A - Fc2 plasmids (Invivogen, San Diego, USA). These plasmids are cloning plasmids with Zeocin selection specifically engineered to facilitate the generation and secretion of Fc fusion proteins. Both the recombinant genes and plasmids were double digested for 2 hours by EcoRI and BspHI endonucleases (NEB) according to manufacturer's instructions. The double digest was possible because both enzymes are 100% active in the same buffer (Buffer 4 from NEB). The digested plasmid and overlap PCR products were gel purified as described above and then ligated overnight at 4 °C by T4 ligase enzyme (Promega) according to manufacturer's instructions. The equation described by Alexei Gratchev ([www.methods.info / Methods / RNA DNA / ligation](http://www.methods.info/Methods/RNA_DNA/ligation)) was used to calculate the volume of the insert needed for the ligation reaction.

$$L_i = 3 \times L_v \times \frac{S_i}{S_v} \times \frac{I_v}{I_i} \times \frac{V_i}{V_v}$$

$L_i$  = volume of the insert in  $\mu\text{l}$ .

$L_v$  = volume of the vector in  $\mu\text{l}$  to be used.

$S_i$  = Size of the insert

$S_v$  = Size of the vector

$I_v/I_i$  = approximate intensity of the vector to the intensity of the insert on agarose gel.

$V_i/V_v$  = volume of the insert to volume of the vector loaded on the gel.

### **2.9.2 Transformation**

C2925I *dam*<sup>-</sup>/*dcm*<sup>-</sup> competent *E. coli* was first purchased from NEB and maintained and re-prepared using the protocol described by Chung et al (1989). In this method, the *E. coli* were grown in LB (Luria-Bertani) broth to the early exponential phase (OD<sub>600</sub> 0.3-0.4), pelleted by centrifugation at 1000 x g for 10 min at 4 °C and then re-suspended at one-tenth of their original volume in ice-cold transformation and storage solution (TSS). A 0.1 ml aliquot of *E. coli* was then transferred into 15 ml cold polypropylene tube, mixed with the ligation mix, and incubated for 30 min at 4 °C. After incubation, 0.9 ml of TSS with 20 mM glucose was added, and the bugs were grown at 37 °C with shaking (225 rpm) for 1 hr to allow expression of the antibiotic-resistance gene. 1 µl of empty pFUSE-mIgG2A-Fc vector was also transformed and used as positive control to check for transformation efficiency. Transformants were then selected by plating transformed *E. coli* on LB agar plates containing Zeocin (50 µg/ml), and incubated overnight at 37 °C. On next day, at least 3 colonies were picked and screened for the presence of the recombinant vector by colony PCR or by restriction endonuclease digestion and gel electrophoresis of minipreps.

### **2.9.3 Colony PCR protocol**

Colonies were picked up, suspended in 3 ml of LB broth + Zeocin and incubated at 37 °C for 2-3 hours with vigorous shaking. After incubation, the cells were centrifuged at maximum speed at 4 °C for 10 minutes and the supernatant was discarded. The cell pellet was then re-suspended in 50 µl of 10 mM EDTA and heated at 100 °C for 5 minutes. After heating, the lysate was vortex and briefly centrifuged. 1-2 µl of the lysate was taken and used as template for colony PCR. The PCR master mix was set up as follows:

Reagent	Volume	Final concentration
10 X Taq Buffer (Qiagen)	10 $\mu$ l	1X
dNTPS, 10mM (Promega)	1 $\mu$ l	200 $\mu$ M each
Sense primer*	1 $\mu$ l	0.5 $\mu$ M
Anti-sense primer*	1 $\mu$ l	0.5 $\mu$ M
cDNA**	1 $\mu$ l	
TaqDNAPolymerase (Qiagen)	0.5 $\mu$ l	0.02 U/ $\mu$ l
H2O	Up to 50 $\mu$ l	
Total	50 $\mu$ l	

\*Sense Primer = Forward primer for MICA, MICB, ULBP1, ULBP2 or ULBP3.

\*Anti-sense primer = Reverse primer for pFUSE-mIgG2A-Fc vector.

\*\*cDNA = colony lysate

#### 2.9.4 Purification of pFUSE-mIgG2A-Fc plasmids containing recombinant DNA (miniprep)

pFUSE plasmids containing the recombinant (fusion) DNA were purified using Qiaprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Using this kit, the positive colonies on colony PCR were collected and re-suspended in 6 ml of LB broth containing Zeocin and incubated overnight at 37 °C with shaking at 225 rpm. The next day the bacterial cells were centrifuged at 1000 x g at 4 °C for 10 minutes and the pellet was re-suspended in 250  $\mu$ l of Buffer P1. After re-suspension, the bacterial cells were lysed with gentle mixing in 250  $\mu$ l of Buffer P2 in the presence of RNase A. Buffer P2 is a NaOH/SDS buffer in which the SDS (sodium dodecyl sulphate) solubilises the components of the cell membrane, leading to lysis and release of the cell contents while NaOH denatures the chromosomal and plasmid DNAs, as well as proteins. The lysate was then neutralized and adjusted to

high-salt binding conditions in one step by the addition of 350 µl of Buffer N3. The high salt concentration of Buffer N3 causes denatured proteins, chromosomal DNA, cellular debris, and SDS to precipitate, while the smaller plasmid DNA re-natures correctly and stays in solution. After clearing the lysate by centrifugation at 14,000 rpm for 10 minutes, the supernatant containing the plasmid DNA was applied to the QIAprep Spin Column by decanting or pipetting and centrifuged for 1 minute at 10,000 rpm. The QIAprep Spin Column is a silica membrane column that allows only the DNA to adsorb to the membrane under optimized buffer conditions. After centrifugation, the flow-through was discarded and the column was washed with 500 µl of Buffer PB and centrifuged again for 1 minute. The salts were removed by a wash step with 750 µl of buffer PE. Any residual wash Buffer should be removed from the column and this can be achieved additional centrifugation for 1 minute. Plasmid DNA was then eluted from the QIAprep column with 50 µl of Buffer EB or water and the purified plasmid DNA was then checked for the presence of recombinant (fusion) DNA by endonuclease digestion and gel electrophoresis.

### **2.9.5 Plasmid Maxi-prep and DNA sequencing**

Bulk cultures of pFUSE-mIgG2A-Fc plasmids containing recombinant NKG2D ligands - Fc genes (MICA/B, ULBP1, ULBP2 and ULBP3 recombinant genes) were purified using the QIAGEN Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions. The purified plasmids were quantified by spectrophotometry and sent to Source BioScience LifeSciences (Cambridge, UK) for DNA sequencing. The resulting sequences were then verified with the NCBI BLAST programme.



### 2.9.6 Transfection

After DNA sequence verification, CHO cells and HEK - 293 cells were transfected with hNKG2D ligand - mFc fusion plasmid constructs and the positive clones were selected. Transfectants were generated using non liposomal reagents, electroporation or a combination of cell - specific reagent and electroporation. With non liposomal reagents, CHO or HEK - 293 cells were cultivated in 75 cm<sup>2</sup> tissue culture flasks as described in Section 2.2.2. 1-2 days after, when the cells reached 90% confluence, transfection was performed using 20 µg of plasmid DNA and 20 µl of jetPRIME (Polyplus transfection<sup>TM</sup>, USA) for 4 hours according to the manufacturer's recommendations. The transfection medium was then removed and cells were incubated at 37 °C with 20 ml fresh culture medium for 72 - 96 hours. Cell supernatants were harvested and kept stored at - 20°C. Stable transfectants were also selected after plasmid linearization with NotI enzyme (NEB) and purification by phenol extraction and ethanol precipitation in 12-well tissue culture plate in a medium containing 400 µg/ml of Zeocin (Invitrogen). The selective medium was changed every 2-3 days and clones resistant to the Zeocin antibiotic emerged after 2-3 weeks. These resistant clones were transferred into 75 cm<sup>3</sup> tissue culture flasks and allowed to grow and expand in the Zeocin selective medium. Cell supernatants were then collected and stored at - 20 °C until used.

Electroporation was used for transient transfection of CHO cells. After trypsinization, a suspension of 1x 10<sup>6</sup> cells/ sample in 500 µl Ham's F-12 culture medium alone (without FCS and antibiotic) was placed in 4 mm electroporation cuvettes (Bio-Rad). This culture medium was used as electroporation buffer. 100 µg of Maxi-prep purified plasmid DNA was then added to the cell suspension and the cuvette was connected to the electroporator (Bio-Rad). The cells were subjected to a

high-voltage electrical pulse of 290 V and 960 $\mu$ F capacitance. After electroporation, the cells were then allowed to recover briefly on ice for 5 minutes before they were placed in 20 ml of normal growth medium. The cell supernatant was collected 72 - 96 hours later and stored at - 20 °C until used.

The combination of cell - specific reagent and electroporation was another method used for transfection of CHO cells. 1-2 days prior to transfection the CHO were cultivated in 75 cm<sup>2</sup> tissue culture plates in complete Ham's F-12 medium until reaching 80-90 % confluency. On the day of transfection, these cells were trypsinized, counted and aliquoted into 1x10<sup>6</sup> cells / sample. These aliquots were then centrifuged, the supernatant removed completely and the cells re-suspended in 100  $\mu$ l of nucleofactor® Solution (Lonza, UK) / sample. The nucleofactor® Solution was made of 4.5 volumes of nucleofactor® Solution to one volume of nucleofactor® Supplement. The cell suspension was then mixed with 2 $\mu$ g of recombinant pFUSE-mIgG2A-Fc plasmid containing the recombinant hNKG2D ligand – mFc gene and transferred into a certified 2 mm electroporation cuvette (Lonza, UK). The cuvette was then placed into the electroporator (Bio-Rad) and the appropriate pulse (960  $\mu$ F capacitance, 290 V) was applied. Following electroporation, the cells were transferred with sterile pipettes into a 75cm<sup>2</sup> tissue culture flask containing 20 ml complete Ham's F12 medium. 72-96 hours later, the cell supernatant was collected and stored at - 20°C until used.

### **2.9.7 Purification of NKG2D ligand-Fc fusion proteins**

NKG2D ligand-Fc fusion proteins were purified from cell supernatants by affinity chromatography using a Pierce® Chromatography Protein A/G Cartridge (Thermo Fischer, USA) according to the manufacturer's protocol. Using a 20 ml

syringe, the cartridge was first washed with 5-10 ml of Thermo Fisher Washing/Binding Buffer, pH 7.2 (BupH™ Phosphate Buffered Saline) at a flow rate of ~ 1ml/minute. Supernatant from transfectant CHO or HEK - 293 cells was then taken out from the -20 °C freezer, warmed to 37 °C in a water bath and transferred into a clean 50 ml tube and diluted with an equal volume of BupH™ Phosphate Buffered Saline. This adjusts the sample to the ionic strength and pH of the Washing/ Binding Buffer and allows maximum binding when applying to the protein A/G resin. The supernatant was then pumped through the protein A/G cartridge at a flow rate of 1 ml/minute and the effluent was discarded. Supernatant taken from untransfected CHO or HEK-293 cells was also used as negative control. Following the addition of the supernatant, the cartridge was washed again with 5-10 ml of Thermo Fisher Washing/Binding Buffer at a flow rate of ~1 ml/minute and then eluted with approximately 2-5 ml of Thermo Fisher ImmunoPure® IgG Elution Buffer (pH 2.8). Eluted fractions of 0.5-1 ml were collected and immediately adjusted to physiological pH by adding 100 µl of the Neutralization Buffer (1 M Tris at pH 8.7) per 1 ml of elute. The eluted fractions were stored at -20 °C until use. The integrity of the proteins was then verified by SDS-PAGE on 10 % acrylamide gels and staining with Coomassie Blue or by Western blotting.

## **2.10 SDS-PAGE and Western blot**

The eluted fractions collected from the supernatant of transfected cell lines were analysed for the NKG2D-fusion proteins secretion. On the day of experiment the samples were thawed to room temperature and their volume was reduced to ~ 10-15 µl by centrifuging in vacuum, and mixed with equal volume of 1X SDS sample buffer. The mixture was then heated to 100 °C for 5-10 minutes, sonicated 3-4 times and then

loaded onto an SDS-PAGE gel. The SDS-PAGE gel was made up as 10 % resolving gel and 4.5% stacking gel and prepared as follows:

<b>Reagent</b>	<b>10% resolving gel</b>	<b>4.5% stacking gel</b>
<b>De-ionized H<sub>2</sub>O</b>	2816 $\mu$ l	3575 $\mu$ l
<b>1M Tris HCl, pH 8.7</b>	3750 $\mu$ l	-
<b>1M Tris HCl, pH 6.7</b>	-	625 $\mu$ l
<b>Polyacrylamide (30% stock)</b>	3333 $\mu$ l	750 $\mu$ l
<b>10% (w/v) SDS (sodium dodecyl sulphate)</b>	100 $\mu$ l	50 $\mu$ l
<b>10% (w/v) ammonium persulphate (Sigma)</b>	100 $\mu$ l	50 $\mu$ l
<b>TEMED (Tetramethylethylenediamine), Sigma</b>	15 $\mu$ l	8 $\mu$ l

The resolving gel was poured into the gel cassette (Invitrogen) and the cassette was filled with SDS-PAGE resolving gel to a level which allows the comb to be inserted with about 5mm between the bottom of the wells and the top of the resolving gel. 0.5 ml of H<sub>2</sub>O was overlaid the gel to ensure a flat interface between the resolving and stacking gels. The gel was then left for about 30 minutes to solidify. After setting the gel, cassette was inverted to drain the H<sub>2</sub>O and the stacking gel was poured into the gel cassette to the top. The comb was inserted and the stacking gel was allowed to polymerize for at least 1 hour at room temperature or left overnight at 4 °C. The comb was carefully removed to prevent the disruption of the gel and the gel cassette was put in an XCell SureLock™ Mini-cell (Invitrogen) containing SDS running buffer. Using special gel loading tips or a micro-syringe the samples, the controls and protein ladder (NEB, UK) were loaded into the wells. The gel was then electrophoresed at 180 V until the dye molecules (the “migration front”) reached the bottom of the gel. If only SDS-PAGE was used, the acrylamide gels were analysed for

the secretion of hNKG2D ligands - mFc fusion proteins by staining with Coomassie Brilliant Blue. If Western blotting carried out, the gels were transferred to PVDF (Polyvinylidene fluoride) membrane (Millipore) in 1X western blot transfer buffer at 30 V for 1 hour. Good electrophoretic transfer was confirmed by the complete transfer of molecular weight markers below 100 kDa when the membrane was stained with 0.1% (w/v) Ponceau S stain in 5% (v/v) acetic acid. Pre-stained markers of greater than 100 kDa may not transfer completely from the gel onto the membrane. After staining with Ponceau S, the PVDF membrane was washed with 1X TBS with 0.075% Tween 20 (Sigma-Aldrich) to remove the stain and then blocked with Western blot blocking buffer (1X TBS, 5% milk and 0.075% Tween 20 ) for 1 hour at room temperature. The membrane was briefly washed 2 times with 1X TBS with 0.075% Tween 20 for 30 seconds to remove excess blocking buffer and then incubated with the primary mouse anti-MICA monoclonal antibodies (Santa Cruz biotechnology, USA) overnight at 4 °C with shaking. Antibodies were diluted to the recommended concentration in a 1% milk/1X TBS incubation solution. On the next day the membrane was washed two times in Western blot washing buffer for 5 minutes on a shaker and then incubated with biotinylated monoclonal secondary antibody (goat anti-mouse HRP-labelled streptavidin, Sigma-Aldrich, UK) at the recommended dilution of (1:10000) in a 1% milk/1X TBS for 1 hour at RT with agitation and then washed as above. For the detection of blotted proteins, enhanced chemiluminescence (ECL) Kit (Millipore) was used. This kit consists of two detection reagents which are mixed in equal quantities just before use e.g. 800 µl of each. Any excess washing solution is shaken off the blot and 1.6 ml volume of working reagent was added to protein surface of the membrane and incubated for 1-5 minutes. The membrane was then blot dried and sealed in a plastic wrap and the outside corners of membrane were

marked with pen and the covered membrane was placed in a developing cassette. Membranes were exposed to X-ray film in a dark room and developed. Exposure times were determined by experience and they varied between 2 seconds and 5 minutes.

## 2.11 Real-time IL-6 PCR quantification

IL-6 mRNA expression was analysed using semi-quantitative real-time PCR with GAPDH mRNA as the internal reference gene. Total RNA (100 ng) from PBMCs or purified NK cells was reverse transcribed into cDNA using the method described previously. Primer sequences used in this experiment for the real-time amplification of IL-6 and GAPDH were acquired from published sequences (Table 2.2).

**Table 2.2: Oligonucleotides used in real-time PCR**

Primer Name	Sequence	Primer length	Product size	Reference
IL-6 primer (1) sense	5' GGTACATCCTCGACGGCATCT3'	21 bp	81 bp	Keller et al,2003
IL-6 primer (1) anti-sense	5' GTGCCTCTTTGCTGCTTTCAC3'	21 bp	81 bp	Keller et al,2003
IL-6 primer (2) sense	5' CATTGTGGTTGGGTCAGG 3'	19 bp	99 bp	Liebertz et al, 2010
IL-6 primer (2) anti-sense	5' AGTGAGGAACAAGCCAGAGC3'	20 bp	99 bp	Liebertz et al, 2010
IL-6 primer (3) sense	5' CTGCAGCCACTGGTTCTGT 3'	19 bp	143 bp	Lechner et al, 2010
IL-6 primer (3) anti-sense	5' CCAGAGCTGTGCAGATGAGT 3'	20 bp	143 bp	Lechner et al, 2010
IL-6 primer (4) sense	5' TCTCCACAAGCGCCTTCG 3'	18 bp	193 bp	Verhoog et al, 2011
IL-6 primer (4) anti-sense	5' CTCAGGGCTGAGATGCCG 3'	18 bp	193 bp	Verhoog et al, 2011
Pre-optimized IL-6 primers			107 bp	Qiagen (QT0008372)
GAPDH sense	5' CATGGGTGTGAACCATGAGAA 3'	21 bp	133 bp	Iwuchukwu et al, 2011
GAPDH anti-sense	5' GGTTCATGAGTCCTTCCACGAT 3'	21 bp	133 bp	Iwuchukwu et al, 2011.

Both the IL-6 and GAPDH reactions were set up using SensiMix™ SYBR Green NO ROX kit (BioLine, UK). This kit consists of a 2X master mix, contains hot start DNA polymerase, SYBR Green I dye, MgCl<sub>2</sub>, dNTPs, and buffer. The cDNA and primers were added to this mix before transfer to the real-time PCR tubes. 20 µl PCR reactions were prepared as follows:

Reagent	Volume	Final Concentration
2X SensiMix™ SYBRGreen NO ROX	10 µl	1x
25 µM IL-6 sense primer	0.5 µl	250 nM
25 µM IL-6 anti-sense primer	0.5 µl	250 nM
Template (PBMCs or NK cell cDNA)	2 µl	100 ng and more
H <sub>2</sub> O	Up to 20 µl	

Using a Corbett Research loading block, the real-time PCR reactions were manually loaded into 0.1 ml real-time PCR tubes (Qiagen, UK). The reactions were then run on a Rotor Gene 2000 Real-Time cycler (Corbett, Australia). After using different PCR parameters to set up the sample/or machine-specific protocols, the following optimised cycling conditions were used for the PCR reactions:

Cycles	Temperature	Time	Notes
1	95 °C	10 minutes	Initial activation of polymerase
50	95 °C	15 seconds	
	60 °C	15 seconds	T <sub>m</sub> of primers
	72 °C	20 seconds	Acquiring at the end of the step

At the end of the reaction, PCR products were mixed with 6X orange G loading dye (NEB, UK) and ran on a 2% agarose gel stained with ethidium bromide.

Fluorescence graphs and melting curves were obtained from each sample via Rotor Gene 5 software accompanying the Rotor Gene 2000 Cyclor. Only those primers with only one band in the gel picture and one peak in the melting curve were used for further experiments.

## **2.12 Transmigration assays**

A modified Boyden chamber was used to study CD4<sup>+</sup> T lymphocyte migration in response to IL-6 secreted by NK cells. CD4<sup>+</sup> T lymphocytes and NK cells from healthy donors were purified from PBMCs by positive selection using a CD4<sup>+</sup> positive selection kit (Stem Cell® Technologies) and NK isolation kit (Miltenyi Biotec) respectively. After purification, NK cells were activated by co-culture with HeLa cells in a ratio of 5:1 plus IL-2 in a dose of 100 U /ml as described in Section 2:3. Both NK and CD4<sup>+</sup> T cells were then incubated at 37 °C overnight in RPMI medium containing 10% FCS. On the next day, 24-well Transwell plates (Corning) with two different pore size inserts (8 micron and 3 micron) were used. Transwell inserts were coated first with 100 µl fibronectin in 1X PBS (Sigma-Aldrich) / well at a density of 5µg/cm<sup>2</sup> for at least 45-60 minutes before seeding the cells. In the first well, CD4<sup>+</sup> T lymphocytes were seeded into the upper compartment at a density of 2 x 10<sup>5</sup> cells /100 µl of culture medium. The purified NK cells from same donor (same concentration as the CD4<sup>+</sup> cells) suspended in 600 µl of culture medium were then placed in the lower compartment. In the second well, the CD4<sup>+</sup> T cells were seeded in the upper compartment and NK cells from same donor and same concentration as CD4<sup>+</sup> T cells were placed in the lower compartment but in the presence of human anti-IL-6 antibody (clone # 6708, R & D System) at a dose of 0.15 µg / ml for each 2.5 ng / ml of human IL 6 as recommended by manufacturer). In the third well, 2 x 10<sup>5</sup> CD4<sup>+</sup> T



cells were added to the upper compartment and 20 ng / ml of IL-6 (ebioscience) in 600  $\mu$ l culture medium was placed in the lower compartment of Transwell well (positive control). In the last well, the CD4<sup>+</sup> T lymphocytes were placed in the upper compartment and only culture medium in the lower chamber. This well acts as a negative control. Transwell plates were then incubated in tissue culture incubator at 37 °C for 4 hours. Following incubation, CD4<sup>+</sup> T lymphocyte migration in response to NK cells was assessed by counting the absolute number of CD4<sup>+</sup> T lymphocytes in the lower compartment by flow cytometry using flow count beads and the results were presented as the percentage of migration of CD4<sup>+</sup> T cells.

### **2.12.1 Calculation of the absolute number of migrated CD4<sup>+</sup> T lymphocytes**

Absolute cell counts can be obtained by adding a known number of flow cytometry cell counting beads to the sample analysed by flow cytometry. These beads are derived from a calibrated suspension of microspheres that are brightly fluorescent across a wide range of excitation and emission wavelengths and contain a known concentration of microspheres. For absolute counts of the migrated CD4<sup>+</sup> T - lymphocytes, all the cells in the lower compartment of the Transwell wells were mixed with 25  $\mu$ l of CountBright Absolute Counting Beads (Invitrogen) prior to the collection. The cells and the beads were then collected, washed twice with 1X PBS and stained with mouse anti-CD4<sup>+</sup> FITC antibody. After 45-60 minutes incubation on ice, the cells were washed twice with FACS buffer. Following the final wash the supernatant was discarded and the cells were re-suspended in 1 ml of FACS buffer. The samples were then run on the flow cytometer. The forward scatter threshold was set low enough to include the microspheres on the forward scatter vs linear side scatter plot. By gating the CountBright™ absolute counting beads and the cells in lower

compartment separately, the absolute counts of CD4<sup>+</sup> T lymphocytes after the migration could be calculated using the following formula:

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

Where:

A = number of cell events

B = number of bead events

C = assigned bead count of the lot (25000 beads/25  $\mu\text{l}$ )

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

Therefore, the total number of migrating CD4<sup>+</sup> T cells in the lower compartment:

**Absolute number of CD4<sup>+</sup> T cells in lower compartment/  $\mu\text{l}$  x volume of sample in lower compartment (600  $\mu\text{l}$ )**

### 2.13 Statistical analysis

None parametric version of the paired t - test, Wilcoxon signed rank test, was employed to compare means. This test does not assume a Gaussian (normal) distribution of the data. In all experiments presented in this thesis the sample sizes were small (< 12) where the parametric tests (t test and paired t test) are not recommended and may be misleading. However, it should be taken into considerations that the non parametric tests are less powerful than parametric tests and this must be considered when interpreting the statistical differences between samples. P values < 0.05 were regarded as statistically significant.

## 2.14 Compositions of solutions, buffers and stains

### 1X Phosphate Buffered Saline (PBS)

8 g of NaCl.  
0.2 g of KCl.  
1.44 g of Na<sub>2</sub>HPO<sub>4</sub>.  
0.24 g of KH<sub>2</sub>PO<sub>4</sub>.  
distilled water (ddH<sub>2</sub>O) to a total volume of 1 litre.  
pH to 7.4 with HCl.  
Sterilized by autoclave.

### FACS Buffer

1X PBS  
0.5- 1% Bovine Serum Albumin (BSA)  
0.1% Sodium Azide

### MACS Buffer

1X PBS  
0.5% BSA  
2mM EDTA  
Filtered through 0.22 µm filter

### 50X TAE (Tris-Acetate-EDTA) Buffer

242 g Tris Base  
100ml 0.5M EDTA pH 8.0  
57.2ml Glacial acetic acid  
Up to 1000 ml with distilled water (ddH<sub>2</sub>O)

### 1X TAE Buffer

20mL 50X TAE buffer  
980mL ddH<sub>2</sub>O

### SDS-PAGE Running Buffer (10X)

10 g SDS  
144 g Glycine  
30.3 g Tris  
Up to 1000 ml with ddH<sub>2</sub>O

### SDS-PAGE Running Buffer (1X)

100 ml 10X SDS-PAGE Running Buffer  
900 ml ddH<sub>2</sub>O

### Western Blot Transfer Buffer

7.2 g Glycine  
1.53 g Tris  
100 ml Methanol  
Up to 500 ml ddH<sub>2</sub>O

### TBS (10X)

12.1 g Tris  
87.7 g NaCl  
pH 8.0 with HCl  
Up to 1000 ml with ddH<sub>2</sub>O

**Western Blot Washing Buffer (1X TBS, 0.075% Tween 20)**

100 ml 10X TBS  
750 µl Tween 20  
Up to 1000 ml with ddH<sub>2</sub>O

**Western Blot Blocking Buffer (1X TBS, 5% (W/V) Marvel, 0.075% Tween 20)**

20 ml of Western Blot Washing Buffer  
1 gm Marvel (Cow milk)

**Western Blot Antibody Buffer**

20 ml of Western Blot Washing Buffer  
0.1 g Marvel

**1X SDS Sample Buffer**

63 mM Tris HCl  
10% Glycerol  
2% SDS  
0.1% 2-Mercaptoethanol  
0.0025% BromophenolBlue  
pH 6.8

**5X RNA Loading Buffer**

8 µl saturated aqueous Orange G solution  
40 µl 0.5 M EDTA, pH8.0  
360 µl 37% formaldehyde  
1ml 100% glycerol  
1542 µl formamide  
2ml 10x FA Gel Buffer  
RNAse-free water to 5 ml

**ELISA Wash Buffer**

1X PBS  
0.05% Tween 20

**ELISA Stop Solution**

2N H<sub>2</sub>SO<sub>4</sub>

**Ponceau S Staining Solution (0.1%(w/v) Ponceau S in 5%(v/v) acetic acid)**

1g Ponceau S  
50ml acetic acid  
Up to 1000 ml with ddH<sub>2</sub>O

**Coomassie Brilliant Blue Staining solution**

40% methanol  
10% acetic acid  
0.025% Coomassie Brilliant Blue R-250  
filtered through Whatman #1 paper.

**Freezing Solution**

90% foetal calf serum (FCS)  
10% dimethyl sulphoxide (DMSO)

**TSS (Transformation and Storage Solution)**

85% LB broth (autoclaved)  
10% (w/vl) PEG (polyethylene glycol)  
5% (v/v) DMSO  
20-50 mM Mg<sup>2+</sup> (MgSO<sub>4</sub> or MgCl<sub>2</sub>), final pH of 6.5

# Chapter 3

## Interleukin-6 Secretion by Peripheral Blood Natural Killer Cells in Healthy Individuals



2012

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### **3.1 Introduction**

#### **3.1.1 The significance of IL-6 secretion by NK cells in inflammation and autoimmunity**

Interleukin-6 (IL-6) has attracted considerable interest in the study of the pathobiology of many chronic inflammatory and autoimmune disorders. Up-regulation of IL-6 production has been observed in a variety of chronic inflammatory and autoimmune diseases such as rheumatoid arthritis (Nishimoto, 2006 ; Lipsky, 2006 ; Park and Pillinger, 2007 ; Cronstein, 2007), systemic sclerosis (Hasegawa et al, 1998 ; Sato et al , 2000 ; Barnes et al, 2011) , systemic lupus erythematosus (SLE) (Linker-Israeli et al, 1999) , ankylosing spondylitis (Falkenbach et al, 2000) , thyroiditis (Bartalena et al, 1993 ) , type I diabetes, (Jain et al, 2001) , mesangial proliferative glomerulonephritis (Horii et al, 1989), and psoriasis (Grossman, 1989). In Rheumatoid arthritis (RA), several studies have reported that the blood as well as the affected tissues of these patients contains highly elevated levels of IL-6 and it has been shown that increased IL-6 serum levels indicate a poor prognosis (Madhok et al, 1993; Stuart et al, 1995; Usón et al, 1997; Nishimoto et al, 2004). The source of IL-6 in these patients has been a matter of intensive investigation. Many studies have proposed that type II synoviocytes are the primary source of IL-6 in the synovial fluid of patients with RA (Guerne et al, 1989; Miyazawa et al, 1999). However, there is increasing evidence that other cells such as natural killer (NK) cells could contribute to the production of this cytokine (Barakonyi et al, 2004; Jost, 2007). Since these cells appear very early at the inflammation site, as cells contributing to innate immunity, the production of IL-6 cytokine by NK cells during the early stage of the immune response has the potential of influencing the later stages of the immune response. Thus NK cell-mediated IL-6 secretion may provide a critical link between the innate and



adaptive immune response of the host. Furthermore, changes in this pathway in various autoimmune diseases, for example RA, may be relevant for the pathogenesis of these disorders.

Recent findings clearly indicate that IL-6 is a key factor involved in the regulation of T cell differentiation and activation (Dienz and Rincon, 2009). IL-6 can act as a co-stimulatory molecule for resting T- cell activation and proliferation through a mechanism independent of IL-2 gene expression (Lotz et al, 1988). Antigen specific CD4<sup>+</sup> T lymphocytes have been shown to expand more vigorously *in vivo* when IL-6 is present due to reduced apoptosis suggesting that IL-6 may have anti-apoptotic properties thus increasing effector/memory T cell survival (Rochman et al, 2005; Dienz and Rincon, 2009). In addition, IL-6 activates T-helper 2 (Th2) cytokine production in CD4<sup>+</sup> T lymphocytes via the transcription factor CREB (Rincón et al, 1997). These include IL-4, IL-5, IL-6 and IL-13. It also promotes autocrine IL-4 production which further enhances Th2 differentiation through an auto-feedback loop (Rincón et al, 1997). Furthermore, IL-6 stimulation has been linked to increased migration of activated CD4<sup>+</sup> T cells *in vitro* which could explain their inability to enter the infected tissues in the absence of IL-6 during an infection (Weissenbach et al, 2004; Dienz and Rincon, 2009). Other studies have shown that the *de novo* differentiation of Th17 cells from naïve mouse CD4<sup>+</sup> T cells is achieved by the simultaneous treatment with IL-6 and low doses of transforming growth factor (TGF)β during antigen stimulation (Bettelli et al , 2006 ; Mangan et al , 2006 ; Veldhoen et al, 2006). In particular, IL-6 trans-signalling appears to promote the maintenance of IL-17-secreting T lymphocytes in inflamed tissues (Jones et al, 2010 ; Neurath et al, 2011) and regulates chronic inflammation *in vivo* (Rabe et al, 2008 ; McLoughlin et al, 2005 ; Neurath et al, 2011).

NK cells were first described as cytotoxic cells eliminating transformed and virally infected cells (Kiessling et al, 1975; Herberman et al, 1975; Messina et al, 1985). However, it has become clear that NK cells interact with various components of the immune system and therefore have the potential to function as regulatory cells. These regulatory functions include assisting in dendritic cell (DC) maturation (Moretta et al, 2006; Lünemann et al, 2009; Lucas et al 2010), regulating T-cell functions and proliferation (Bell, 2007; Lünemann et al, 2009), limiting adaptive immune responses and regulating autoimmune responses in a variety of experimental models as well as in humans with common autoimmune diseases (Lünemann et al, 2009). Several studies have shown that NK cells constitute a significant fraction of the infiltrating cells in a range of CD4<sup>+</sup> driven autoimmune diseases and these cells are predominantly of CD56<sup>bright</sup> phenotypes (Pridgeon et al, 2003; Schepis et al, 2009; Poli et al, 2009 ; Conigliaro et al, 2011). Taking into consideration that cells with this phenotype are mainly cytokine secretors (Cooper et al, 2001a; Strowig et al, 2008) , together with the fact that IL-6 is a powerful regulator of CD4<sup>+</sup> T cell response (Dienz and Rincon, 2009) one can speculate that these NK cells could be a source of secretion of IL-6 in these patients. Furthermore, the preliminary findings in our laboratory that the NK cells isolated from the blood of patients with autoimmune diseases produce significantly more IL-6 after appropriate stimulation than the NK cells isolated from the blood of healthy donors raise the possibility that NK cells could augment the T-cell response in these patients via IL-6 secretion.

The concept of the present study came after a number of preliminary observations about IL-6 expression/secretion by NK cells in our laboratory. In DNA array experiments, IL-6 mRNA was expressed in NK cells isolated from the peripheral blood of healthy individuals and RA as well as the synovial fluid (SF) of the patients

(data not published). IL-6 protein was also detected in the supernatants taken from co-cultures of NK cells and the conventional K562 target cells irrespective of whether the NK cells were isolated from the blood of healthy donors or patients, or the SF (Jost, 2007). It is important to point out that in contrast to peripheral blood NK cells, most rheumatoid synovial fluid NK cells are of a CD56<sup>bright</sup> CD16<sup>dim/-</sup> phenotype (Pridgeon et al, 2003). As mentioned earlier, these cells are primarily cytokine secreting cells. As a result it has been suggested that cytokine production by these cells may play a role in the initiation and maintenance of the disease.

### **3.1.2 The aims of this chapter**

Based on the preliminary information on the secretion /expression of IL-6 by NK cells, it was decided to study further the ability of PBNK cells to secrete IL-6 cytokine following different stimuli. This study was conducted with normal healthy donors to gain an insight into IL-6 secretion by NK cells. To approximate physiological conditions under which cytotoxicity would occur inside the body, the NK cells were stimulated with target cells, K562 and HeLa cells. These two cell lines activate NK cells through two different mechanisms. K562 erythroleukemia cells lack the expression of MHC class I molecules (Lozzio and Lozzio, 1979) and hence they are good targets for the NK cells that can easily recognise and kill them according to the “missing self” principles (Kärre et al, 1986; Ljunggren and Kärre, 1990). In contrast, HeLa cells express a full set of MHC class I molecules, so they elicit normal KIR-mediated inhibitory signals in the NK cells. However, these cells express the ligands for the dominant activating NKG2D receptors on NK cells, MICA protein (Groh et al, 1999; Zhang et al, 2001). This ligand-receptor interaction activates NK cells and induces cytotoxicity despite the presence of MHC class I molecules (Bauer et

al, 1999). Using these two cell lines allowed us to study the activating pathway and the receptors involved in the IL-6 secretion by NK cells. However, there is a possibility that these cell lines also secrete IL-6 as well. To eliminate the possibility of IL-6 being derived from the target cells PBNK cells were also stimulated by other methods such as using IL-2 and non-specific chemical stimuli such as phorbol diesters. Establishing the timing of IL-6 production following triggering of the NK cells was another aim of this chapter. The final aim was to investigate if the stimulus for this secretion is via soluble mediators or a direct cell-to-cell interaction. This was achieved by using a Transwell plate.

## **3.2 Results**

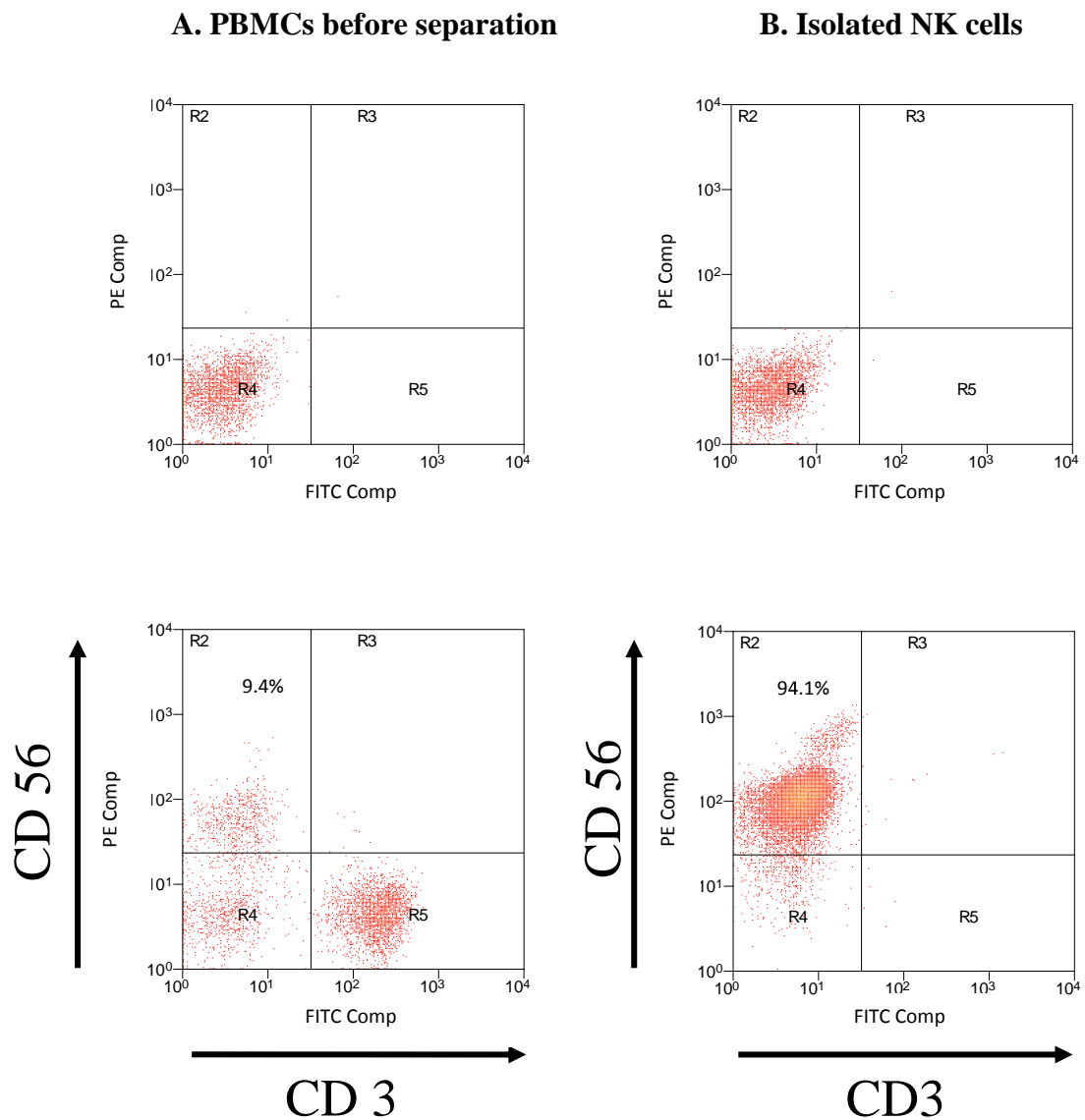
### **3.2.1 NK cell magnetic selection (negative vs. positive selection)**

As described in Chapter 2, human PBMCs were isolated from peripheral blood by density centrifugation over a step gradient using Ficoll-Paque™ and NK cells were purified from the PBMCs by negative (magnetic) selection using the MACS MicroBeads system. Briefly, the PBMCs were first stained with a cocktail of biotin-conjugated monoclonal anti-human antibodies against antigens not expressed by NK cells. After 10 minutes incubation on ice, the cells were coupled with a cocktail of anti-biotin micro beads. Following a second incubation, the cells were passed through a MACS column placed in a strong magnetic field that retained the undesired cells in MACS column while NK cells passed through the column and were collected as an enriched, untouched cell fraction. This system yields a high purity of untouched NK cells as assessed by flow-cytometry (Figure 3.1). Since a high level of NK cell purity was required throughout this study, this method was used for purification of NK cells from PBMCs to eliminate any potential bystander effect of contaminating cells.

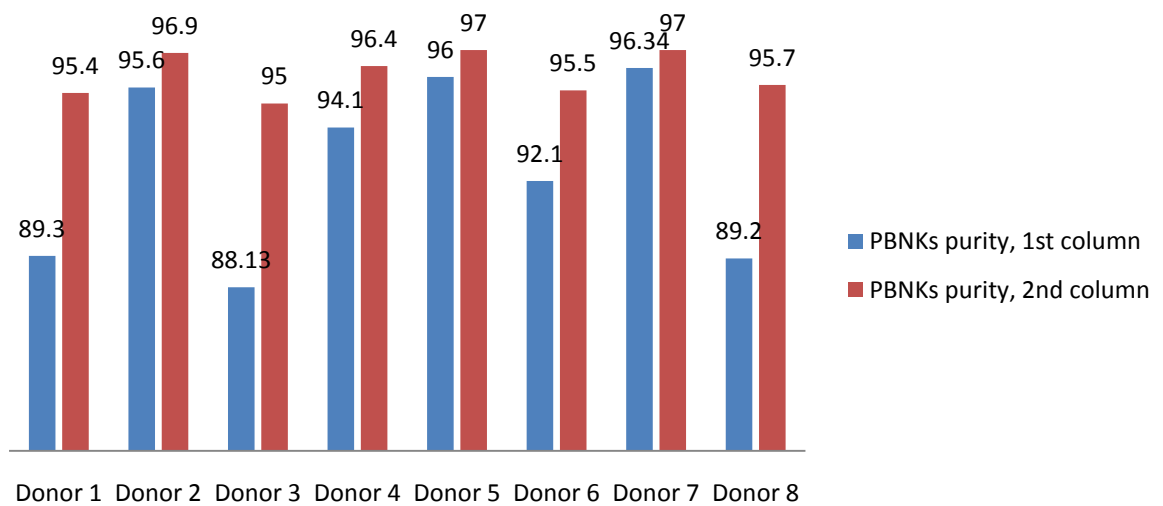
Direct magnetic labelling of the NK cell surface antigen CD56 is often used for the purification of NK cells from PBMCs (positive selection). Although this method is faster than negative selection and can produce high level of pure CD56<sup>+</sup> cells, many disadvantages to positive selection limited its use in this study. The main disadvantage was that the CD56 surface marker is not only expressed by NK cells but also a small proportion of T cells (NKT cells and some CD8<sup>+</sup> T-cells) also express this surface marker. Thus, using positive selection up to 50% of the isolated CD56<sup>+</sup> cells are CD3<sup>+</sup> T cells (data not shown). In addition beads cannot be removed easily and must be carried along with the cells. It was feared that antibody binding to the positively selected NK cells may interact with the remaining beads and affect their subsequent functional responses.

### **3.2.2 Assessment of PBNK cell purity after negative selection**

As many cells are capable of IL-6 synthesis, including T-lymphocytes and monocytes, and some of these cells might have been trapped during NK cell selection, it was necessary to exclude the possibility that IL-6 was produced by these cells and not the NK cells. Following isolation, flow cytometric analysis was performed on each sample for the quantitation of NK cells (defined as CD56<sup>+</sup> CD3<sup>-</sup> cells). The average purity of NK cells after isolation was 92.59% (range 88.18% - 96.34%). On average, the cell pool contained 1.44% CD56<sup>+</sup>CD3<sup>+</sup> cells (range 0.20%-2.62%). The average T cell (CD56<sup>-</sup> CD3<sup>+</sup>) contamination was 0.74 (range 0.11- 1.99). Purities of  $\geq 95\%$  were obtained from all the samples after passing the eluted NK cell fractions over a second LS MACS column. A summary of the separation performance for the isolation of PBNK cells is shown in Figure 3.2.



**Figure 3.1: Purity of PBNK cells after immunomagnetic negative selection as assessed by flow cytometry (dot plot profiles).** **A:** PBMCs before magnetic separation. R4 in the top picture represents to total unstained PBMCs fraction. R2 in the bottom picture represent the PBNK cell fraction after staining with CD3 FITC and CD56 PE monoclonal antibodies. **B:** PBNK after purification. R4 in the top picture represents the PBNK cell fraction in an unstained sample while R2 in the bottom picture represent the PBNK cell fraction after magnetic separation. The gate was set by forward and side scatter to exclude dead cells, debris, and cell aggregates. The purity of PBNK cells after isolation in this sample was 94.1 %.



**Figure 3.2: Purity of isolated CD56<sup>+</sup> CD3<sup>-</sup> PBNK cells after negative selection using MACS MicroBeads System expressed as percentage of CD56<sup>+</sup> CD3<sup>-</sup> cells within the live cell gate.** The PBNK cells were isolated by magnetic depletion of non-NK cells using eight healthy donors. The purity after isolation ranged from 88.13% to 96.34% (average was 92.59%). However, purity of  $\geq 95\%$  was obtained from all these samples after passing the eluted NK cells over a second MACS column.

### **3.2.3 Secretion of IL-6 by purified PBNK cells**

After isolation the resting PBNK cells were activated and tested for the production of IL-6 using different approaches. These included co-culturing with target cells such as K562 or HeLa cells, stimulation with cytokines (IL-2) at 2 different concentrations and using non specific chemical mediators (e.g. phorbol myristate acetate [PMA]) together with ionomycin). After activation 100 µl of supernatants were collected at 1, 3, 6, 18 and 24 hrs and frozen at - 80 °C until further analysis. Since the aim was to find out how the NK cells contribute to inflammation via the IL-6 secretion during the early stages of the immune response, time points as early as 1 and less than 24 hrs were chosen. Supernatants from target cells alone and from non-activated (resting) PBNK cells were also collected at the same time points and compared with those taken from the activated NK cells. The IL-6 in the supernatants was then assayed using a commercially available IL-6 ELISA kit as described in Chapter 2.

#### **3.2.3.1 Endogenous IL-6 secretion by K562 and HeLa cell lines**

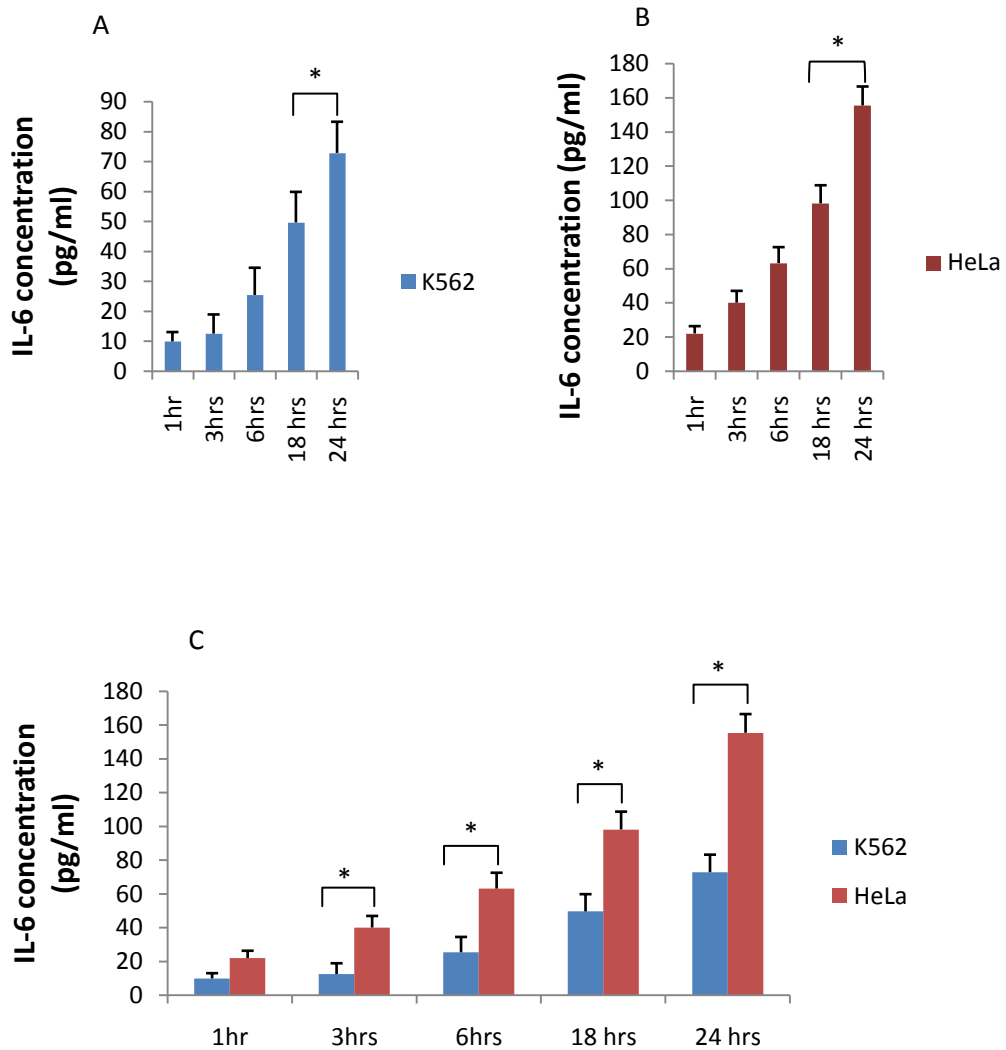
The identification of the cells responsible for cytokine production in cultures containing a mixed cell population remains one of the main limitations of ELISA experiments. To investigate the possibility that the detected IL-6 was produced by K562 or HeLa cells and not the purified PBNK cells during the co-culture experiments, supernatants of wells containing these target cells alone were analysed as negative controls. The results are shown in Figure 3.3. As can be seen from this figure, it is clear that both K562 and HeLa cell lines spontaneously produced IL-6 (Figures 3.3 A and B). The IL-6 production by these cell lines increased with time as the cytokine being detected as early as 1 hr, increasing further at 3 and 6 hrs, and reaching significant levels at 18 and 24 hrs ( $p < 0.05$ ). HeLa cells produced significantly more



IL-6 than K562 cells at the same duration of culture (Figure 3.3 C). Again, this accumulation during the culture period seems to indicate a non-stimulated spontaneous IL-6 release by both of the used cell lines.

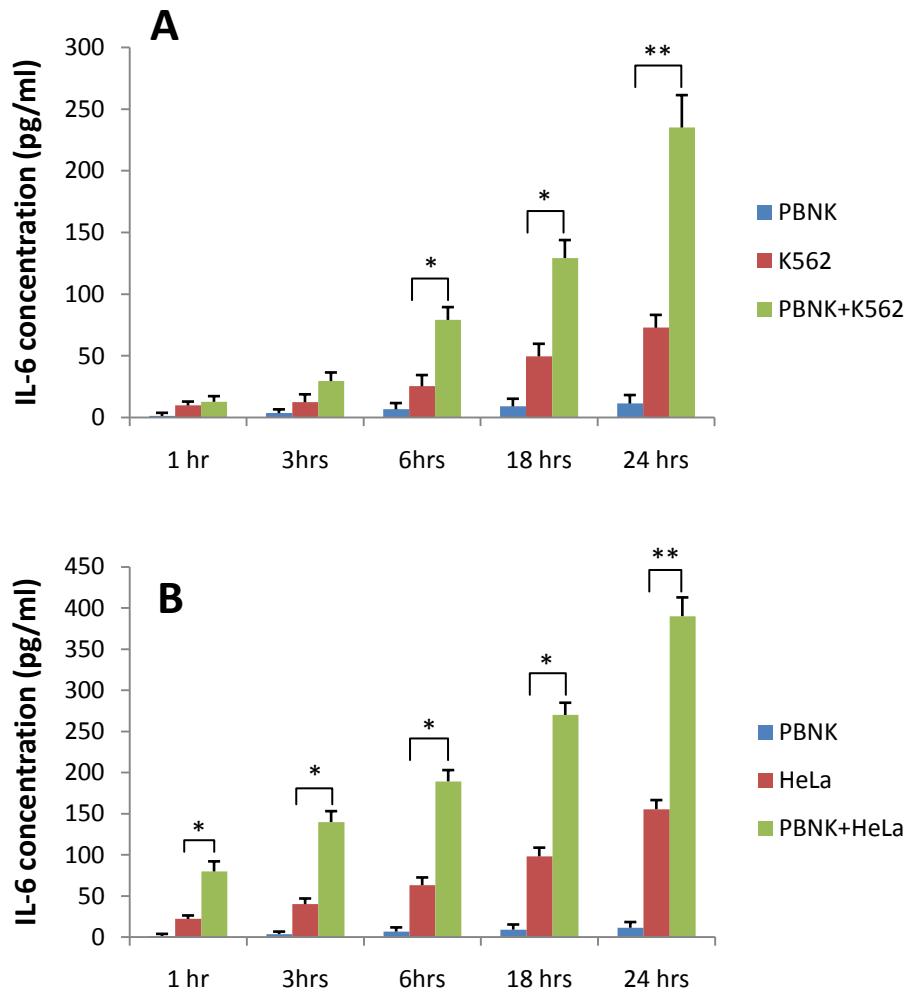
### **3.2.3.2 PBNK and target cell co-cultures produce more IL-6 than cultures containing target cells or PBNK cells alone**

After conducting these control experiments we then measured the IL-6 cytokine levels in supernatants taken from co-cultures of effector cells (PBNK) + target cells (K562 or HeLa) and compared them with those obtained from effector cells or target cells alone. K562 cells were co-cultured at a concentration of  $1 \times 10^5$  cells with the same number of purified PBNK cells in 96-well round bottom tissue culture plates. Since HeLa cells are much larger than K562, the cell concentration was adjusted to obtain effector: target (E: T) ratio of 5:1 to compensate for this difference. Therefore, only  $2 \times 10^4$  of HeLa cells were co-cultured with  $1 \times 10^5$  purified PBNK cells. Cultures of K562 or HeLa cells and PBNK cells alone at the same concentration as in the co-cultures were also used as negative controls. Supernatants were then collected at 1, 3, 6, 18 and 24 hrs after the plating of the cells and analysed for IL-6 by ELISA. The results of these experiments are shown in Figure 3.4. Six hrs after initiating the stimulation of PBNK cells with K562 targets the IL-6 concentration increased significantly compared with K562 alone ( $p < 0.05$ ) while IL-6 concentration in PBNK + HeLa co-cultures was significantly higher as early as 1 hr ( $p < 0.05$ ).



**Figure 3.3: Endogenous production of IL-6 protein by K562 and HeLa cell lines.**

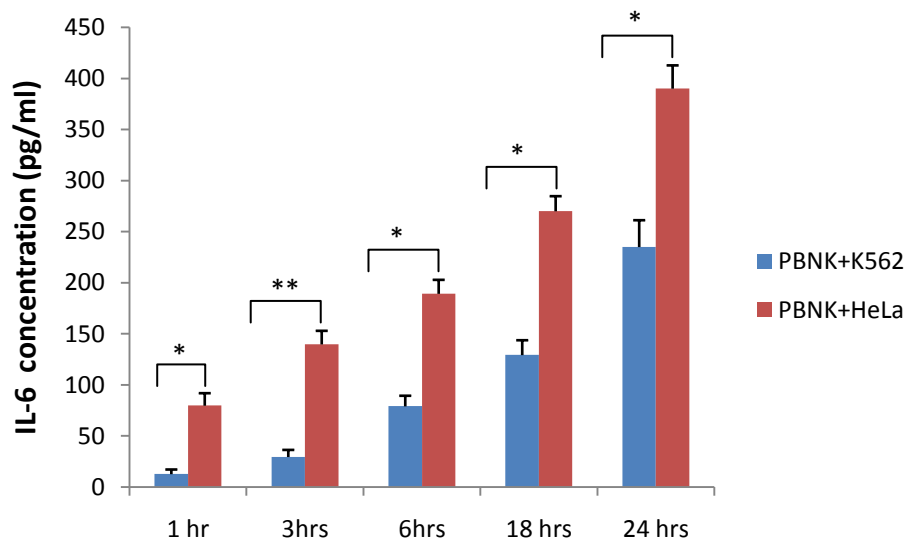
$1 \times 10^5$  K562 cells and  $2 \times 10^4$  HeLa cells were plated in 96 well plates. Supernatants were harvested at 1, 3, 6, 18 and 24 hrs and analyzed for IL-6 production by ELISA. As shown in Figures A and B both cell lines spontaneously produced IL-6. HeLa cells produced significantly more IL-6 than K562 at the same time points (Figure C). All measurements were performed in duplicates and presented as means of three independent experiments. Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*,  $p < 0.05$ .



**Figure 3.4: Increased production of IL-6 following the activation of NK cells with cellular targets. A:** The mean IL-6 concentrations (pg/ml) in the supernatants taken from wells containing K562, PBNK and NK+ K562 co-cultures at different time points. The IL-6 increased significantly in the co-culture supernatants at 6 hrs following stimulation with the K562s compared to the supernatants from the K562s or PBNK cells alone (n= 8, p <0.05). **B:** Comparison of the mean IL-6 concentrations (pg/ml) in the supernatants in wells containing HeLa, PBNK and HeLa + NK co-cultures at different time points. IL-6 increased significantly in the co-culture supernatants as early as 1 hr following stimulation with the HeLa compared to the supernatants from the HeLa or PBNK cells alone (n=8, p <0.05). The measurements were performed in duplicates and the results are representative of three independent experiments. Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*, p<0.05, \*\*, p<0.01. Significant difference at p < 0.05.

### **3.2.3.3 PBNK + HeLa co-cultures produce more IL-6 than PBNK+K562 co-cultures**

Next IL-6 concentrations were compared in the supernatants taken from the co-cultures of purified PBNK and K562 cells with those obtained from co-cultures of purified PBNK + HeLa cells. Again,  $1 \times 10^5$  PBNK cells were co-cultured with the same number of K562 targets in 96-well round bottom tissue culture plate. Only  $2 \times 10^4$  HeLa cells were co-cultured with  $1 \times 10^5$  PBNK cells. The time points when IL-6 levels were tested in the supernatants were 1, 3, 6, 18 and 24 hrs after plating the cells. The results are shown in Figure 3.5. From this figure it is clear that the IL-6 concentration in the supernatants taken from PBNK + HeLa co-cultures was significantly higher than those taken from PBNK+ K562 co-cultures at the same time points. However, we should mention that these results were expressed as means  $\pm$  SD of three independent experiments performed with 8 healthy donors and there were some individual variations between the donors regarding the IL-6 secretion in the co-cultures.



**Figure 3.5: Comparison of IL - 6 concentrations (pg / ml) in the co-culture supernatants (PBNK + K562 vs PBNK + HeLa) as determined by ELISA.** NK cells were isolated from the PBMCs of eight healthy donors by negative selection and co-cultured with K562 cells (E: T ratio was 1:1) or HeLa cells (E: T ratio 5:1). Supernatants were taken at 1, 3, 6, 18 and 24 hrs and IL-6 cytokine concentration was determined in the supernatants by ELISA. The PBNK + HeLa co-cultures produce significantly more IL-6 than PBNK + K562 co-cultures at all time points. Results were representative of three experiments and presented as means  $\pm$  SD. Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*,  $p < 0.05$ , \*\*,  $p < 0.01$ . Significant difference at  $p < 0.05$ .

#### **3.2.3.4 Secretion of IL-6 by PBNK cells after stimulation with PMA and ionomycin**

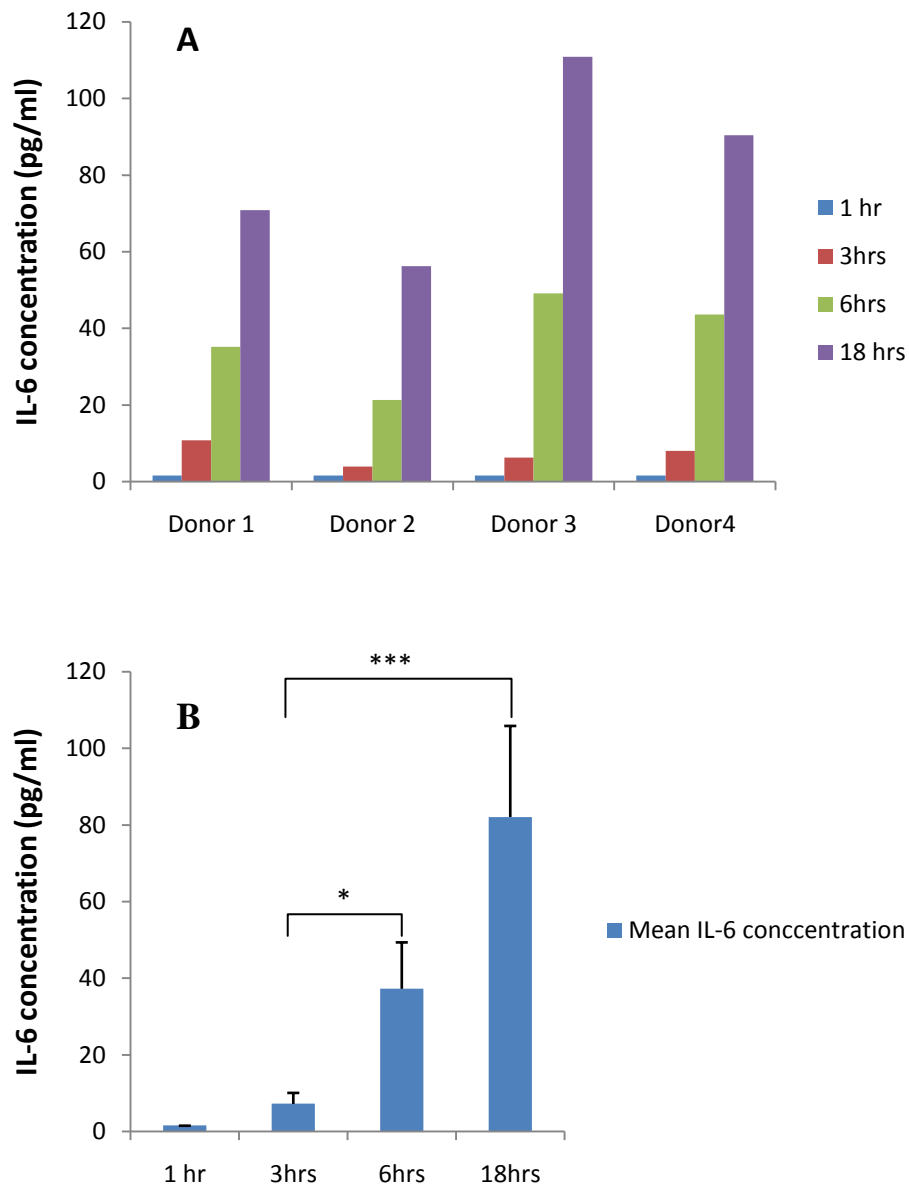
Utilizing co-cultures it was observed that more IL-6 cytokine was produced in co-cultures than the effector (PBNK cells) alone or target cells (K562 cells or HeLa cells) alone. This suggested that both PBNK and target cells could contribute to the IL-6 production. However, the co-culture experiments cannot exclude the possibility that the target cell were the source of IL-6 and not the PBNK cells. To eliminate this possibility and prove that NK cells could produce this cytokine, pharmacological stimulation of PBNK cells was also carried out. Human PBNK cells from 4 healthy donors, at the same density as in the co-cultures ( $1 \times 10^5$  cells), were washed twice with 1X PBS, cultured in 96-well round bottom plate and stimulated with potent non-specific chemical stimulators (PMA in a dose of 10 ng/ml and ionomycin in a dose of 1  $\mu$ g/ml). To determine timing of cytokine release, 100  $\mu$ l of cell-free supernatants were collected at 1, 3, 6 and 18 hrs. The results are depicted in Figure 3.6 A and summarized in Figure 3.6 B. IL-6 concentration was significantly increased as early as 6 hours following stimulation with PMA and ionomycin ( $p < 0.05$ ) with the concentration increasing further at 18 hours.

#### **3.2.3.5 Secretion of IL-6 by PBNK cells after stimulation with IL-2**

It has been observed that stimulation of NK cells with IL-2 promotes their cytotoxicity and enhances the production of different cytokines including interferon gamma (IFN- $\gamma$ ) (Weigent et al, 1983; Trinchieri et al, 1984; Ortaldo et al, 1984; Kehri et al, 1988; Armant et al, 1995; Son et al, 2001). However, little is known about the secretion of IL-6 by IL-2 activated NK cells. IL-2 in a dose of 50- 100 U / ml is known to induce NK activation in both primary NK cells and NK cell lines. In this study, a

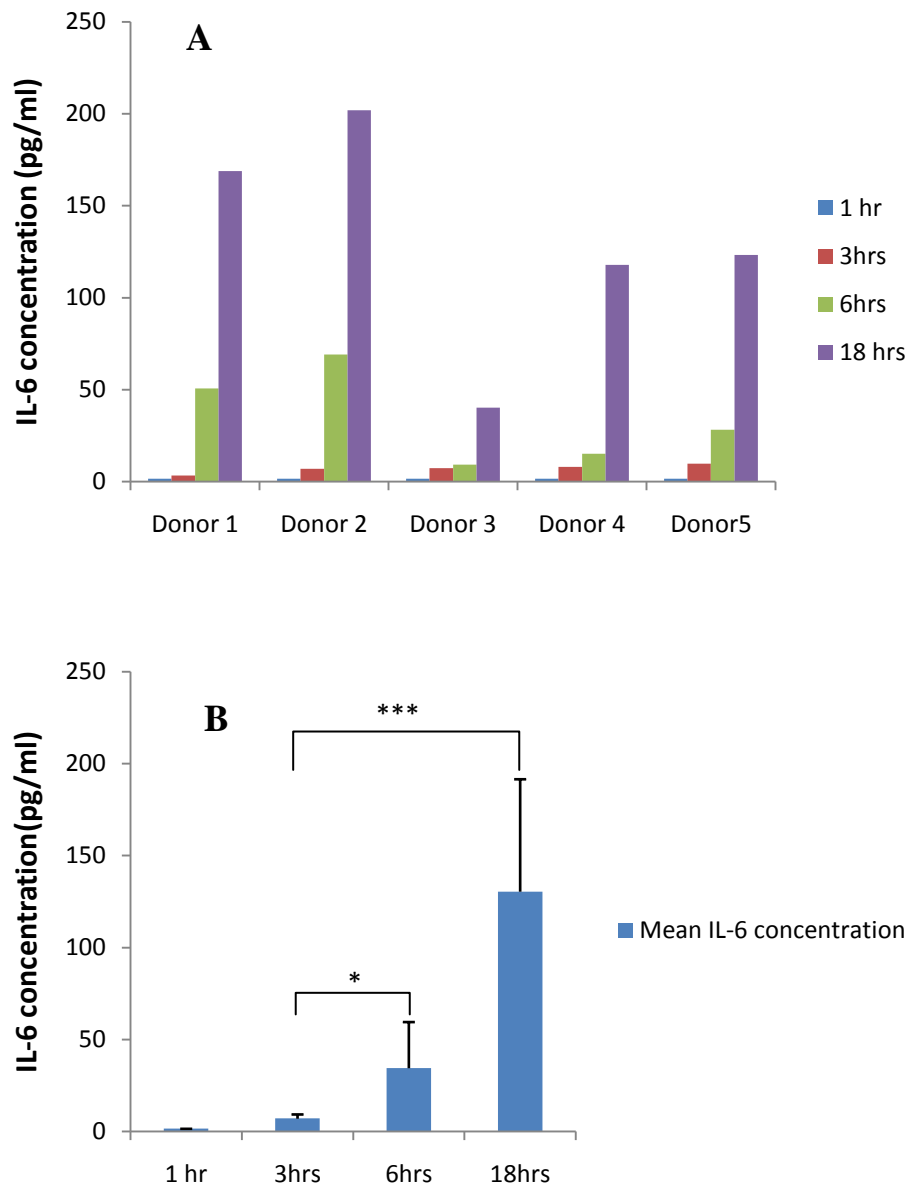
preliminary experiment was carried out using  $1 \times 10^5$  purified NK cells from a healthy donor stimulated with recombinant human IL-2 (rhIL-2) at a dose of 100 U / ml. 100  $\mu$ l of supernatants were collected at 1, 3, 6 and 18 hrs and the IL-6 concentration was determined by ELISA. Little IL-6 was produced by PBNK cells in response to this dose of rIL-2 at all time points with concentrations ranging from 2.7 pg/ml at 1 hr to 12.4 pg/ml at 18 hrs. These figures were not significantly different from those obtained with non-activated (resting) PBNK cells.

Next, we have examined the ability of IL-2 to enhance IL-6 cytokine secretion by PBNK cells at higher doses. Doses from 200 U /ml up to 1000 U/ml have been used for activation of resting NK cells in some studies (Kehri et al, 1988; Weil-Hillman, 1990; Sarina et al, 1997). Stimulating resting PBNK cells from 5 healthy donors with 500 U/ml of rhIL-2 was tried next. Supernatants were then collected at same time points as in the previous experiment. The results are shown in Figure 3.7 A and summarized in Figure 3.7 B. As shown in this figure there was a significant amount of IL-6 produced; the concentrations were higher than in the PMA experiment.



**Figure 3.6: The IL-6 production by PBNK cells following activation with PMA and ionomycin.** **A)** PBNK cells were purified from 4 healthy donors by negative selection and stimulated with PMA (10 ng/ ml) and ionomycin (1  $\mu$ g g/ml). Following incubation, the supernatants were collected at 1, 3, 6 and 18 hrs and analysed for IL-6 levels. **B):** The mean  $\pm$  SD for the 4 samples at each of the four time points was calculated. The IL-6 concentration started to increase significantly at 6 hrs after stimulation with PMA and ionomycin ( $P < 0.05$ ). Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*,  $p < 0.05$ , \*\*\*,  $p < 0.001$ . Significant difference at  $p < 0.05$ .



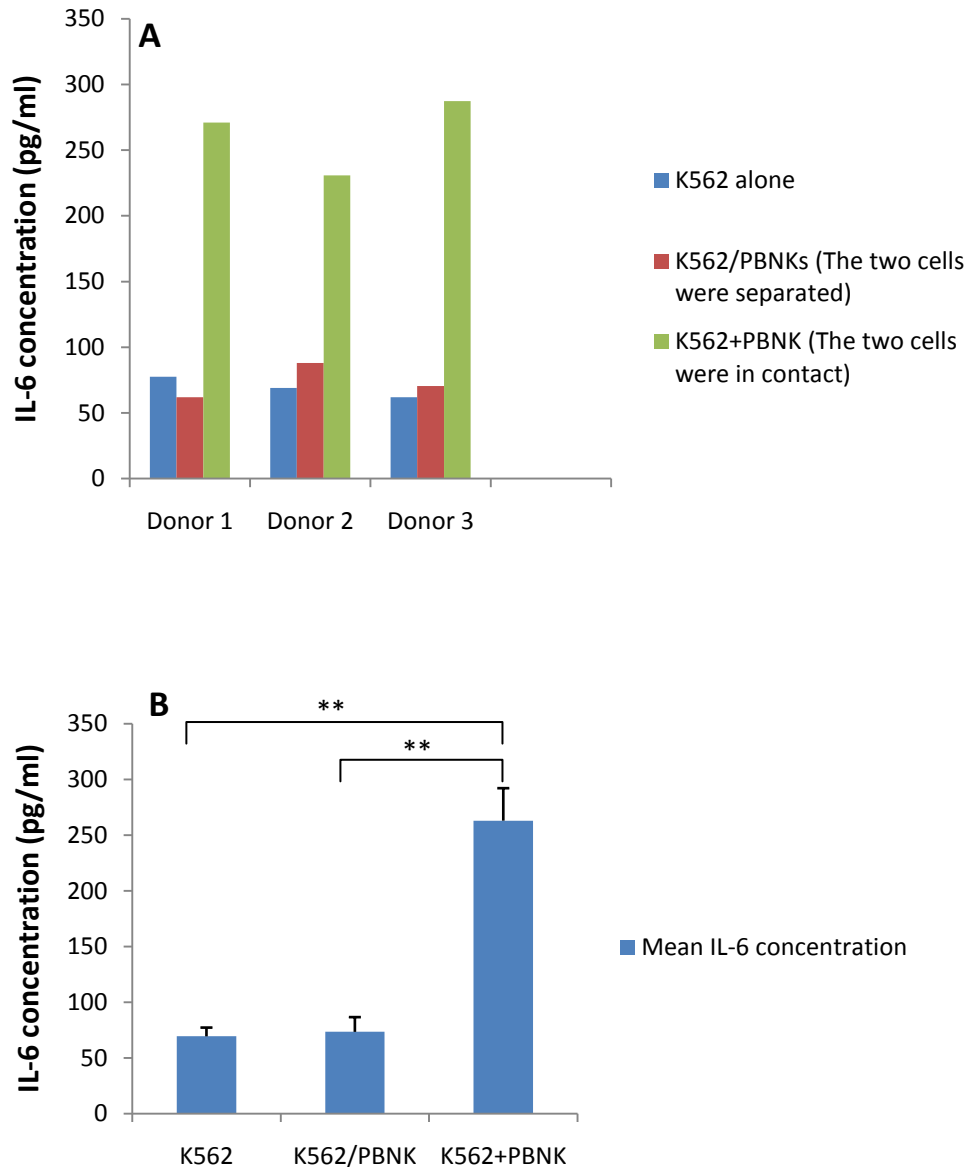


**Figure 3.7: IL-6 secretion by PBNK cells after stimulation with rhIL-2.** **A)** PBNK cells were isolated from 5 healthy donors and stimulated with IL-2 at a dose of 500 U/ml. Following incubation, the supernatants were collected at 1, 3, 6 and 18 hrs and analysed for IL-6 protein by ELISA. Despite some inter-individual variability at 6 and 18 hrs, IL-6 was secreted by NK cells isolated from all donors tested. **B)** The mean  $\pm$  SD for the 5 samples at each of the four time points was calculated. Again, the IL-6 concentration started to increase significantly at 6 hrs after stimulation with IL-2 ( $P < 0.05$ ). Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*,  $p < 0.05$ , \*\*\*,  $p < 0.001$ . Significant difference at  $p < 0.05$ .

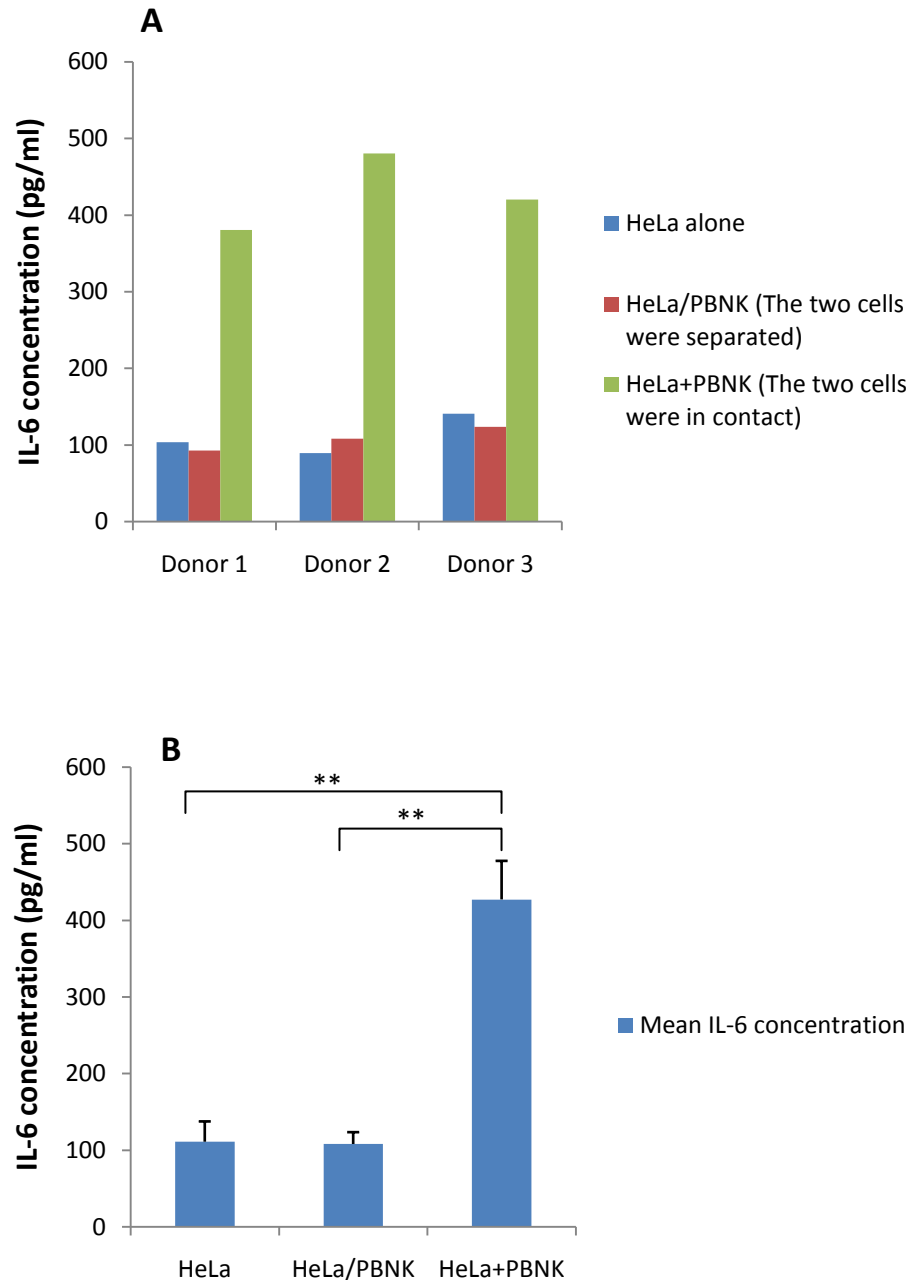
### **3.2.4 Direct PBNK-target cell contact is necessary for the initiation of IL-6 production upon target cell recognition**

To analyse whether IL-6 secretion in the co-culture experiments was the result of direct cell-cell interactions between PBNK cells and the target cells or was induced by the presence of soluble mediators, the following experiment was devised: co-culture experiments were set up where the target cells were separated from PBNK cells using Transwell inserts. The pores in the inserts used would allow the free transit of soluble mediators, such as cytokines or chemokines, but not the cells themselves. The details of this experiment were discussed in Chapter 2. Briefly, a 24-well Transwell plate with 0.4  $\mu\text{m}$  pore size insert was used.  $1 \times 10^5$  of K562 cells (or  $2 \times 10^4$  HeLa cells) suspended in 600  $\mu\text{l}$  of complete culture medium (complete RPMI or DMEM medium) were placed in the lower compartment. PBNK cells at a concentration of  $1 \times 10^5$  cells suspended in 100  $\mu\text{l}$  culture medium were then placed in the Transwell insert and therefore separated from the targets by the membrane. As controls, two additional wells were prepared. One of these, the positive control, had the PBNK cells and the targets mixed together (same concentration as in the co-culture experiments) in the lower compartment while the upper compartment received only 100  $\mu\text{l}$  of culture medium. A Transwell insert was still present to eliminate the possibility that chemicals eluting from the membrane could inhibit IL-6 production. In the negative control well, only the target cells ( $1 \times 10^5$  K562 or  $2 \times 10^4$  HeLa cells) were added to the lower compartment and the upper insert compartment received only culture medium. These controls would indicate if the insert could affect IL-6 production due to the presence of chemicals in the membrane/insert. The experiment was conducted with cells from 3 healthy donors. Supernatants from the lower compartment were collected and assayed for the induction of IL-6 secretion after 24-

hour incubation. The results are shown in Figures 3.8 and 3.9. As can be seen from these Figures, when the PBNK and target cells were separated from each other (i.e. no direct physical contact between them), the concentration of IL-6 in the K562 or HeLa cells compartments was not significantly different from that of the negative controls (target cells alone) ( $p > 0.05$ ). However, in the co-cultures of NK cells and K562 or HeLa cells, where cellular contact was maintained, the levels of IL-6 production were significantly increased ( $p < 0.01$ ). These results clearly indicate under normal or physiological conditions soluble factors do not affect IL-6 secretion by NK cells and that direct physical contact between NK cells and target cells is necessary for the triggering IL-6 production.



**Figure 3.8: The effect of direct cell-cell interaction on the initiation of IL-6 production by PBNK cells.** Transwell inserts with 0.4  $\mu\text{m}$  pore size were used. In the first well the PBNK cells in the upper compartment were separated from K562 cells in the lower compartment by Transwell membrane. In the second well, the PBNK cells were mixed with the K562 cells in the lower compartment and only culture medium was placed in upper compartment. In the third well, K562 cells were placed in lower compartment while only culture medium was placed in upper compartment. The assay was performed on purified PBNK cells from 3 healthy donors. **A:** Shows the results obtained with the individual donors. **B:** The mean  $\pm$  SD for the 3 samples. The IL-6 concentration significantly increased in the co-culture wells over that seen in the other two groups ( $P < 0.01$ ). The error bars denote the SD; \*\*,  $p < 0.01$ .



**Figure 3.9: The effect of direct cell-cell interaction on the initiation of IL-6 production by PBNK cells.** Same experimental setup as in Figure 3.8, but using HeLa as target cells. **A:** Individual results with purified PBNK cells from 3 healthy donors. **B:** The mean  $\pm$  SD for the 3 sample groups was calculated. The IL-6 concentration significantly increased in the co-culture group than other two groups ( $P < 0.01$ ). Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*\*,  $p < 0.01$ .

### 3.3 Discussion

#### 3.3.1 Cytokine and chemokine secretion by PBNK cells

Natural Killer (NK) cells are an important element of innate immunity and have both regulatory and effector functions. Previous studies have demonstrated that NK cells are not a homogenous group of innate lymphocytes. Instead, at least two distinct subsets of NK cells are found based on the density of surface expression of CD56 and CD16 (FC $\gamma$ RIII) (Cooper et al, 2001*a*; Berahovich et al, 2006; Caligiuri, 2008; Moretta, 2010). The CD56<sup>dim</sup> CD16<sup>bright</sup> cells express low levels of CD56 and high levels of CD16. They represent approximately 90% of NK cells in normal peripheral blood and function primarily as potent cytolytic effector cells (Cooper et al, 2001*a*; Lünemann et al, 2009). In contrast CD56<sup>bright</sup> CD16<sup>dim/-</sup> cells constitute less than 10% of peripheral blood NK cells but are enriched in secondary lymphoid tissues (Cooper et al, 2001*a*; Lünemann et al, 2009; Poli et al, 2009). For many years, it was thought that CD56<sup>dim</sup> CD16<sup>bright</sup> cells are predominantly cytotoxic while CD56<sup>bright</sup> CD16<sup>dim/-</sup> cells are mainly cytokine secretors. This based on the findings that upon exogenous monokine stimulation with IL-12, IL-15 and IL-18, CD56<sup>dim</sup> CD16<sup>bright</sup> cells secrete only low levels of cytokines while CD56<sup>bright</sup> CD16<sup>dim/-</sup> NK cells produce large amounts of various cytokines including IFN- $\gamma$ , TNF- $\alpha$ , GM-CSF, IL-10 (Diefenbach et al, 2001 ; Béziat et al, 2010,) and chemokines such as macrophage inflammatory protein (MIP)-1 family members and RANTES (Diefenbach et al , 2001). These cells become cytotoxic only after prolonged activation (Strowig et al, 2008). However, in contrast to monokine stimulation, evidence accumulated during the past few years indicate that the CD56<sup>dim</sup> cells may be more important than CD56<sup>bright</sup> cells for cytokine and chemokine secretion following target cell recognition (Juelke et al, 2010; Fauriat et al, 2010). Fauriat et al (2010) demonstrated that in

response to target cell recognition (K562), CD56<sup>dim</sup> rather than CD56<sup>bright</sup> NK cells, were primarily responsible for the production of cytokines and chemokines, including MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, IFN- $\gamma$  and TNF- $\alpha$ .

### **3.3.2 IL-6 Secretion by PBNK cells**

This work was initiated after a number of observations about IL-6 expression / secretion by NK cells in our laboratory. Firstly, in quantitative PCR array experiments IL-6 mRNA expression was seen both in NK cells isolated from the blood of healthy donors and the synovial fluid of RA patients. Secondly, using a multiple bead assay, significant IL-6 production was seen when the NK cells (isolated from the blood of the control individuals, or patients suffering from RA or systemic sclerosis) were co-cultured with the K562 cell line. However, the quality of the data in these experiments was only preliminary. Based on these findings it was decided to investigate the ability of PBNK cells to secrete IL-6 further.

Production of IL-6 by NK cells is rather a novel concept. Intensive search in the literature revealed that this was only reported in one publication. Barakonyi et al (2004) studied cytokine production following the engagement of CD160, an Ig-like activating NK cell receptor whose expression is restricted to the CD56<sup>dim</sup> CD16<sup>bright</sup> subset. Binding the ligand (HLA-C) or a mAb to this receptor induced PBNK cells to produce IFN- $\gamma$ , TNF- $\gamma$ , and IL-6. This CD160-mediated cytokine production was negatively controlled by the co-engagement of CD158b inhibitory receptor. However, the NK cells in this study were co-stimulated with both rhIL-2 and targets cells and therefore it is unclear which one was relevant for the IL-6 production. Although the authors failed to demonstrate the significance of this IL-6 secretion by NK cells, they provided evidence that the NK cells have the potential to secrete this cytokine.

Moreover, this study supported the concept that that CD56<sup>dim</sup> CD16<sup>bright</sup> could be a major source of pro-inflammatory cytokines upon target cell recognition. Nevertheless, these results need to be confirmed by other groups independently.

Together with TNF- $\alpha$  and IL-1, IL-6 is considered a major cytokine important in the protection from pathogens during an infection (Dienz and Rincon, 2009). Since NK cells are involved in the early defence mechanisms against infection, therefore, the concept of IL-6 secretion by NK cells fit with the first line defence role of these cells. Furthermore, IL-6 has been shown to profoundly influence the adaptive immune response by determining CD4<sup>+</sup> T cell differentiation (Rincón et al, 1997; Dienz and Rincon, 2009). Given that NK cells appear at the inflammation site at the early stages of the process where the number of other cells with a potential to secrete IL-6 is low, it is possible that NK cell-mediated IL-6 secretion is essential in orchestrating and potentiating the later stages of the adaptive immune response. Moreover, IL-6 is also known to play an important role in disease progression and tissue damage in some of CD4<sup>+</sup> driven autoimmune diseases such as multiple sclerosis (Sfrent-Cornateanu et al , 2006 ; Barnes et al, 2011) and RA (Nishimoto N , 2006 ; Park and Pillinger , 2007 ; Srirangan, 2010). This fact, together with the preliminary findings in our laboratory that the NK cells isolated from the blood of patients with autoimmune diseases produce significantly more IL-6 after appropriate stimuli than the NK cells isolated from the blood of healthy donors (data not shown), raise the possibility that NK cells could regulate the T-cell response in these patients via IL-6 secretion.



### **3.3.3 Secretion of IL-6 by PBNK cells upon target cell recognition**

To gain more insight into IL-6 production by NK cells, their ability to secrete this cytokine following the recognition of cellular targets was studied using two different cell lines. The widely used NK cell target cell line K562 was selected for these experiments because these cells lack the expression of MHC class I molecules, which normally inhibit NK cell activity (Powell et al, 1989; Nishimura et al, 1994) and therefore, in keeping with the “missing self hypothesis”, are killed by NK cells (Kärre et al, 1986; Ljunggren and Kärre, 1990). On the other hand, HeLa cells express MHC class I molecules, which could interact with various inhibitory KIRs (two and three domain, long cytoplasmic tailed forms of CD158 and others). However, due to the expression of ligands that interact with the dominant activating receptor, NKG2D, on the NK cells, these targets are still killed very efficiently (Pende et al, 2001).

One confounding factor was that control experiments showed that IL-6 was constitutively secreted by both K562 and HeLa cells. This finding is not new for K562 cells; a search in the literature showed that IL-6 secretion by these cells had previously been detected by others (Navarro et al, 1991). However, the observation with HeLa appears novel. Nonetheless, as shown here the secretion of IL-6 in the co-cultures of purified PBNK cells and K562 and HeLa cells was unquestionably raised compared to the K562 and HeLa control cultures. The signalling mechanisms activated in the NK cells in the presence of K562 cells, which lack the expression of MHC class I molecules and the class I positive HeLa cells may be completely non-overlapping. It is not just that K562 cells fail to engage the various inhibitory class I receptors resulting in the lack of the inhibitory signals provided by ITIM motifs of these receptors. In the absence of class I molecules stimulating receptors such as KIR2Ds and KIR3Ds (also recognise HLA-A,-B,-C locus proteins), and CD94/NKG2C, CD94/NKG2E and

CD94/NKG2H (recognise HLA-E locus proteins), will not be engaged either. On the other hand, with the class I positive HeLa cells one would predict KIR2DL and KIR3DL mediated inhibitory signalling events. However, HeLa cells express ligands for the dominant activating receptor, NKG2D. These include MICA (Groh et al, 1999; Zhang et al, 2001), weak expression of MICB proteins (Schrambach et al, 2007; Butler et al, 2009) and possibly weak expression of various forms of ULBPs as well (Butler et al, 2009). Binding of these proteins to NKG2D receptors on the surface of NK cells results in NK cell activation that cannot be overridden by the inhibitory signals elicited via the MHC class I binding KIRs and CD94/NKG2A (B) heterodimers. Comparing NK + K562 and NK + HeLa co-cultures is somewhat difficult since HeLa cells are physically much larger than K562. Thus, the number of NK cell interacting with a given target cell, and possibly the time the NK cells spend attached to the targets as well, is likely to be different. The work presented here tried to compensate for this difference in size by conducting the experiments with 5 times fewer HeLa cells, effectively increasing E: T ratios.

NK + HeLa co-cultures produced significantly higher IL-6 levels than NK + K562 cultures at the same time points. Moreover, the IL-6 production was detectable sooner, as early as 1 hr in NK + HeLa co-cultures, compared to 6 hrs with NK + K562 cells. Whether these differences were due to the physical characteristics of the two target cell lines or result from the engagement of distinct signalling pathways (or the combination of both) remains to be determined. Also, at this point it is unclear if IL-6 production in the NK + K562 and the NK + HeLa cultures represents the convergence of two signalling pathways that are initiated at the cell surface via the engagement of two different receptors, or results from signalling via the same receptor irrespective of the target cells used, which will require further clarification.

### **3.3.4 IL-6 secretion by NK cells in response to IL-2 cytokine and non-specific chemical activators (PMA+ ionomycin)**

Since both target cells used in the co-culture experiments were able to produce IL-6 themselves, hypothetically it was possible that the increase seen in the co-cultures was due to elevated cytokine production by K562 or HeLa cells after the addition of NK cells. Since IL-6 secretion by NK cells is not a widely accepted concept, it was important to show that the NK cells themselves were able to produce this cytokine.

#### **3.3.4.1 The IL-2 response**

It is known that the stimuli able to increase the NK cell cytotoxicity are also up-regulate cytokine biosynthesis by these cells (Perussia, 1996). Therefore, purified NK cells were preincubated in the presence of IL-2, the cytokine that activates NK cells and markedly enhances their cytotoxic potential (Vivier et al, 2008). At first, activation of NK cell cytokine secretion was tried at a dose of 100 U/ml of IL-2. However, in a single preliminary experiment, negligible amounts of IL-6 similar to that secreted by resting PBNK cells, were expressed in the supernatants after 1, 3, 6 and 18 hrs. Next, NK cells were activated with a high non-physiological dose of IL-2. 500 U/ml of rhIL-2 was tested and the experiments were carried out on PBNK cells collected from 5 healthy donors. In all 5 samples IL-6 was significantly detected in the supernatants collected after 6 and 18 hrs with highest concentration at 18 hrs. This suggested that the 100U/ml dose of IL-2 used in the preliminary experiment may have been insufficient to induce IL-6 secretion by NK cells. The need for a rather high dose of IL-2 may be explained by the distribution of IL-2 receptor subunits on NK cells. Nagler et al (1990) and Baume et al (1992) have demonstrated that only CD56<sup>bright</sup>

subset, which constitute <10% of peripheral blood NK cells, carry the high affinity IL-2R $\alpha\beta\gamma$  (CD25) in addition to the free IL-2Rp75 chains. The vast majority of NK cells (CD56<sup>dim</sup>) express only the intermediate affinity IL-2 $\beta\gamma$  receptor on the cell surface. Thus, the need for a high IL-2 concentration in the IL-6 secretion experiments is consistent with the assumption that the process involves the CD56<sup>dim</sup>, primarily cytotoxic, NK cell subset.

#### **3.3.4.2 The PMA + ionomycin response**

To investigate further the ability of NK cells to produce IL-6 non-specific chemical activation was also used. Our data revealed that stimulation with PMA + ionomycin was effective in inducing IL-6 production by NK cells. A significant increase of IL-6 concentration was observed as early as 6 hrs following stimulation. PMA acts as a direct agonist of protein kinase C (PKC) which plays a vital role in the cascade of events leading to cell activation. Treatment of a variety of cells with phorbol esters activates the expression of a range of early response genes through protein kinase C-dependent pathways (Blumberg, 1988). The protein kinase C (PKC) enzymes are known for their long-term activation. They remain activated after the original activation signal or the Ca<sup>2+</sup> wave is gone (Klann et al, 1991; Sessoms et al, 1992). This is presumably achieved by the production of diacylglycerol from phosphatidylinositol by a phospholipase; fatty acids may also play a role in long-term activation (Nishizuka, 1995). The relatively rapid expression of IL-6 cytokine following treatment of NK cells with PMA + ionomycin might be, at least in part, due to the presence of regulatory sequence elements in the corresponding IL-6 gene promoter through mechanisms that are protein kinase C-dependent. However, the amount of IL-6 in the tissue culture supernatants was noticeably less after PMA +

ionomycin exposure than that seen after co-cultures of NK cells and target cells, or that resulting from high dose IL-2. Thus, protein kinase C is unlikely to be the only pathway that is responsible for the secretion of this cytokine in NK cells following target cell recognition.

### **3.3.5 The Transwell plates result**

Transwell plates were used to investigate whether IL-6 production by NK cells upon target cell activation was mediated by soluble mediators or cell-cell contact. Separating NK cells and target cells abolished increases in cytokine production proving that direct interaction between NK cells and target cells is necessary for triggering IL-6 production by NK cells, as the semi-permeable membrane of Transwell chambers allows for the free passage of soluble factors but prevents direct cell contact.

In conclusion, the results presented in this chapter demonstrate that, although the IL-6 is secreted by K562 and HeLa cell lines, the concentration of IL-6 is significantly higher when these target cells are co-cultured with PBNK cells. These findings suggest that the NK cells can contribute to IL-6 secretion. This notion was confirmed by the observation that the NK cells secrete IL-6 when activated with PMA + ionomycin or high doses of IL-2. The dose of IL-2 required for effective IL-6 secretion suggests that the IL-6 is derived from NK cells that express receptors with only intermediate affinity for IL-2. The direct physical interaction between NK cells and target cells, probably via membrane bound receptor-ligand interaction, is necessary for triggering IL-6 production by NK cells, as it demonstrated by Transwell experiments. Given that NK cells appear at inflammation sites at the earliest stages of

the process, where the number of other cells with a potential to secrete IL-6 is low, it is possible that NK cell-mediated IL-6 secretion is essential in orchestrating and potentiating the later stages of the adaptive immune response.

# Chapter 4

## Involvement of Different Activating Receptors in Regulating IL-6 Production by Natural Killer Cells



2012

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## **4.1 Introduction**

The work presented in the previous chapter has shown that NK cells have the potential to secrete IL-6 following different stimuli. Although this secretion can be initiated with high doses of cytokine (IL-2) or with non specific chemical mediators (PMA+ ionomycin) these kinds of stimuli are unlikely to occur inside the body. In contrast, activation via target recognition is a common event under physiological conditions. The results of Transwell experiments clearly indicated that direct physical contact between NK cells and target cell (NK cell receptor-ligand interaction) was necessary for IL-6 secretion by these cells.

### **4.1.1 Specific receptor engagement and the cytokine secretion by NK cells**

NK cells mediate protection against viruses and intracellular pathogens through direct lysis of infected cells and by secretion of cytokines that shape the subsequent adaptive immune response (Papazahariadou et al, 2007; Lanier, 2008). These NK cell functions are finely regulated by array of surface receptors inducing inhibitory and activating signals (Lanier, 1998; Long, 1999; Moretta et al, 2001; Vivier et al, 2004; Moretta et al, 2004; Lanier, 2005) as well as by the presence of soluble mediators (cytokines). The role of cytokines in the initiation of cytokine secretion by NK cells has been extensively studied. Many of these studies have shown that NK cells express a number of monokine receptors including IL-2R (Caligiuri et al, 1990; Nagler et al, 1990; Baume et al, 1992), IL-1 $\beta$ R (Cooper et al, 2001*b*), IL-12R (Wang et al, 2000), and IL-18 R (Kunikata et al, 1998). In addition IL-15 can induce its effect on NK cells through the common IL-2R  $\beta\gamma$  subunit (Carson et al, 1994). These receptors are distributed on both NK cell subsets but with different densities. Stimulation of these receptors by IL-2, IL-12, IL-15 and IL-18 alone or in

combination, results in expression / or secretion of cytokines including IFN- $\gamma$ , TNF- $\alpha$ , GM-CSF, IL-4, IL-10 and IL-13 (Carson et al, 1997; Fehniger et al, 1999; Lauwerys et al, 2000) and chemokines such as MIP-1 family members (Bluman et al, 1996; Fehniger et al, 1999) by NK cells.

On other hand, relatively less is known about cytokine production upon specific engagement of NK cell receptors (Fauriat et al, 2010). A number of structurally distinct receptors have been implicated in the activation of NK-cell effector functions despite the expression of MHC class I molecules. Depending on the way these receptors signal, they have been grouped into three categories (Bryceson et al, 2006): receptors that signal through immunoreceptor tyrosine-based activation motif (ITAM) containing subunits (e.g. CD16, NKp46, NKp44 and NKp30), the DAP10-associated receptor NKG2D, and several other receptors (e.g. 2B4, DNAM-1) that signal via other different pathways. Review on these receptors was described in Chapter 1. While the role of NK cell receptors in the initiation of NK cell cytotoxicity is well known, minimal requirements for induction of cytokine secretion upon engagement of these receptors with their monoclonal antibodies (mAbs) or ligands on target cells has still not been established clearly. Some studies have demonstrated that NK cells can release cytokines on recognition of targets (Bancroft et al, 1993; Tsutsui, et al, 1996) but the mechanism behind this remains unclear.

CD16 is a 50-80 kDa glycoprotein that is expressed in two distinct (CD16a and CD16b) isoforms (Schumann et al, 1994). Cross-linking of CD16 or of the activating forms of CD94/NKG2 and activating KIR receptors on NK cells leads to production of GM-CSF, TNF- $\alpha$ , IFN- $\gamma$  and IL-2 by NK cells (Mandelboim et al, 1998; Márquez et al, 2010).

Despite their major role in triggering NK-mediated killing of most tumour cell lines, evidence accumulated in recent years has suggested broader functions of natural cytotoxicity receptors (NCRs) that could involve cytokine secretion. Studies have shown that cross-linking human NKp46 or NKp30 can induce IFN- $\gamma$  cytokine secretion by PBNK cells (Andre et al, 2004). This effect is synergized when these receptors are co-stimulated with NKG2D, 2B4, CD2 and, to a lesser extent, with DNAM-1 (Bryceson et al, 2006). The mAb-specific engagement of NKp30 was found to trigger the production of IFN- $\gamma$ , TNF- $\alpha$ , MIP-1  $\alpha$ , MIP-1 $\beta$ , and GM-CSF cytokines by human decidual NK cells (dNK) cells (El - Costa et al , 2008). Moreover, mouse splenic and intestinal NK cells can secrete IFN- $\gamma$  and IL-22 in response to NKp46 stimulation (Satoh-Takayama et al, 2009).

NKG2D (CD314) is a lectin-like homodimeric receptor that recognizes a variety of stress-induced self-ligands, such as MICA, MICB and ULBP molecules in humans (Bauer et al, 1999; Steinle et al, 2001) as well as the H60, Rae1 and MULT molecules in mice (Cerwenka et al, 2000). Besides its expression on NK cells, NKG2D is also detected on CD8<sup>+</sup> T cells and  $\gamma\delta$  T cells, and can be up-regulated by IL-15 (Bauer et al, 1999). NKG2D signalling is achieved through membrane association with signalling adaptor. The adaptor that associates with human NKG2D is the DAP10 transmembrane adaptor, which bears a YxxM motif and activates the phosphatidylinositol 3-kinase pathway (Wu et al, 1999). Cross-linking human NKG2D receptors with their specific ligands (but not with mAbs) is sufficient to induce IFN- $\gamma$  secretion by human NK cells (Billadeau et al, 2003; Andre et al 2004).

#### **4.1.2 Aims of this Chapter**

Here it was addressed how specific engagement of activating receptors recognizing non-MHC class I molecules, namely CD16, NKG2D (CD314), NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3) regulated IL-6 mRNA expression and protein production by purified resting human PBNK cells. These aims were achieved by inducing NK cell activation with immobilized antibodies against these activating receptors and assessing the effect of the engagement of these receptors on PBNK IL-6 gene expression at transcription level in different time points by quantitative real time PCR (qPCR). IL-6 secretion by NK cells was also measured in the supernatants at the same time points by ELISA. In addition the data obtained from these various receptors on the induction of IL-6 cytokine transcripts and protein production by PBNK cells were directly compared, thereby providing some insight into the IL-6 cytokine profile induced by these different receptors.

#### **4.2 *In vitro* stimulation of PBNK cells**

##### **4.2.1 Preparation of plastic-immobilized antibodies**

100 µl (~10µg/ml) of anti CD16 monoclonal antibody (clone 3G8, gift from Inflammation Research Unit, University of Liverpool) were immobilized directly to 96-well polystyrene tissue culture flat bottom plates. 3G8 is a mouse anti-human CD16 that specifically binds to the transmembrane form of the IgG Fc receptor (FcγRIIIa). Anti-NKG2D monoclonal antibody (clone 149810), anti-NKp46 (clone 195314), anti-NKp44 (clone 253415) and anti-NKp30 (clone 210845), (all from R & D Systems, UK) were diluted in carbonate buffer (eBioscience) to approximately 10 µg / ml and immobilized directly to 96-well flat bottom polystyrene tissue culture plates (Loza et al, 2003). These antibodies were used in the activation of resting NK

cells and stimulation of IL-6 cytokine production by these cells. Following the addition of antibodies to the wells, the plates were incubated for 18 hrs at 4 °C. On the next day, the wells were washed two times with 1X PBS to remove unbound antibodies and filled with 200 µl of complete RPMI medium containing the effector NK cells.

#### **4.2.2 Preparation of effector cells**

Human NK cells from 10 healthy donors were isolated from fresh or short term PBMCs cultures by negative selection using an NK cell isolation kit (Miltenyi Biotec, Germany) as described in Chapter 2. For CD16, NKG2D, NKp46 and NKp30 activation, NK cells from each healthy donor were added to the wells containing immobilized antibodies at a concentration of  $1 \times 10^5$  cells / well and the plate was incubated at 37° C for 1, 3, 6 and 24 hours in a humidified incubator. For NKp44 activation, since this receptor is a marker for activated NK cells, and is not expressed by resting cells, the NK cells were pre-activated with recombinant human IL-2 (R & D Systems, UK) at a dose of 100 U / ml for 24 hrs prior to adding them to the wells containing the immobilized NKp44 antibody. This was followed by a further 1, 3, 6 and 24 hours incubation at 37° C in a humidified incubator. Wells containing resting NK cells without antibody activation were used as negative control. Supernatant was collected from each well at 1, 3, 6 and 24 hrs and stored at -20 °C until tested for IL-6 production using a human IL-6 ELISA kit (eBioscience). At the same time, the NK cells from each well were collected for RNA extraction, reverse transcription and quantitative PCR (qPCR).

### **4.2.3 RNA extraction from PBNK cells**

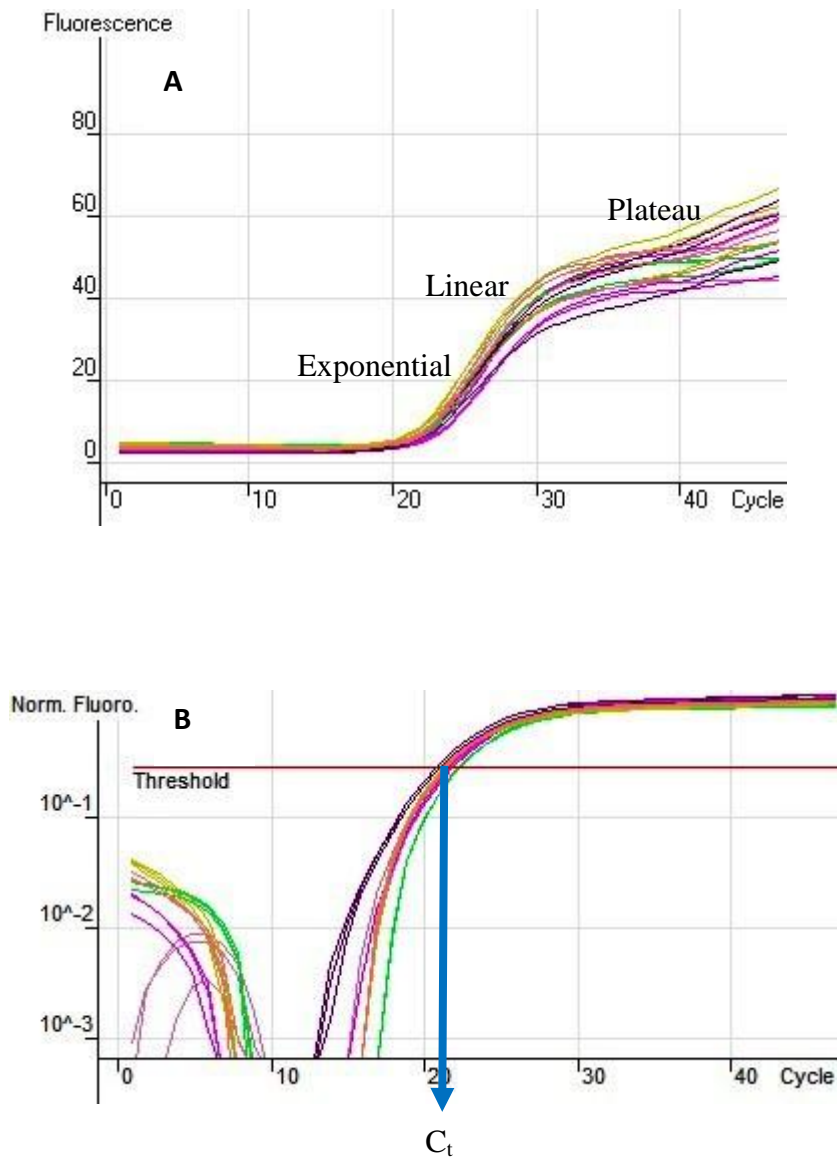
After activation with immobilized anti- CD16, anti-NKG2D, anti-NKp46, anti-NKp44 and anti-NKp30 antibodies, the NK cells ( $1 \times 10^5$  cells / well) were collected from the tissue culture plates at 1, 3, 6 and 24 hrs. Resting NK cells from the same donors were also extracted at the same time points and used as negative controls. Total RNA was then extracted from both the activated and resting NK cells using the QIAamp RNA Blood Mini Kit (Chapter 2). Since the isolated RNA was then used in qPCR that are sensitive to very small amounts of DNA, so more RNA clean- up was necessary. Contaminants were removed by further washing with RWI and RPE buffers. High-quality RNA was then eluted in water. The concentration and purity of the RNA was determined by measuring the absorbance at 260 nm (A260) and 280 nm (A280) in a spectrophotometer as described in Chapter 2. Following extraction and quantification, the RNA was either stored at  $-80\text{ }^{\circ}\text{C}$  or immediately reversed transcribed into complementary DNA (cDNA) as described in Chapter 2. However, in most cases the volume of the master mix was scaled up or down depending on the amount and volume of the extracted RNA. cDNAs from both the activated and resting NK cells were then used to assess IL-6 mRNA expression by real-time PCR.

### **4.2.4 Real-time quantitative PCR (qPCR)**

A basic PCR reaction can be broken up into three phases ([www.applied biosystems.com](http://www.appliedbiosystems.com)): exponential phase in which the accumulating products double at every cycle (assuming 100% reaction efficiency); linear phase in which the reaction components are becoming consumed and the reaction is slowing; and finally the plateau (end-point) phase in which the reaction is stopped and, if left long enough, the PCR products will begin to degrade (Figure 4.1 A). Real-time chemistries allow for



the detection of PCR amplification during the exponential phase of the reaction because this phase provides the most accurate and precise data for quantitation. Within the exponential phase, the real-time PCR instrument calculates two values (Figure 4.1 B): The threshold line is the level of detection at which a PCR reaction reaches a fluorescent intensity above background; and the cycle threshold (Ct) which is the PCR cycle at which the sample reaches a fluorescent intensity above background. Measuring the kinetics of the reaction in the exponential phase of PCR provides a distinct advantage over traditional PCR detection. Traditional methods need post reaction analysis (e.g. use of agarose gels) for the detection of PCR amplification at the final phase or end-point of the PCR reaction. In addition, using real-time PCR the results can be tracked in “real-time” thus combining the amplification and the detection in one step.



**Figure 4.1: A) The three different phases of PCR reaction.** In the exponential phase the reaction components are in excess, there is an exact doubling of product each cycle, and the reaction is specific and precise. In the linear phase the reaction components are being consumed, amplification slows, and the reactions become highly variable. The final phase of PCR amplification is the plateau (end-point) phase. Here, the reaction is complete and no more products are being generated. The real time PCR takes its measurements during the exponential phase, while traditional PCR takes its measurements during the plateau phase. **B)** The real-time PCR reaction showing the threshold line and the cycle threshold ( $C_t$ ). The  $C_t$  is the PCR cycle at which the fluorescence intensity of the sample increases above the background level. The threshold was calculated by the Rotor-Gene 5 software.

#### **4.2.4.1 Quantification assays in real-time PCR**

There are two methods used for quantification of gene expression in real-time PCR. The first method is the absolute quantification assay which uses a standard curve of serially diluted standards of known quantities to determine the quantity of unknown samples (Wong et al, 2005). Absolute quantification results are reported as an absolute quantity (copies, ng, pg, etc.) extrapolated from the standard curve. The second method is the relative quantification (also known as comparative quantification) of gene expression which allows quantifying differences in the expression level of a specific gene between different samples (Cikos et al, 2007). The data output is expressed as a fold-change or a fold-difference of expression levels. Both absolute and relative quantification use the Ct values obtained during real-time PCR. In absolute quantification the Ct values of test samples are compared to those of the standards of known quantity plotted on a standard curve. Relative quantification involves comparing the Ct values of the test samples to those of control samples. This analysis yields a ratio rather than absolute quantity e.g. the relative amount (fold difference) of a target nucleic acid in target vs. control samples.

#### **4.2.4.2 Methods of monitoring PCR amplification in “real-time”**

##### **1. Fluorescent dyes**

Intercalating fluorescent dyes represent the simplest and cheapest way to monitor the real-time PCR reaction. The fluorescence of these reporter dyes increases as the PCR product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase.

SYBR-Green I is the most commonly used fluorescent dye in real-time PCR with excitation and emission maxima of 494 nm and 521 nm, respectively (Ponchel et al, 2003). It exhibits little fluorescence when it is free in solution, but its fluorescence increases up to 1000-fold when it binds double stranded DNA. As the number of copies of DNA increases during the reaction the fluorescence increases. Therefore, the overall fluorescent signal from a reaction is proportional to the amount of double stranded DNA present. Ethidium bromide can also be used for the detection of double stranded DNA but its carcinogenic nature renders its use restrictive. Although these double stranded DNA-binding dyes provide the simplest and cheapest option for real time PCR, the principal drawback of DNA-binding dyes is their lack of specificity. As they bind to any double stranded DNA the presence of nonspecific products in a real-time PCR reaction may contribute to the overall fluorescence and affect the accuracy of quantification.

## **2. TaqMAN hydrolysis probes**

In TaqMan® experiments, three oligonucleotides are used: forward and reverse primers specific to the target of interest and a fluorogenic probe. This probe is an oligonucleotide with a fluorescent reporter dye attached to the 5' end such as 6 - carboxyfluorescein (FAM), and a quencher dye attached to the 3' end such as 6-carboxy-tetramethyl-rhodamine (TAMRA). The oligonucleotide sequence of the probe is complementary to the target sequence. Initially the probe is not hydrolyzed and the quencher and the reporter fluorophore remain in proximity to each other. This proximity however, does not completely quench the fluorescence of the reporter dye and background fluorescence is observed (Leutenegger, 2001). During PCR the probe anneals specifically between the forward and reverse primer to an internal region of

the PCR product and has a melting temperature 10°C higher than that of the primers (Leutenegger, 2001). Binding of the TaqMan probe prior to the primers is crucial because without it PCR products would be formed without generation of fluorescence intensity and thus without being detected. The 3' phosphate group (P) prevents extension of the TaqMan probe. During extension the 5' exonuclease activity of the polymerase cleaves the probe, releasing the reporter molecule away from the close vicinity of the quencher. The fluorescence intensity of the reporter dye, as a result, increases. This process repeats in every cycle and does not interfere with the accumulation of PCR product. In real-time PCR, fluorescent primers and probes offer two main advantages over fluorescent dyes. First, they specifically detect the target sequence so nonspecific products do not affect the accuracy of quantification. Second, they allow multiplex reactions to be performed in a single tube.

### **3. Hybridisation probes (FRET probes)**

The hybridization probe system consists of four oligonucleotides: forward and reverse primers specific to the target of interest and two fluorophore - labelled hybridisation probes. The up-stream (donor) probe is labelled with a fluorophore at the 3' end of oligonucleotide sequence while the downstream (acceptor) probe is labelled with a fluorophore at the 5' end (Okamura et al, 2006). The two fluorophores are carefully chosen so that the emission spectrum of one overlaps significantly with the excitation spectrum of the other. Interactions between the excited states of these fluorophore molecules lead to the transfer of excitation energy from one (the donor) dye molecule to the other (acceptor molecule) (Juskowiak, 2011). This process is called Fluorescence Resonance Energy Transfer (FRET) and it is a distance - dependent (typically 10-80 nucleotides) (Lorenz et al, 2006). During PCR the two

different oligonucleotide probes hybridize to adjacent regions of the target DNA such that the fluorophores, which are coupled to the oligonucleotides, are in close proximity in the hybrid structure in a head-to tail arrangement. The donor fluorophore (F1) is excited by an external light source then passes part of its excitation energy to the adjacent acceptor fluorophore (F2). The excited acceptor fluorophore (F2) emits light at a different wavelength which can then be detected and measured (Howell, 2006).

#### **4. Other primer- and probe- based methods**

Several other fluorescent primer- and probe-based chemistries have been devised for monitoring DNA/RNA amplification in real-time. These include molecular beacons, minor groove binder (MGB) - eclipse probes, amplifluor assays, scorpion primers, LUX primers, and ozyme primers. Molecular beacons are single-stranded bi-labelled fluorescent probe held in a hairpin-loop conformation (around 20 to 25 nucleotide) with complementary stem sequences (around 4 to 7 nucleotides) at the 5' and 3'ends of the probe (Li et al, 2008). A fluorescent reporter molecule is attached to the 5' end of the molecular beacon, and a quencher is attached to the 3' end. Formation of the hairpin therefore brings the reporter and quencher together, so no fluorescence is emitted. During the annealing step of PCR, molecular beacons hybridize to their target sequence causing the hairpin-loop structure to open and the stem to denature. The reporter and quencher are thus separated. As the quencher is no longer close enough to absorb the emission from the reporter dye, the dye is allowed to fluoresce and the PCR instrument detects the increase of emitted energy. The measured fluorescent signal is directly proportional to the amount of target DNA. The MGB-eclipse probe system consists of three oligonucleotides: forward and reverse gene-specific primers and the eclipse probe. The eclipse probe has a complementary

sequence to the target DNA. The 5' end of the probe is attached to the quencher molecule and the MGB peptide moiety, while the reporter dye is attached to the 3' end of the probe (Cloud et al, 2005). The un-hybridized probe adopts a random three-dimensional conformation that brings the reporter and quencher together, so quenching occurs. During PCR, the probe anneals to the target with the help of MGB, thus the probe becomes linearized. This leads to the separation of the reporter dye from the quencher to produce fluorescence.

#### **4.2.4.3 IL-6 real-time PCR primers**

Optimal design of the PCR primers is crucial for accurate and specific quantification using real-time PCR. For the assay described herein, detection is based on the binding of the SYBR Green I dye into double stranded IL-6 PCR products. As discussed above, while this assay is cheaper than probe-based methods, it loses the additional level of specificity introduced by the hybridisation of the PCR product to a specific fluorescent TaqMan probe. The sensitivity of detection with SYBR-Green I dye may therefore be compromised by the formation of primer-dimers, lack of specificity of the primers, primer concentration and the formation of secondary structures in the PCR product. All of these factors could potentially lead to the creation of unexpected double stranded DNA product, which would incorporate SYBR-Green I dye and register a fluorescent signal.

Throughout this study IL-6 mRNA expression in CD16-, NKG2D-, NKp46-, NKp44- and NKp30- activated PBNK cells was analyzed by real-time PCR from 10 healthy donors using SYBR-Green I dye. The levels of IL-6 mRNA in un-stimulated (resting) NK cells were chosen as control. Gene expression levels in both activated and resting NK cells were normalized to GAPDH (glyceraldehyde-3-phosphate

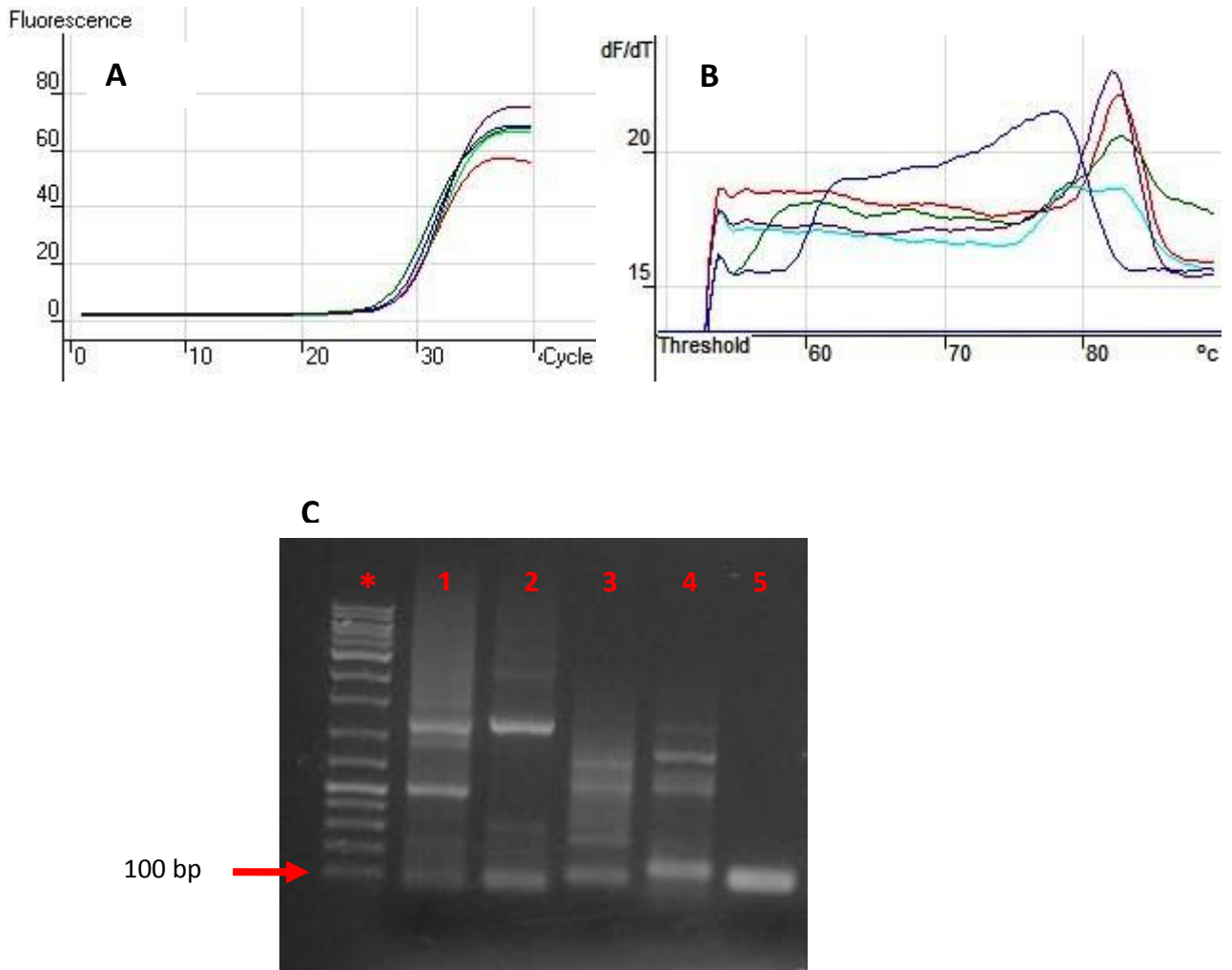
dehydrogenase) mRNA and relative quantification was performed by a relative quantification method. Primer sequences used in this experiment for the real-time amplification of IL-6 and GAPDH were acquired from published papers (Chapter 2, Section 2.11). All IL-6 and GAPDH primers were tested to meet at least two critical criteria: single - amplicon specificity and consistently high amplification efficiencies. The single - amplicon specificity was experimentally validated with two quality control assays, a melt curve analysis and agarose gel electrophoresis. Both must verify that a single IL-6 or GAPDH gene specific product is produced. The second criterion to pass was an amplification efficiency  $> 90\%$ .



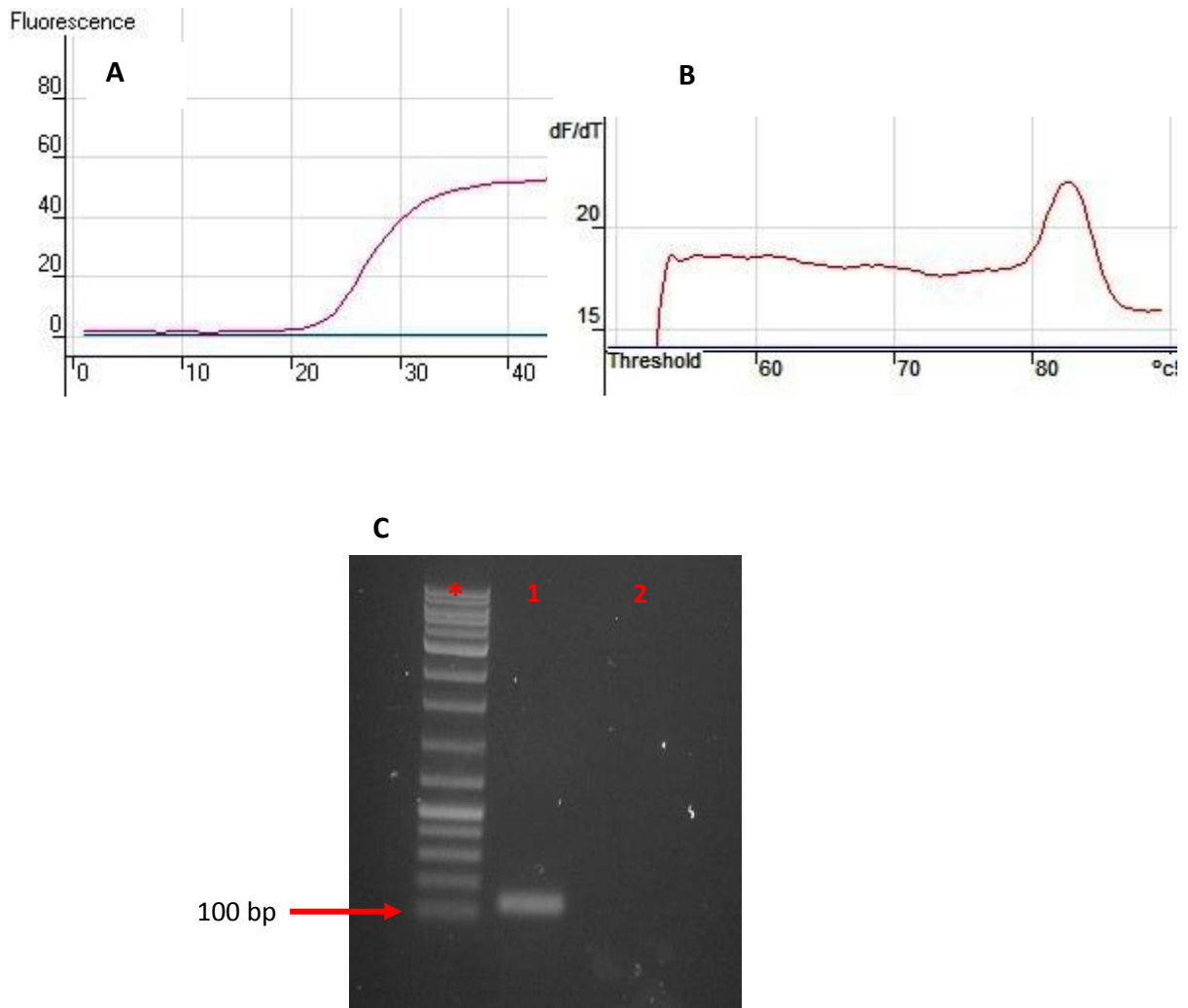
## **4.3 Results**

### **4.3.1 Checking primers for single - amplicon specificity**

To test the performance and validity of IL-6 and GAPDH primers, a preliminary primer check was performed. Using 1 µl (~100-200 ng) of PBMCs cDNA as template and 2 X SensiMix™ SYBR Green NO ROX kit (BioLine, UK), 20 µl real-time PCR reactions (one for each IL-6 primer set and one for GAPDH with its negative control) were prepared and run as described in Chapter 2. The PCRs were run in two independent experiments and at the end of the cycling programme the melting curves of the PCR reactions were checked. Ideally, melting curve should contain a single peak with no shoulders, and the agarose gels of the amplified product should reveal a single band corresponding to the predicted amplicon length. Figure 4.2 shows the melting curves and agarose gel electrophoresis for the IL-6 primers used for the optimisation steps. Apart from the pre-optimized primer from Qiagen, all other primer sets gave rise to melting curves with more than one peak and more than one band on agarose gel indicating either the production of primer dimers or nonspecific products. The primer dimers may be eliminated by optimizing primer concentration or changing thermocycling parameters. Many attempts were made to optimize primer concentrations and cyclic parameters but all these attempts failed. Since these were very time consuming procedures, all primers with more than one peak during melting curve analysis were excluded and the pre-optimized primer set (Qiagen) was selected for the final experiments. Regarding the specificity of the GAPDH primer set the melting curve clearly indicated a single peak and demonstrated that the GAPDH PCR product had a specific melting temperature at ~82 °C (Figure 4.3 A & B). Subsequently, the agarose gel electrophoresis showed that the band was specific with the expected length of 133 bp (Figure 4.3 C).



**Figure 4.2: IL-6 primers test.** Four IL-6 primer sets (selected from published papers) and one pre-optimized IL-6 primer set (purchased from Qiagen) were tested using 1  $\mu$ l (~100-200 ng) of PBMC cDNA and 2 X SensiMix<sup>TM</sup> SYBR Green NO ROX kit. **A)** Real-time fluorescent detection of the IL-6 amplicon. **B)** Dissociation (melting) curves of the different IL-6 PCR reactions. A single peak suggested the absence of non-specific amplification products. **C)** Agarose gel electrophoresis of real-time PCR products. Lane \*: 100-10000 bp DNA Ladder; lane 1: IL-6 primer 1 (expected product size is 80 b.p); lane 2: IL-6 primer 2 (expected product size is 99 b.p); lane 3: IL-6 primer 3 (expected product size is 143 b.p); lane 4: IL-6 primer 4 (expected product size is 193 b.p); lane 5: commercial pre-optimized IL-6 primer (expected product size is 107 b.p). Apart from the pre-optimized IL-6 primer set, all other primers gave rise to non specific PCR products. These primers were excluded from the final study.



**Figure 4.3: Quality control of the GAPDH primer set.** The GAPDH primer set was tested using 1  $\mu$ l (~100-200 ng) of PBMC cDNA and the 2 X SensiMix<sup>TM</sup> SYBR Green NO ROX kit. Real-time PCR was run in 20  $\mu$ l volumes with 1  $\mu$ l of forward and 1  $\mu$ l of reverse primers. PCR conditions were as follows: 95°C for 10 min, 40 cycles of [95°C for 15 seconds, 60°C for 15 seconds and 72°C for 20 seconds]. The melting of the PCR product was performed from 55°C to 95°C, rising in 0.5°C increments. **A)** Real-time fluorescent detection of GAPDH amplicon. **B)** Melting curve for GAPDH PCR reaction. The single peak suggests a single size product. **C)** Agarose gel electrophoresis of the GAPDH real-time PCR products. The left lane shows the 100-10000 bp molecular weight ladder. Lane 1 shows the amplicon (133 bp). No amplification was found in negative control (no template DNA sample, lane 2).

### 4.3.2 Testing the primers amplification efficiency

The typical method to analyze real-time PCR data is the Livak method (Livak and Schmittgen, 2001). This is also referred to as the  $2^{-\Delta\Delta CT}$  method. It uses only Ct values and consists of three main steps:

1) Normalization of the Ct value of gene of interest to that of reference gene, for both the test sample and the control sample. This is  $\Delta CT$ :

$$\Delta Ct (\text{test}) = Ct (\text{Gene of interest, test}) - Ct (\text{reference gene, test})$$

$$\Delta Ct (\text{control}) = Ct (\text{Gene of interest, control}) - Ct (\text{reference gene, control})$$

2) Calculation of the difference between the  $\Delta CT$  of the test sample and the  $\Delta CT$  of the control. This is called  $\Delta\Delta Ct$ :

$$\Delta\Delta Ct = \Delta Ct (\text{test}) - \Delta Ct (\text{control})$$

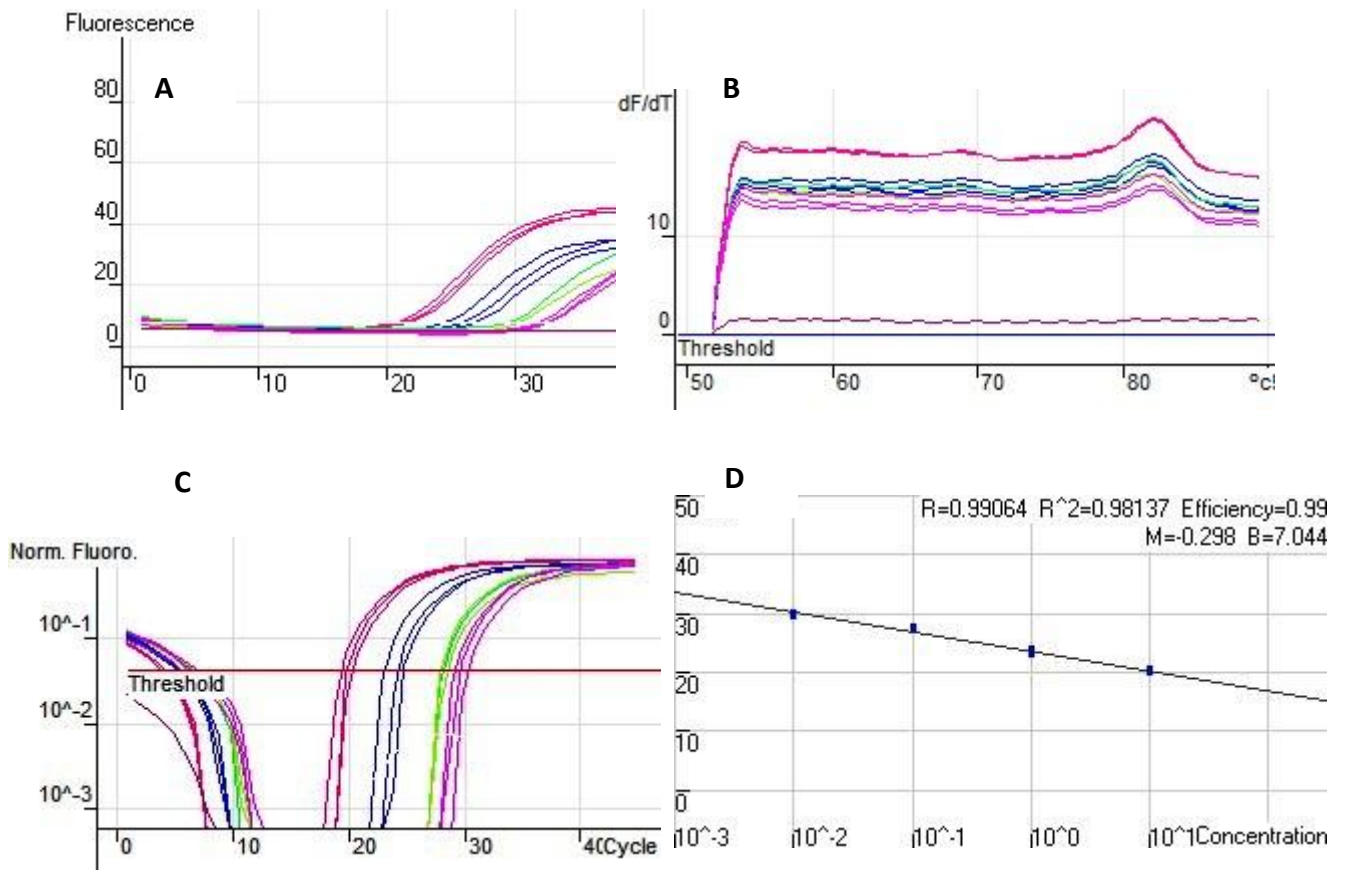
3) Calculation of the expression ratio:

$$\text{Expression ratio (folds)} = 2^{-\Delta\Delta Ct}$$

However, this method often assumes that both the gene of interest and the reference gene PCR assays have 100% amplification efficiency (a two-fold increase in the amount of the amplicon during each cycle). Since 100% efficiency is rarely seen in practice, therefore the error rate in the fold difference increases exponentially. PCR amplification efficiencies between 90-110% are generally considered acceptable and enable PCR assays to accurately analyze gene expression using the Livak method (Dorak, 2011). To determine the PCR amplification efficiency a calibration standard curve is required. The cDNA template of known concentration is serially diluted. After the end of the cycling, the dilution and Ct values are exported to Microsoft Excel and a standard curve is drawn by plotting the Ct values versus the log<sub>10</sub> dilution values

(linear regression) and the slope is calculated from the standard curve. The efficiency of the reaction can be calculated by the following equation:  $E = 10^{(-1/\text{slope})} - 1$ . Slopes of -3.1 to -3.6 in the Ct Vs. log-template amount standard curve correspond to a PCR efficiency of 90-110%. The closer the slope is to -3.33, the closer the amplification efficiency is to the theoretical 100% (Dorak, 2011). If the PCR efficiency is < 90%, the quantitative real-time PCR should be further optimized or alternative primers designed. If the efficiency is > 110%, running another standard curve experiment with a minimum of 3 replicates and a minimum of 5 logs of template concentration is recommended. Nowadays most of real-time PCR machines are provided with software that can generate the PCR efficiency calculations rapidly and automatically.

The preliminary primer test experiment has indicated all the IL-6 primers gave rise to non specific bands apart from the pre-optimized primers from Qiagen. Since these primers are pre-optimized by the company for the IL-6, therefore no PCR amplification efficiency test was carried out on these primers. However, the GAPDH reference gene primers were evaluated for their amplification efficiency. Here 4 serial 10 fold dilutions of PBMCs cDNA were made. Each dilution was prepared in triplicates and the PCR was run using the same parameters as in the previous experiment. As can be seen from Figure 4.4, the amplification efficiency of the GAPDH PCR reaction was 99%,  $R=0.99064$ ,  $R^2 = 0.98137$ . Therefore, these primers were used for the final experiments.



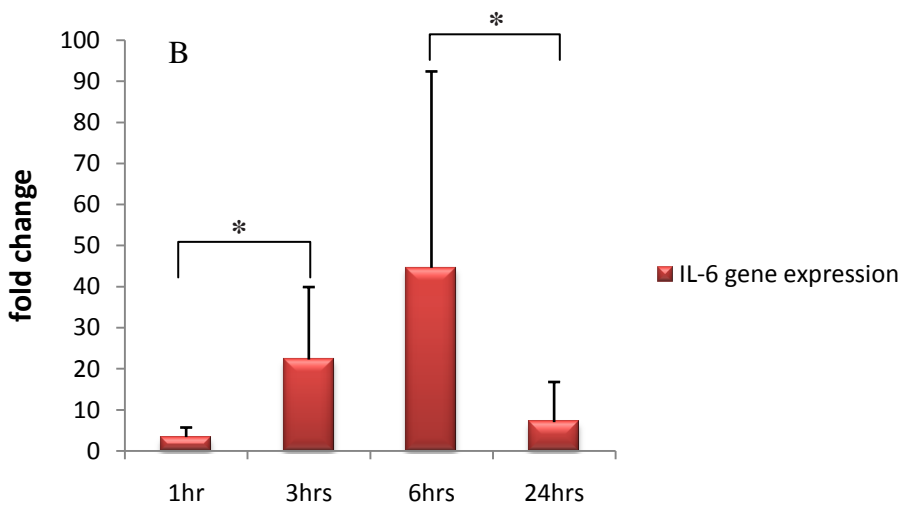
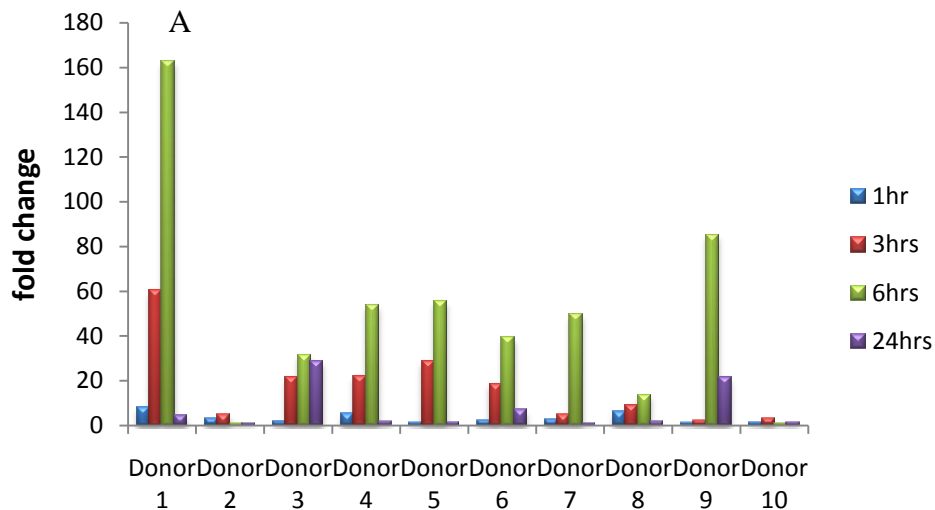
**Figure 4.4 Amplification efficiency of the GAPDH primer set using SensiMix™ SYBR Green NO ROX kit.** **A)** Real-time amplification curve from a 10-fold dilution series of a GAPDH target amplified from PBMCs cDNA with starting amounts of 100 ng. GAPDH was amplified in four triplicate reactions using the GAPDH experimental primer set. Real-time PCR was run in 20  $\mu$ l of final volume with 1  $\mu$ l of forward and 1  $\mu$ l of reverse primers. The PCR conditions were as follows: 95°C for 10 min, 40 cycles of [95°C for 15 seconds, 60°C for 15 seconds and 72°C for 20 seconds]. **B)** Melting curve analysis. Single peaks on the melting curve of all samples indicated that no contaminating DNA, primer dimers or non-specific products were present in the reaction. **C)** Log-transformation of the fluorescence measurements and the points of intersection of the amplification curves and the threshold. The threshold was determined by the Rotor Gene 5 software. **D)** Standard curve of the GAPDH amplification. The curve generated by the Rotor Gene 5 software provided with the Rotor Gene 2000 Thermocycler based on the log dilution (X-axis) and Ct values (Y-axis). The efficiency of the PCR reaction was 99%. The correlation coefficient of the GAPDH standard was  $R = 0.99$ .

### **4.3.3 Involvement of different NK cell activating receptors in IL-6 mRNA expression and protein secretion**

#### **4.3.3.1 Stimulation via CD16**

##### **1. Engagement of CD 16 induced IL-6 mRNA expression in PBNK cells**

First we tested whether the CD16 cross-linking on PBNK cells can stimulate IL-6 gene expression at a transcriptional level in these cells. PBNK cells were stimulated with anti-CD16 antibodies (clone 3G8) as described in Sections 4.2.1 and 4.2.2, and mRNA expression was studied after 1, 3, 6 and 24 hours. Quantitative analysis was performed by comparing the IL-6 signal obtained from CD16-activated NK cells to that obtained from resting PBNK cells. Gene expression levels from both activated and non-activated PBNK cells were normalized to the house keeping gene (GAPDH signal) at same time points. Relative quantification was then performed using the  $\Delta\Delta CT$  method. The results are shown in Figure 4.5 A and summarized in Figure 4.5 B. It is clear that 1 hour post stimulation, IL-6 mRNA expression was already more than 3-fold enhanced compared to the pre-stimulation levels ( $3.4 \pm 2.3$  fold greater than resting). At 3 hours, the IL-6 mRNA expression significantly increase with more than 22 fold enhanced to pre-stimulation level ( $22.3 \pm 17.65$  fold greater than resting,  $p = 0.022$ ). In most cases, the most prominent increase was found 6 hours after stimulation exceeding the pre-stimulation level more than 44-fold ( $44.6 \pm 47.8$  fold greater than resting). However, this increase, when compared to that at 3 hours was not statistically significant ( $p = 0.064$ ). IL-6 mRNA expression clearly peaked around this time as at 24 hours the levels were dropping compared to 6 hours, although, at least in some donors, they were still above background ( $7.01 \pm 9.77$  fold greater than resting,  $p = 0.013$ ).

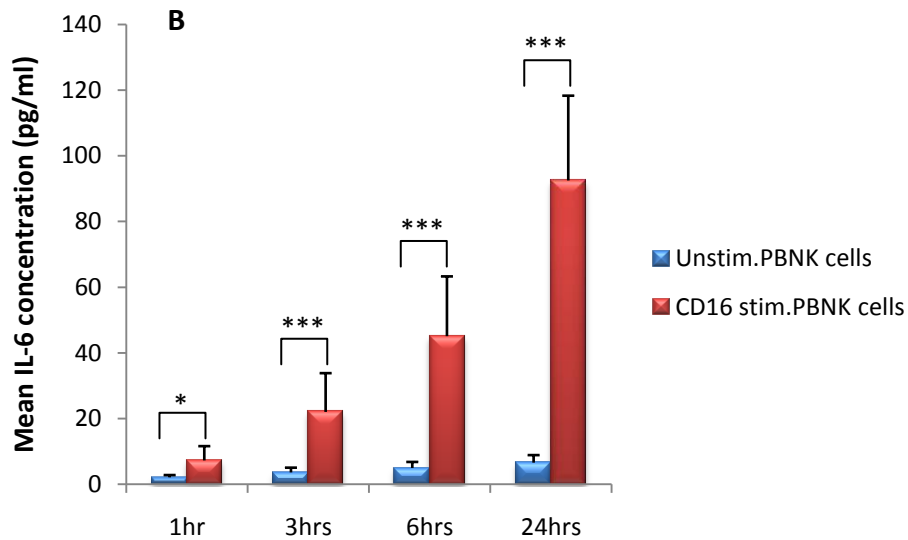
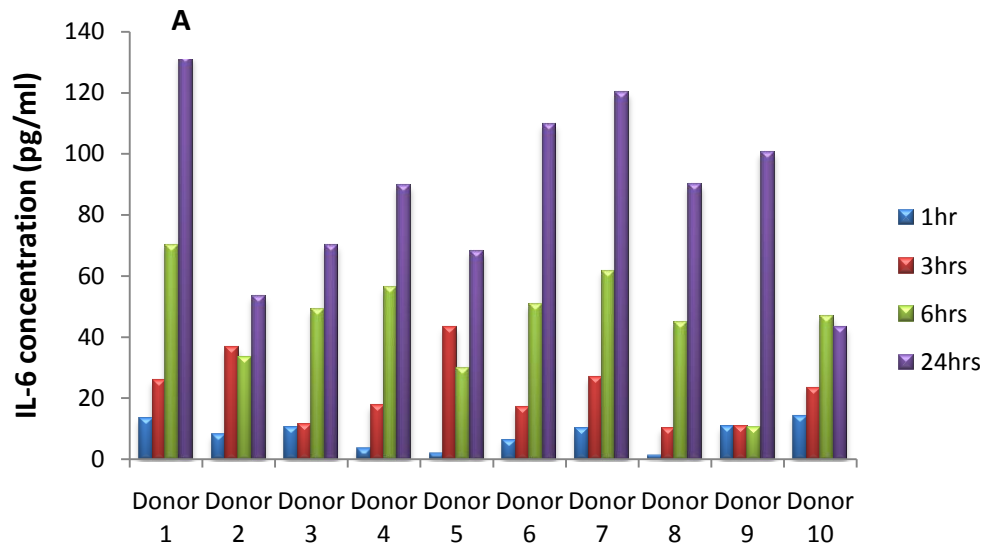


**Figure 4.5: Effect of CD16 stimulation on IL-6 gene expression by PBNK cells at transcriptional level.** Purified NK cells from 10 healthy donors were incubated with immobilized anti-CD16 antibodies (3G8) in a 96-well tissue culture plate. The NK cells were then collected at 1, 3, 6 and 24 hrs and analyzed in triplicates for IL-6 mRNA expression. In all samples, gene expression levels were normalized to GAPDH mRNA and relative quantification was performed using the  $\Delta\Delta CT$  method. **A)** In most cases, the most prominent increase in IL-6 mRNA expression was found at 6 hours (only two cases at 3 hours). **B)** The mean values and SD of the 10 independent experiments. IL-6 mRNA started to increase significantly at 3 hours ( $p < 0.05$ ) following stimulation. The most prominent increase was noticed at 6 hrs while a significant reduction of IL-6 mRNA expression was observed at 24 hours post stimulation ( $p < 0.05$ ). Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*,  $p < 0.05$



## **2. Engagement of CD16 induced IL-6 protein secretion by PBNK cells**

The IL-6 protein secretion by PBNK cells from the same donors used in the above experiment was also studied after exposure of these cells to immobilized CD16 mAb as described above. The quantity of IL-6 released by these cells into the medium was then determined by enzyme-linked immunosorbent assay at 1, 3, 6 and 24 hours (Figure 4.6A). It was found that the engagement of CD16 receptors on the surface of NK cells by their mAb significantly enhanced IL-6 synthesis in a time-dependent fashion. By 1 hour after exposure CD16 stimulated cultures synthesized almost 4 times more IL-6 ( $7.28 \pm 4.34$ ) than was produced by identical cultures of non-stimulated NK cells ( $2.11 \pm 0.70$ ),  $p = 0.010$  (Figure 4.6 B). At 3 hours post stimulation, the amount of synthesized IL-6 was increased up to 22 pg/ml ( $22.04 \pm 11.79$ ) in comparison to less than 4 pg/ml ( $3.62 \pm 1.45$ ) seen without stimulation ( $p < 0.001$ ). The secretion of IL-6 by CD16 activated NK cells continued to increase thereafter and reached about 48 % of maximum at 6 hours. However, in contrast to IL-6 mRNA expression which inhibited at 24 hours, the IL-6 protein secretion showed a maximum at 24 hours post stimulation ( $92.48 \pm 25.80$ ).

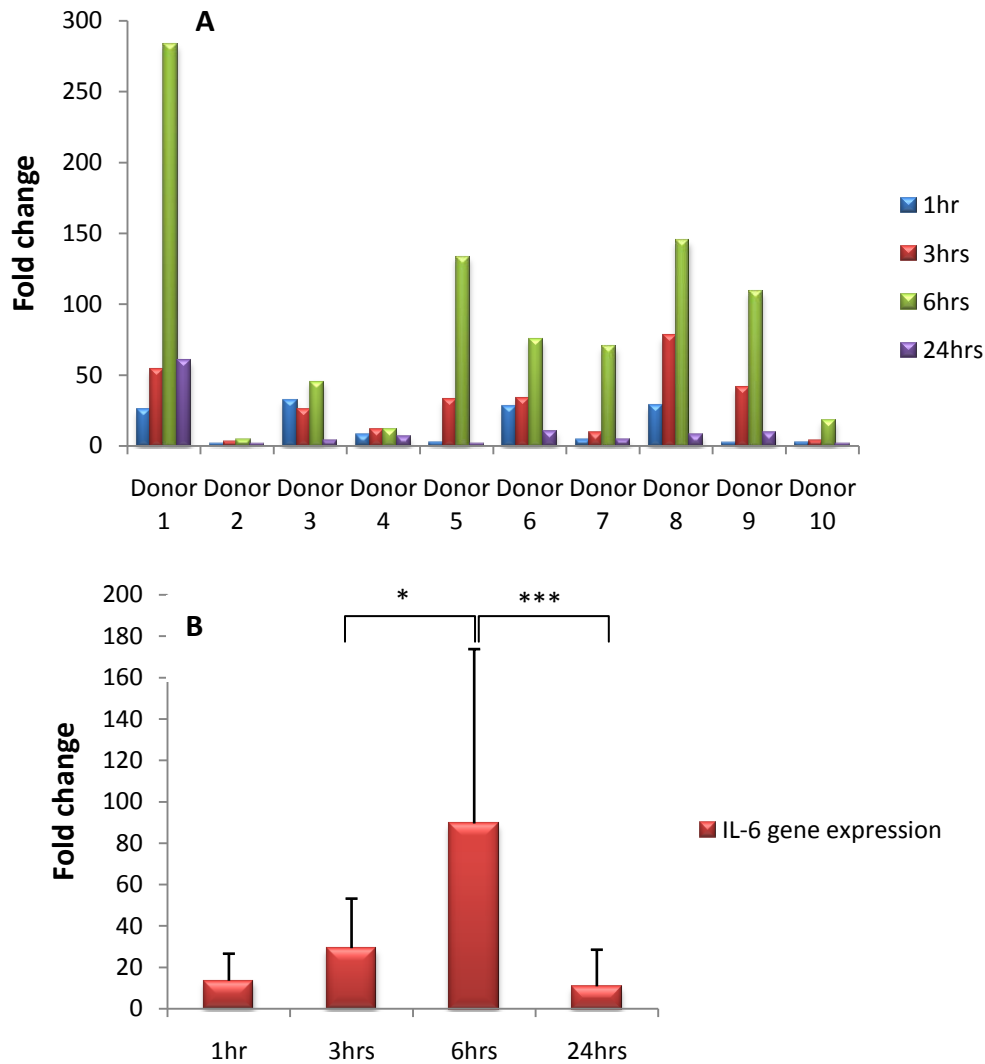


**Figure 4.6: IL-6 protein secretion by CD16 activated PBNK cells.** **A)** The PBNK cells from 10 healthy donors (same donors as in real-time PCR experiment) were purified by negative magnetic selection and then stimulated with immobilized anti-CD16 mAb in a 96-well plate. IL-6 accumulation in the medium was measured by ELISA at 1, 3, 6 and 48 hours after stimulation. **B)** The mean values and S.D. of 10 determinations from 10 independent experiments are shown and compared to mean values obtained from resting NK cells from same donors. Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Significant difference at  $p < 0.05$ .

#### **4.3.3.2 Stimulation via NKG2D**

##### **1. Engagement of NKG2D receptors induced a greater increase in IL-6 transcript level in PBNK cells compared to CD16**

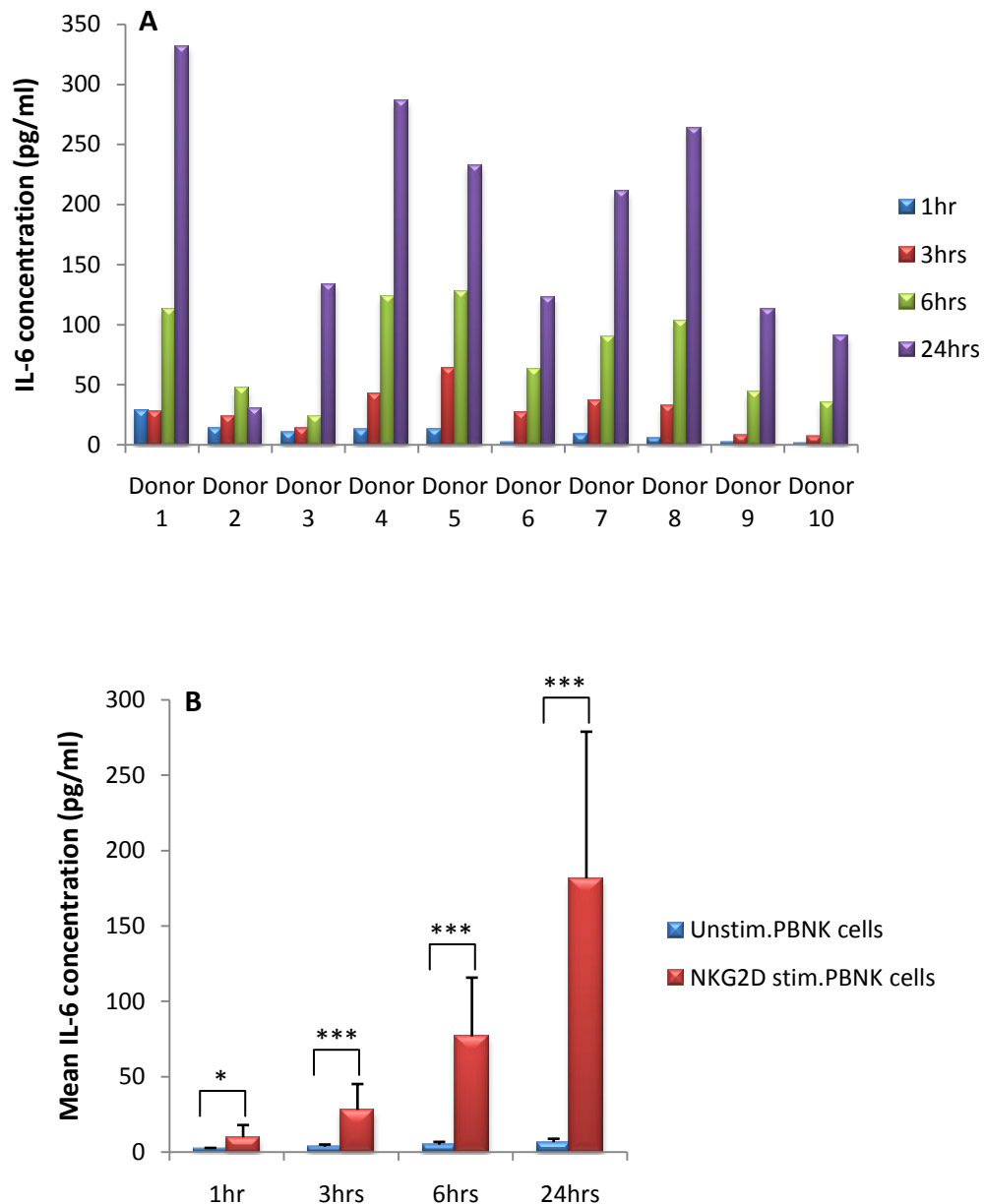
We then assessed the effect of the engagement of another NK receptor, NKG2D, on IL-6 mRNA expression (Figure 4.7 A). Purified PBNK cells from 10 healthy donors were cultured in 96-well tissue culture plate in the presence of immobilized anti-NKG2D mAbs as described in Section 4.2.1 and 4.2.2. The IL-6 transcripts synthesized by these cells were determined by real-time PCR at 1, 3, 6 and 24 hours using the same experimental approach as with CD16. Despite some inter-individual variations, activation of NK cells through NKG2D receptors produced more IL-6 transcripts than CD16 at all 4 time points. In all cases, the IL-6 transcripts were up-regulated as early as 1 hour ( $13.46 \pm 13.08$  fold greater than resting), increased further at 3 hours ( $29.20 \pm 23.95$  fold greater than resting ) and reached a maximal level at 6 hours ( $89.46 \pm 84.24$  fold greater than resting ) (Figure 4.7 B) . Although NKG2D engagement resulted in the production of more IL-6 transcripts ( $29.20 \pm 23.95$  fold greater than resting) than CD16 engagement ( $22.3 \pm 17.65$  fold greater than resting) at 3 hours, no significant statistical difference was found between 1 and 3 hours ( $p = 0.085$ ). This was due to the fact that NKG2D induce more IL-6 transcripts at 1 hour compared to CD16. However, IL-6 gene expression significantly increased at 6 hours ( $p= 0.043$ ). Again, the expression of the transcript appeared to be transient as the mRNA expression was profoundly lower by 24 hours ( $10.76 \pm 17.7$  fold greater than resting,  $p < 0.001$ ).



**Figure 4.7: The effect of NKG2D stimulation on IL-6 gene expression by PBNK cells.** After purification NK cells from each donor (n=10) were incubated in the presence of an immobilized anti-NKG2D mAb in a 96-well tissue culture plate. The NK cells were then collected at 1, 3, 6 and 24 h and analyzed for IL-6 mRNA expression in triplicates. Results represent the relative fold increase over resting (non-stimulated) PBNK cells in the 10 individuals. Gene expression levels were normalized to GAPDH and relative quantification was performed. **A)** Results of real-time PCR. The most prominent increase in IL-6 mRNA expression was found at 6 hours. **B)** The mean values and SD of the 10 experiments. IL-6 mRNA increased significantly by 6 hours ( $p < 0.05$ ) but started to return to baseline at 24 hours post stimulation ( $p < 0.001$ ). Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*,  $p < 0.05$  ; \*\*\*,  $p < 0.001$

## **2. NKG2D engagement markedly stimulated IL-6 protein secretion by PBNK cells**

Conditioned media of cultured PBNK cells from same 10 healthy donors as in the real time experiment stimulated with immobilized anti-NKG2D mAb were also assayed for the production of IL-6 protein at 1, 3, 6 and 24 hours by ELISA (Fig 4.8A & B). PBNK cells cultured in medium alone produced negligible amount of IL-6 after 1 hour of incubation ( $2.11 \pm 0.70$ ). Cross-linking of NKG2D significantly increased IL-6 production to  $9.89 \pm 8.09$  pg/ml ( $p = 0.0173$ ). A continuous significant response was observed after incubation for 3 hours, in which IL-6 production increased to  $28.04$  pg /ml  $\pm 17.12$  compared to resting NK cells ( $3.62 \pm 1.45$ ,  $p < 0.001$ ). At 6 hours conditioned medium from PBNK cells stimulated with anti-NKG2D mAb possessed significantly greater IL-6 activity ( $76.77 \pm 38.95$ ) than that of un-stimulated PBNK cells ( $4.97 \pm 1.84$ ,  $p < 0.001$ ). In contrast to IL-6 mRNA expression, 24 hours of stimulation with antibodies elicited maximum levels of IL-6 protein ( $181.47 \pm 97.33$ ), which was highly significantly different from non-stimulated cells ( $6.589 \pm 2.30$ ,  $p < 0.001$ ). The amount of IL-6 induced by anti-NKG2D antibodies, however, was substantially more than that elicited by anti-CD16 at all four time points.



**Figure 4.8: The effect of NKG2D engagement on IL-6 secretion by PBNK cells.** **A)** NKG2D-stimulated IL-6 secretion by purified PBNK cells (n = 10).The tissue culture plate was pre-treated with 10  $\mu$ g/ml of anti-NKG2D mAb for 24 hours. Supernatants were collected from a 96 well tissue culture plate at 1, 3, 6 and 24 hours after the addition of the PBNK cells and IL-6 levels were determined by ELISA. **B)** Results from the 10 samples expressed as the mean  $\pm$  SD compared to mean  $\pm$  SD values obtained from resting NK cells from same donors. Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*\*, p < 0.01; \*\*\*,p < 0.001. Significant difference at p < 0.05.

### **4.3.3.3 Stimulation via NKp46**

#### **1. NKp46 stimulation induced IL-6 expression comparable to that seen with NKG2D**

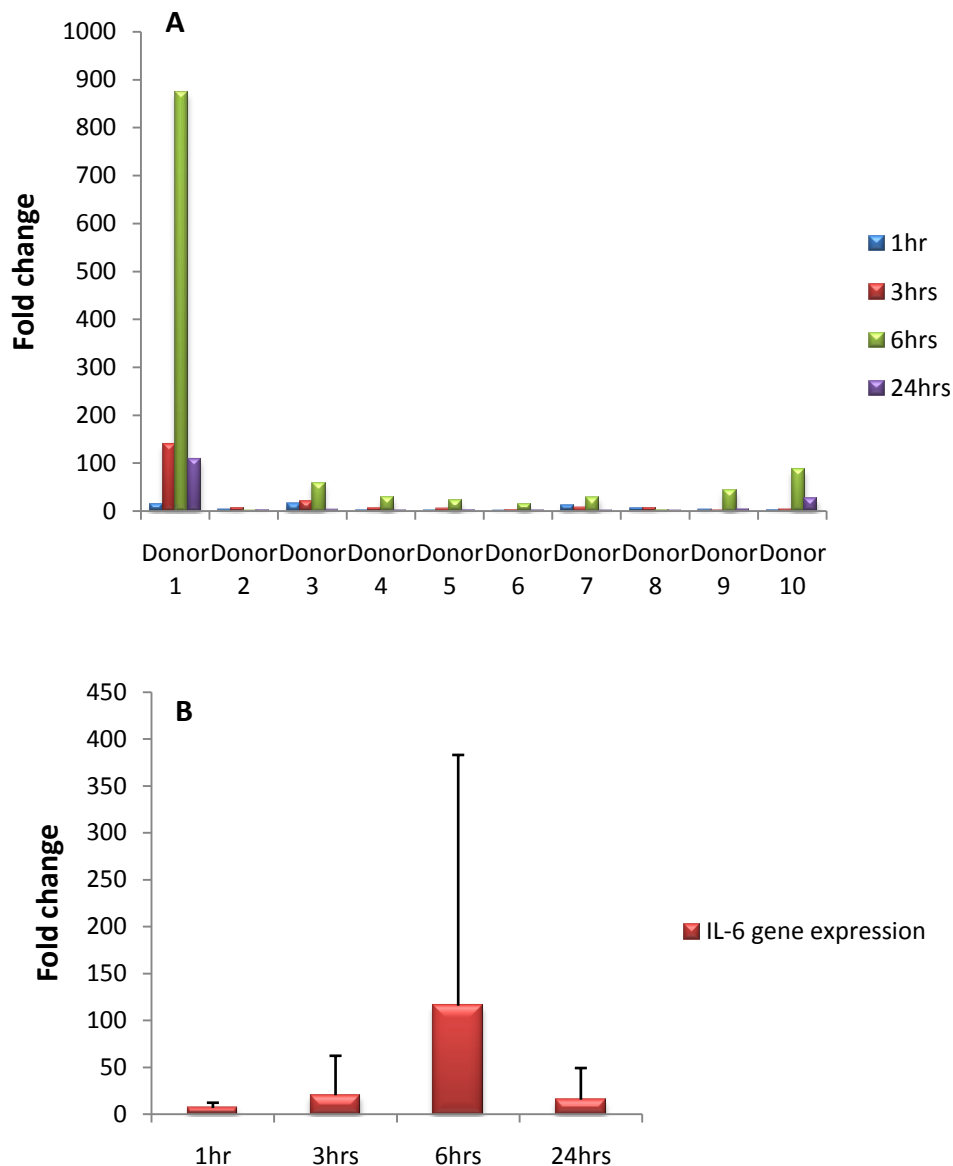
Purified PBNK cells from 10 healthy donors were stimulated with immobilized anti-NKp46 mAb and the IL-6 mRNA expression was assessed by real-time PCR as described previously (Figure 4.9 A). Similar to the results of the anti-CD16 and anti-NKG2D stimulation experiments, the induction of IL-6 mRNA was very rapid, reaching 18% of maximum at 1 hour post-stimulation ( $6.56 \pm 5.55$  fold greater than resting) and peaking between 3 ( $20.12 \pm 42.03$  fold greater than resting) and 6 hours ( $115.58 \pm 267.42$  fold greater than resting) (Figure 4.9 B). IL-6 mRNA levels by 24 hours were lower, comparable to those seen at 1 hour ( $15.42 \pm 33.68$ ) (Figure 4.9 B). No statistically significant difference was found in IL-6 mRNA expression between 1 and 3 hours ( $p = 0.33$ ), 3 and 6 hours ( $p = 0.28$ ), and 6 and 24 hours ( $p = 0.26$ ) respectively. However, it should be noted that production by donor 1 was much higher than anyone else in all experiments and this sample was clearly an outlier.

#### **2. NKp46 cross-linking also induced IL-6 protein production**

After stimulation with immobilized anti-NKp46 mAb, the supernatants from the cultured PBNK cells were harvested at 1, 3, 6 and 24 hours and analyzed for IL-6 protein secretion (Figure 4.10 A). A significant detectable level of IL-6 protein was detected at 1 hours post stimulation ( $12.34 \pm 5.76$ ) in comparison to ( $2.11 \pm 0.70$ ) in non-stimulated cultures,  $p < 0.01$  (Figure 4.10 B). With increasing exposure IL-6 cytokine-secretion progressively increased toward ( $29.49 \pm 26.11$ ) at 3 hours and ( $73.16 \pm 40.03$ ) at 6 hours in comparison to ( $3.62 \pm 1.45$ ) and ( $4.97 \pm 1.84$ ) in non-

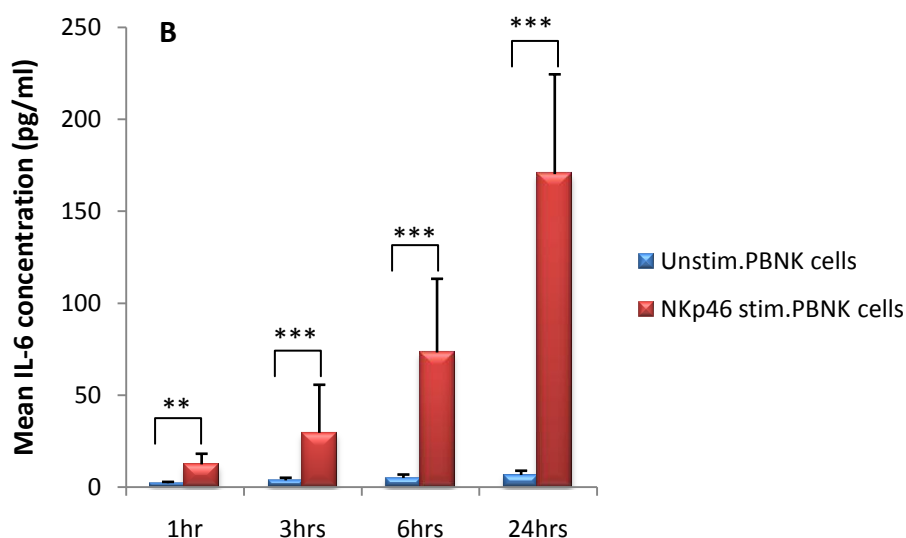
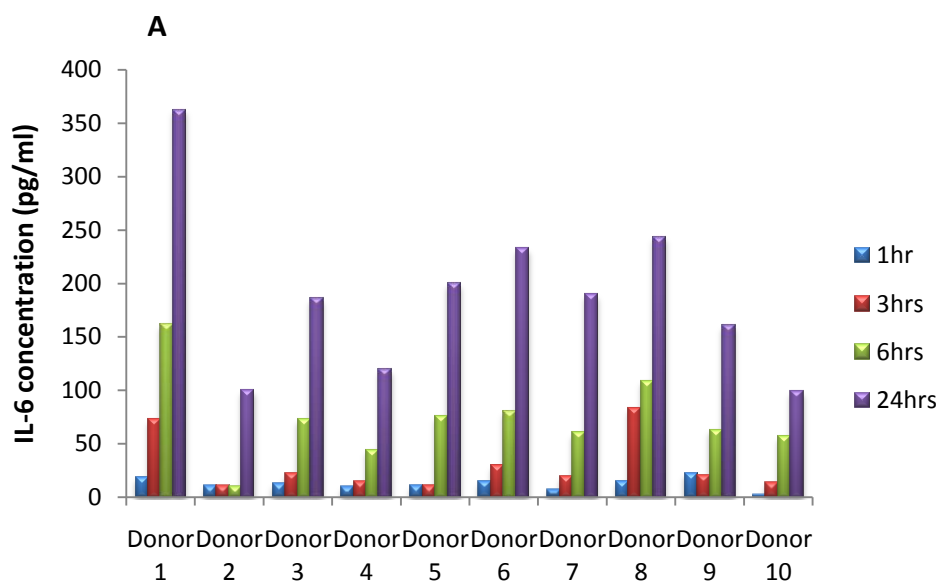
stimulated cells respectively ( $p < 0.001$ ). Similar to the observations with CD16 and NKG2D stimulation, the IL-6 protein secretion reached a peak after 24 h of culture with  $170.09 \text{ pg/ml} \pm 54.24$  in comparison to  $6.59 \pm 2.30$  by non-stimulated cells ( $p < 0.001$ ) (Figure 4.10 B).





**Figure 4.9: IL-6 mRNA expression in PBNK cells after NKp46 stimulation.**

Purified PBNK cells established from 10 healthy donors were incubated in 96-well tissue culture plate in the presence of an immobilized anti-NKp46 mAb (10 $\mu$ g/ml). IL-6 and GAPDH mRNA expression levels were determined (in triplicates) by real-time PCR and compared to those obtained from non-stimulated (resting) NK cells. **A)** Results of real-time PCR quantitation in individual donors. Maximum level of induced cytokine mRNA was seen at 6 hours. **B)** The mean and SD values of the maximum levels of 10 healthy donors. A return towards baseline IL-6 mRNA expression was observed at 24 hours post stimulation.



**Figure 4.10: Kinetics of IL-6 protein production by PBNC cells after NKp46 stimulation.** Purified PBNC cells from 10 healthy donors were cultured in a 96-well plate pre-treated with anti-NKp46 mAb at a concentration of 10 $\mu$ g/ml. The anti-NKp46 mAb was allowed to immobilize on the surface of plate for 18 hrs prior to addition of purified PBNC cells. Supernatants were collected at 1, 3, 6 and 24 after the stimulation. Supernatants from non-stimulated PBNC cells from same donors were taken at the same time points and used as negative control. **A)** IL-6 production by PBNC cells was detectable after 1 hr stimulation with mAb and peaked at 24 hrs. **B)** Mean and SD from all 10 tested individuals compared to non-stimulated samples at the times indicated. IL-6 production by NKp46 stimulated PBNC cells significantly increased as early as 1 hr compared to non-stimulated cells. Error bars denote the SD; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Significant difference at  $p < 0.05$

#### **4.3.3.4 Stimulation via NKp30**

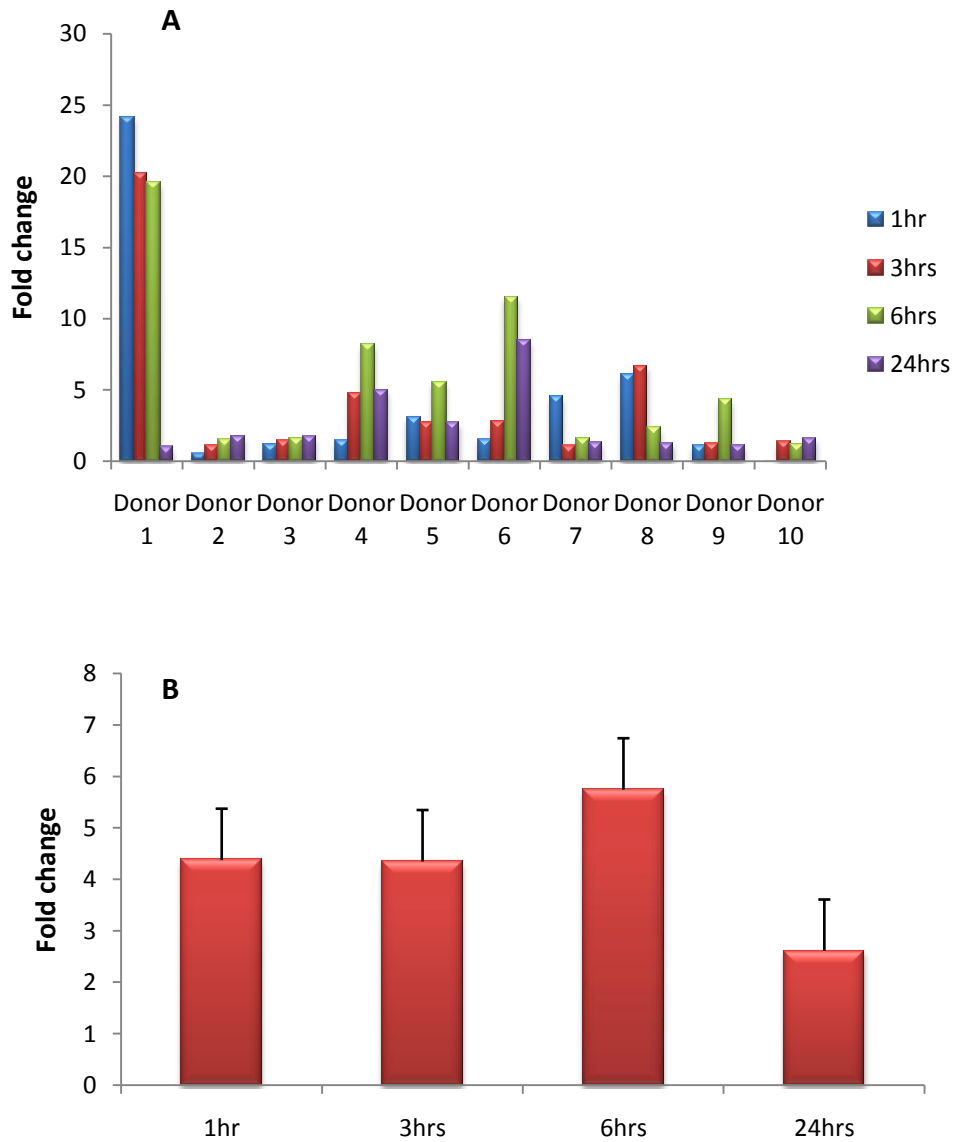
##### **1. NKp30 was less efficient than CD16, NKG2D or NKp46 in stimulating IL-6 mRNA expression**

The effect of the engagement of NKp30 receptors on PBNK cells is shown in Figure 4.11 A and B. Crosslinking NKp30 induced detectable levels of IL-6 mRNA about 4-fold higher than resting at 1 hour ( $4.37 \pm 7.19$ ). However, there was no significant further increase at 3 hours ( $4.35 \pm 5.87$  fold greater than resting,  $p > 0.05$ ) or 6 hours in IL-6 transcript levels ( $5.74 \pm 5.91$  fold change over resting,  $p > 0.05$ ). As with other receptors, IL-6 mRNA expression started to return to baseline levels at 24 hours ( $2.60 \pm 2.38$  fold greater than resting); a feature that seems to be consistent with observations made with all other receptors tested.

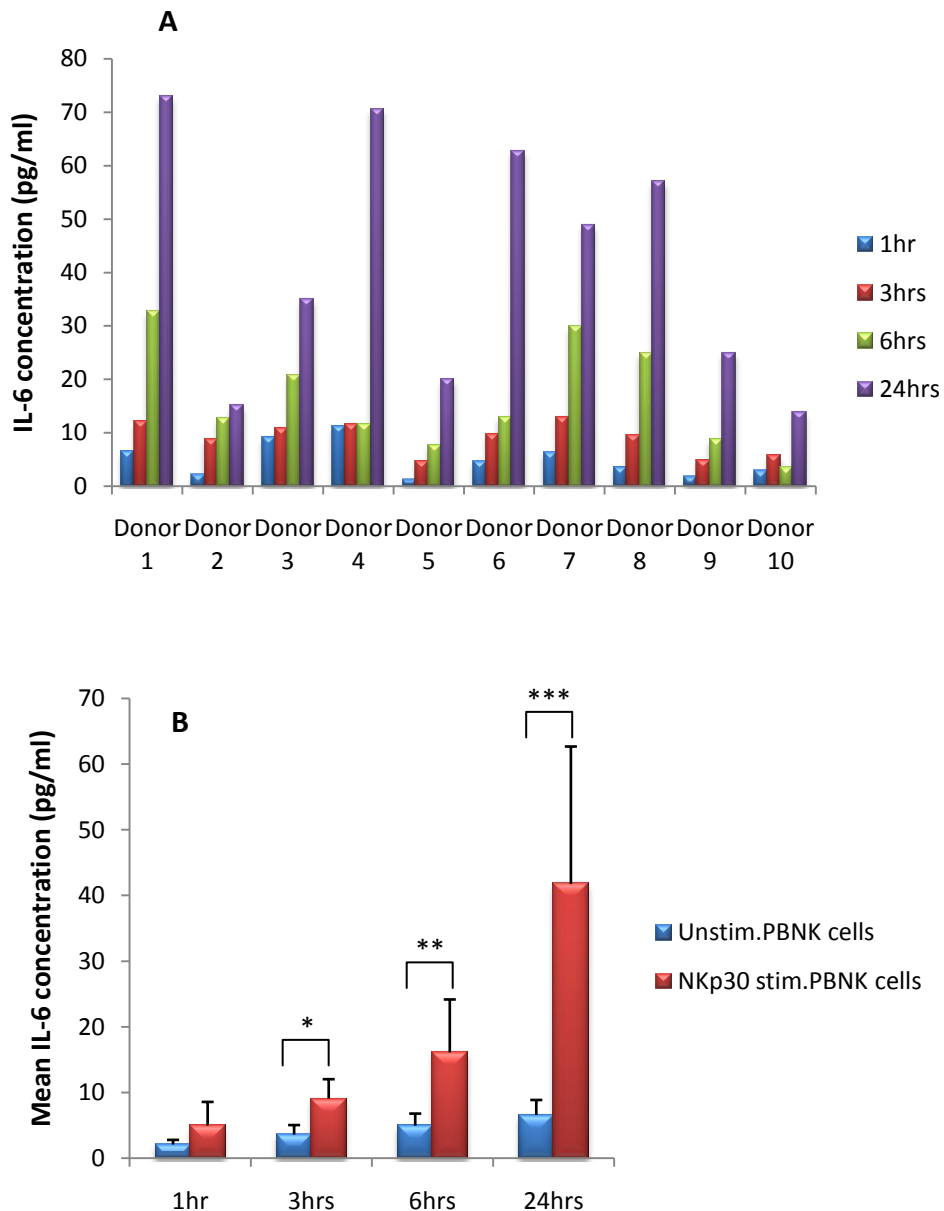
##### **2. A smaller increase in IL-6 transcript levels following NKp30-activation resulted in a limited increase in IL-6 protein secretion**

To analyze whether the considerably reduced IL-6 mRNA response elicited by anti-NKp30 activation affected the cytokine protein secretion profile of these cells, supernatants were collected at the same 4 time intervals and analyzed for IL-6 protein by ELISA (Figure 4.12 A). Although the general pattern of protein secretion was still maintained and increased over time, the lower level of IL-6 transcripts resulted in reduction in the secreted IL-6 protein in NKp30-activated PBNK cell cultures (Figure 4.12 B). Nevertheless, these reduced levels of IL-6 proteins were still higher than those obtained from resting cells at all 4 time points. One hour post stimulation, the mean IL-6 concentration ( $4.98 \pm 3.61$ ) was higher, although not statistically different, than without stimulation ( $2.11 \pm 0.70$ ,  $p > 0.05$ ). Activation for 3 hours with mAb resulted in significantly increased IL-6 concentration ( $9.11 \pm 2.93$ ) as compared to the

control samples ( $p < 0.05$ ). PBNK cells stimulated with mAb for 6 hours showed a greater increase in IL-6 secretion ( $16.12 \pm 8.06$ ) than untreated PBNK cells ( $4.97 \pm 1.8$ ,  $p < 0.01$ ). A peak in IL-6 levels was observed in anti-NKp30- stimulated NK cells at 24 hours ( $41.75 \pm 20.92$ ); a level which was highly significantly different from the non-activated control samples ( $6.59 \pm 2.30$ ,  $p < 0.001$ ).



**Figure 4.11: Effect of NKp30 stimulation on IL-6 mRNA expression by PBNK cells.** Purified PBNK cells from 10 healthy donors were cultured in the presence of immobilized anti-NKp30 mAb (10 $\mu$ g/ml) for the indicated time periods. **A)** Real-time PCR results from 10 individual donors. **B)** The combined mean and SD values from all 10 samples. Compared to anti-CD16, NKG2D and NKp46, anti-NKp30 induced markedly lower increase in IL-6 transcripts. No significant change was found in IL-6 mRNA expression at any of the 4 time points tested.

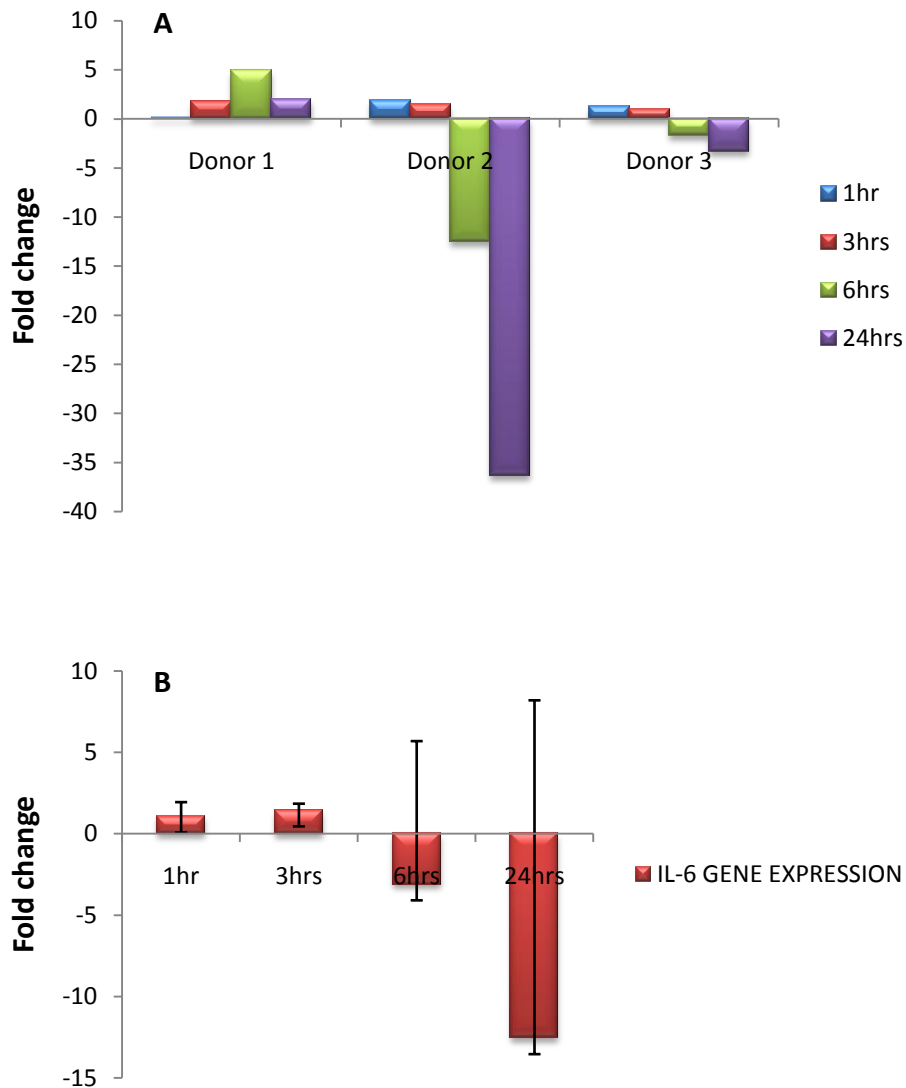


**Figure 4.12: IL-6 secretion by NKp30-activated PBNK cells.** The PBNK cells (n=10) were cultured for 24 hours in 96-well plate in the presence of immobilized anti-NKp30 mAb (10 $\mu$ g/ml). Cell supernatants were collected at the indicated time points to measure the IL-6 concentration by ELISA. Supernatants from resting PBNK cells from the same donors at the same time points were taken as controls. **A)** Results of ELISA experiments from the 10 individual donors. **B)** Mean and SD of the 10 determinations compared to mean and SD obtained from controls. Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \* p < 0.05 \*\* , p < 0.01; \*\*\*, p < 0.001. Significant difference at p < 0.05.

#### **4.3.3.5 Stimulation via NKp44**

##### **1. Cross-linking of NKp44 induced different IL-6 mRNA profiles by NK cells**

The NKp44 receptor is a marker of IL-2 activated NK cells and its expression is confined to these pre-stimulated cells (Vitale et al, 1998). In order to study the effect of the engagement of this receptor on the expression of IL-6 mRNA by PBNK cells, purified PBNK cells from 3 donors were pre-activated for 24 hours with human recombinant IL-2 (hrIL-2) at a dose of 100 U /ml. Following this activation, PBNK cells were washed with 1X PBS and cultured in 96-well plates in the presence of an immobilized anti-NKp44 mAb. Thereafter, the cells were collected at 1, 3, 6 and 24 hours and investigated for IL-6 mRNA expression by real-time PCR. The profile of the accumulation of mRNA encoding the IL-6 was different from that seen in the experiments studying the effects of the other receptors. mAb- mediated cross-linking of NKp44 resulted in a down-regulation of IL-6 mRNA levels rather than increased expression in two of the three volunteers studied (Figure 4.13 A). After 1 hour stimulation, the mean IL-6 transcripts in NKp44-stimulated NK cells showed only two-fold increase over non-stimulated NK cells ( $1.07 \pm 0.86$ ) (Figure 4.13 B). At 3 hours post stimulation, there was no significant further increase ( $1.44 \pm 0.39$  fold greater than resting,  $p = 0.53$ ) while cultures at 6 hours after mAb stimulation clearly demonstrated a decrease in IL-6 mRNA levels ( $-3.10 \pm 8.78$ ) followed by a further decrease at 24 hours ( $-12.55 \pm 20.74$  fold less than control values). However, it is important to mention that these experiments were carried out on cells from only three volunteers and these results may need to be confirmed further.

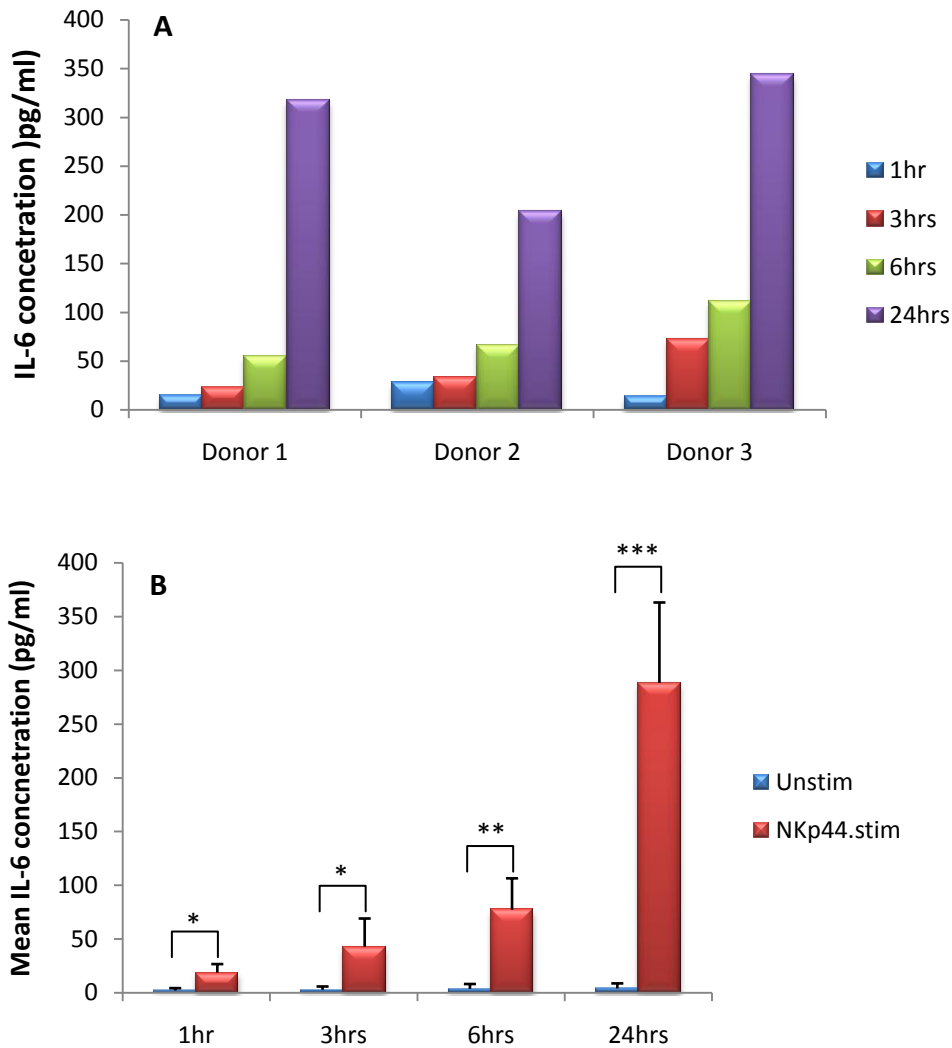


**Figure 4.13: Analysis of IL-6 mRNA expression in NKp44-activated PBNK cells.** PBNK cells from 3 health donors were purified by negative magnetic selection and activated for 24 hours with rhIL-2 at a dose of 100 U/ml. After activation NK cells were washed with 1X PBS and added to 96-well plate at a concentration of  $1 \times 10^5$  cells / well in the presence of immobilized anti-NKp44 mAb (10  $\mu\text{g}$  / ml). Thereafter the cells were collected at 1, 3, 6 and 24 hours and assayed for IL-6 mRNA expression by real-time PCR. **A)** Fold change of NKp44 mRNA levels over control conditions (standardized using the internal reference GAPDH gene) in 3 healthy donors. Two out of the three donors showed down-regulation of IL-6 transcripts over time. **B)** Bars represent mean and SD of the changes from the above three donors at the specific time points indicated. Results demonstrated down-regulation rather than an increased expression with maximum inhibition seen at 24 hours.



## **2. IL-6 protein levels in NKp44- activated NK cells did not run in parallel with IL-6 mRNA measurements**

To verify whether the IL-6 down-regulation at the mRNA level in NKp44-activated PBNK cells was associated with similar alteration of IL-6 bioactivity, the protein concentrations of IL-6 in the supernatant from the same 3 healthy donors were measured at 1, 3, 6 and 24 hours (Figure 4.14 A & B). The results showed that the PBNK cells still secreted high amounts of IL-6 following NKp44 stimulation. One hour post stimulation (when the IL-6 transcription level was only two fold greater than control), IL-6 concentration was about 9 times higher in activated NK cells ( $18.65 \pm 7.90$ ) than in the resting group ( $2.2 \pm 2.23$ ),  $p = 0.022$ . At 3 hours (when the IL-6 mRNA expression in NKp44 activated NK cells was not significantly changed), a consistent significant increase in IL-6 was observed ( $42.72 \pm 26.21$ ) compared to the control resting PBNK cells cultures ( $2.56 \pm 3.12512$ ),  $p = 0.015$ . In contrast to the expression profile of IL-6 mRNA which was down-regulated at 6 hours, the IL-6 protein level continued to increase reaching  $77.03 \pm 29.29$ . This level was prominently higher than the resting level ( $3.44 \pm 4.55$ ),  $p < 0.01$ . The highest level of induction for IL-6 protein was observed at the late stage of stimulation i.e. 24 hours with  $288.05 \pm 74.88$  pg/ml (significantly higher than non-stimulated NK cells,  $p < 0.001$ ). Thus, our results indicated that the cytokine protein levels did not run in parallel with the corresponding mRNA transcription levels throughout the time course examined. This discrepancy was particularly pronounced at 6 and 24 hours.



**Figure 4.14: IL-6 production by PBNK cells after stimulation with immobilized anti-NKp44 mAb.** Human PBNK cells were isolated from PBMCs of 3 healthy donors by negative selection and activated for 24 hours in the presence of 100 U / ml of recombinant IL-2 human (rhIL-2) followed by incubation in the presence of anti-human NKp44 immobilized mAbs at a concentration of 10  $\mu$ g / ml in a 96 well plate. **A)** Results of the ELISA experiments from the 3 individual donors. **B)** Mean and SD values compared to the mean and SD obtained using control cells. Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Significant difference at  $p < 0.05$ .

#### **4.3.4 Direct comparison of IL-6 mRNA expression and protein secretion following activation of PBNK cells via the CD16, NKG2D, NKp46, NKp44 and NKp30 receptor pathways**

In the previous experiments IL-6 gene expression and protein production were measured in cultured PBNK cells (from healthy donors) following *in vitro* mAb cross-linking of CD16, NKG2D, NKp46, NKp44 and NKp30 receptors. Here we summarised the results from direct comparison of our data. When comparing the different receptors with each other, four features were evaluated: the time of induction, the time and the level of maximal accumulation, and the time of inhibition. The time of induction is the first measurable response to stimulation and is defined here by the earliest point in time (within the time frame utilized in this study) with a statistically significant increase in the levels of IL-6 mRNA or protein compared to non-stimulated cells at the same time point. With all receptors studied IL-6 transcripts were detected as early as 1 hour following stimulation (Figure 4.15 A). At this time point anti-NKG2D was the strongest inducer of IL-6 mRNA expression with a more than 13 fold increase ( $13.46 \pm 13.08$ ) followed by anti-NKp46 with more than 6 fold increase ( $6.56 \pm 5.55$ ). Stimulation via CD16 and NKp30 resulted in only a moderate induction of IL-6 mRNA expression with  $3.4 \pm 2.3$  and  $4.37 \pm 7.19$  fold increase respectively. NKp44 cross-linking had only a two fold increase at this time point ( $1.07 \pm 0.86$  fold of control levels).

Maximum expression was observed at 6 hours in all receptors except NKp44. Here anti-NKp46 showed the highest mean levels of IL-6 mRNA ( $115.58 \pm 267.42$  fold increase) followed by anti-NKG2D ( $89.46 \pm 84.24$  fold). CD16 stimulation resulted in a less pronounced increase with  $44.58 \pm 47.80$  fold greater than resting cells. NKp30 binding resulted in only a very small increase ( $5.74 \pm 5.91$ ) while

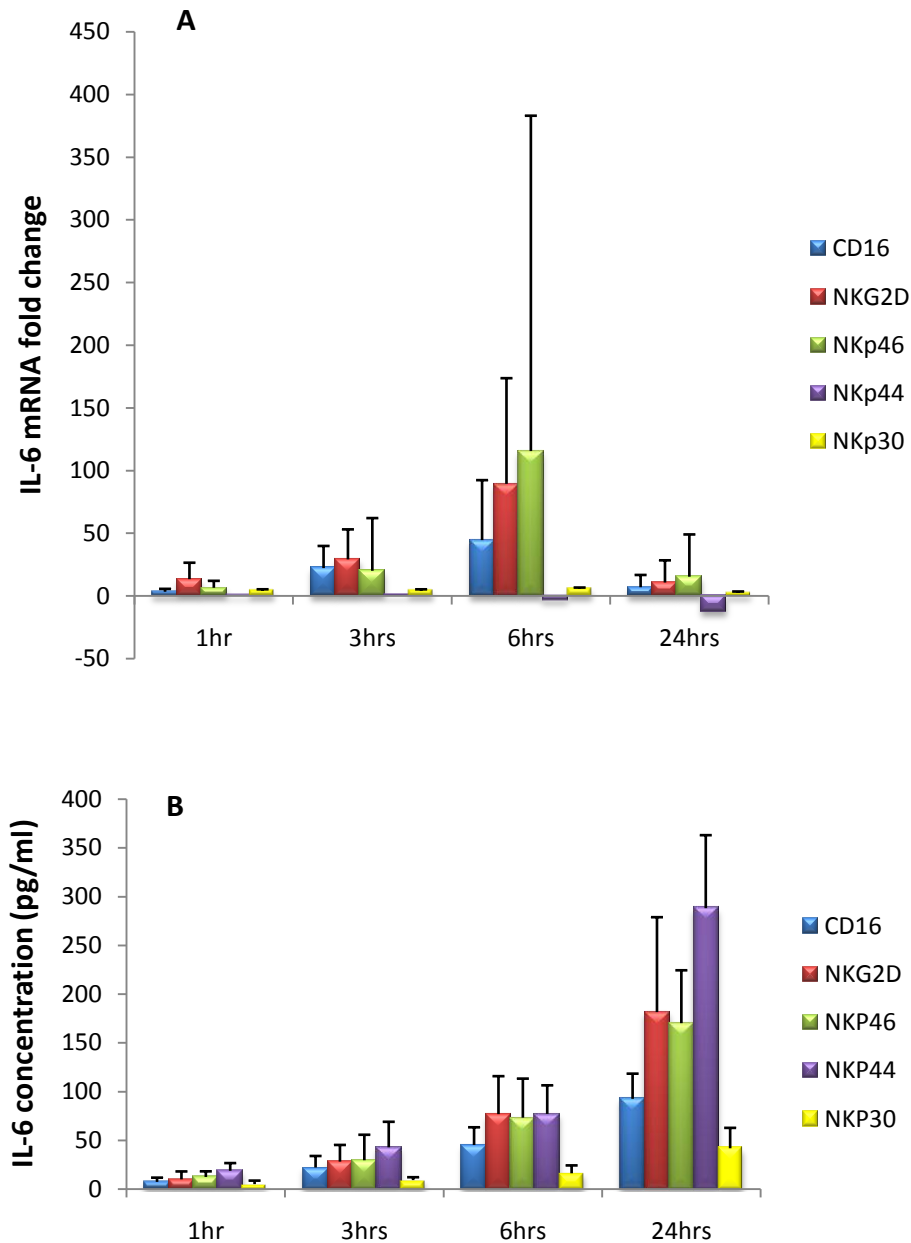
anti-NKp44 induced a different expression profile as it down-regulated IL6 transcripts at 6 hours ( $-3.10 \pm 8.78$ ). With the exception of NKp44 where only 3 donors were examined, the data presented above were derived from the mean  $\pm$  SD of the 10 different donors. When the 10 individuals were compared, there were some differences in maximum production of IL-6 mRNA between NKG2D and NKp46 at 6 hours. While anti-NKp46 stimulated maximum mRNA expression in only 3 individuals, anti-NKG2D stimulated maximum mRNA expression by NK cells in 7 donors (Table 4.1).

**Table 4.1: Maximum IL-6 mRNA expression (fold change) by NKp46 and NKG2D at 6 hours**

	<b>NKG2D</b>	<b>NKp46</b>
<b>Donor 1</b>	283.38	873.09
<b>Donor 2</b>	4.44	1.22
<b>Donor 3</b>	45.25	57.81
<b>Donor 4</b>	11.92	28.70
<b>Donor 5</b>	133.43	21.45
<b>Donor 6</b>	74.88	14.25
<b>Donor 7</b>	70.19	27.28
<b>Donor 8</b>	144.66	86.22
<b>Donor 9</b>	108.63	43.41
<b>Donor 10</b>	17.75	2.31
<b>Average</b>	89.45	115.57

24 hours post stimulation IL-6 mRNA expression showed a tendency to return towards baseline values irrespective of the receptor stimulated. The most pronounced drop (compared to values at 6 hours) was observed with anti-NKp46 antibody followed by anti-NKG2D, anti-CD16 anti-NKp44 and NKp30 antibodies respectively.

Regarding protein secretion (Figure 4.15 B), detectable levels of IL-6 were observed as early as 1 hour post stimulation with all receptors studied. In these experiments anti-NKp44 was the strongest inducer of IL-6 secretion ( $18.65 \pm 7.90$ ) followed by anti-NKp46 ( $12.34 \pm 5.76$ ) and anti-NKG2D ( $9.89 \pm 8.10$ ) respectively. Anti-CD16 induced a smaller increase ( $7.26 \pm 4.34$ ), while anti-NKp30 only caused a small increase ( $4.98 \pm 3.61$ ). A similar consistent increase in IL-6 production was also observed with all receptors at 3 hours. At 6 hours following activation, anti-NKp44 still elicited the highest IL-6 concentration ( $77.03 \pm 29.29$ ). However, this was only marginally different from that observed with anti-NKG2D ( $76.77 \pm 38.95$ ) and anti-NKp46 ( $73.16 \pm 40.03$ ). In comparison anti-CD16 ( $45.09 \pm 18.20$ ) and anti-NKp30 ( $16.12 \pm 8.07$ ) were less potent stimulators. The most prominent increase of IL-6 production was observed at 24 hours in all receptors. Thus, anti-NKp44 stimulated the maximum IL-6 production by PBNK cells at all 4 time points. However, it should be pointed out that this receptor is a marker of IL-2 activated NK cells and the PBNK cells for testing the effect of this pathway were co-cultured with IL-2 for 24 hours before their activation with immobilized anti-NKp44 mAb.



**Figure 4.15: A) Comparison of IL-6 mRNA expression after stimulation via CD16, NKG2D, NKp46, NKp44 and NKp30 receptors. At 1 hour anti-NKG2D was the strongest inducer of IL-6 mRNA expression followed by anti-NKp46. Maximum expression was observed at 6 hours in all receptors studied except NKp44. IL-6 transcripts were returning towards baseline levels at 24 hours except NKp44 where diminishing levels of IL-6 mRNA were observed much earlier, at 6 hours. B) Comparison of IL-6 protein secretion by PBNK cells after stimulation via the different activating receptors at 1, 3, 6 and 24 hours. Anti-NKp44 stimulated maximum IL-6 protein secretion at all 4 time points.**

## **4.4 Discussion**

### **4.4.1 Receptor engagement and cytokine secretion**

NK cells recognize and destroy malignant and virally infected cells and respond to signals generated by activating and inhibitory receptors on their surfaces to constrain killing to appropriate target cells (Reefman et al, 2010). Such responses are not confined to cytotoxic effector mechanisms but also involve the secretion of cytokines and chemokines as immuno-defensive agents that additionally serve to activate resident inflammatory cells and recruit other cells to the site of inflammation. These NK cell-sourced cytokines can also regulate dendritic cells (Zhang et al, 2006), T cells (Scharton et al, 1993; Vankayalapati et al, 2004), and B cells (Zhang et al, 2006). Thus, cytokine production by NK cells influences the innate and shapes the subsequent adaptive immune responses. Although cytokine secretion in response to soluble mediators has been extensively studied, little is known about how cytokines are secreted by NK cells in response to specific engagement of NK cell receptors and what are the minimal requirements for cytokine secretion upon engagement of these receptors (Fauriat et al, 2010).

It was earlier shown that resting PBNK cells are induced to produce IL-6 upon target cell recognition (Chapter 3), although such production could also be induced with high doses of IL-2 or stimulation with phorbol diesters. The major goal of this chapter was to provide an experimental assessment of how specific involvement of different activating receptors, namely CD16, NKG2D, NKp46, NKp44 and NKp30 can regulate IL-6 mRNA expression and protein secretion by resting NK cells. To achieve this, NK cells were purified from fresh or short-term cultured PBMCs by immunomagnetic negative selection. As mentioned before, the purity of these cells was more than 95%. The purified NK cells were cultured in 96 tissue

culture plates in the presence of immobilized mAb to CD16, NKG2D, NKp46, NKp44 and NKp30. The cells and supernatants were collected at 1, 3, 6 and 24 hours and analysed for the IL-6 transcripts by real-time PCR and protein production by ELISA. This allowed identification of multiple pathways that modulate the release of IL-6 cytokine stimulated by these different receptors. The role of activating KIRs was not investigated mainly because of the unavailability of specific agonist KIR receptor antibodies. The data presented here show that cytokine secretion by resting NK cells (in the absence of exogenous cytokines) can be induced by activating receptors.

#### **4.4.2 Real-time PCR considerations: The choice of housekeeping gene**

Relative quantification with real-time PCR has been widely used to estimate the expression level of genes of interest. In this experimental approach the expression of a target gene is measured with respect to an internal reference gene (so-called housekeeping gene). The critical issue that defines the reliability of the obtained data is the choice of the housekeeping gene. The housekeeping gene must meet certain criteria before it can be used as an effective reference gene. Relative gene expression comparisons work best when the gene expression of the chosen endogenous / internal control is more abundant and remain constant in proportion to total RNA among the samples (Dorak, 2011). The most common, but not necessarily the most suitable, choices are housekeeping genes which code for components of ribosomal subunits (18S rRNA), components of the cytoskeleton ( $\beta$ -actin), components of the major histocompatibility complex ( $\beta$ 2-microglobulin), and enzymes of the glycolytic pathway (GAPDH) (Bas et al, 2004).

18S rRNA is a structural RNA for the small component of eukaryotic cytoplasmic ribosomes (Nazar, 2004), one of the basic components of all eukaryotic



cells. Although this gene was considered the most stable housekeeping gene used for normalization in comparative analyses of mRNA expression in some human blood cells such as T lymphocytes (Bas et al, 2004), several factors limit its use in this study. Firstly, because the 18S rRNA samples lack poly-A tails, so they are not reversed transcribed by the oligo dT method (Zhu and Altmann, 2005) that was used for cDNA synthesis in this study. Secondly, as the 18S rRNA is a ribosomal RNA (not mRNA), therefore it may not always represent the overall cellular mRNA population. Thirdly, since it is abundantly expressed, 18S rRNA yields very small (<15) Ct values which is not desirable, especially when the target gene has a low expression level (Dorak, 2011). Lastly, because of the absence of introns in the 18S rRNA molecule, this gene is also susceptible to false results from contaminating genomic DNA.

The  $\beta$ -actin is one of the two non-muscle cytoskeletal actins that are involved in cell motility, structure and integrity (Ng et al, 1985). The  $\beta$ -actin gene is frequently used for determination of the mRNA content and the efficiency of reverse transcription in analysed samples. However, it has been shown that the human genome contains processed  $\beta$ -actin pseudogenes (Ng et al, 1985; Ullmannová and Haskovec, 2003), which can result in the amplification of fragments from genomic DNA displaying the same size as those generated from the cDNA template. Therefore,  $\beta$ -actin is not a suitable control gene for reverse-transcription polymerase chain reaction (RT-PCR) (Selvey et al, 2001). Additionally, similarly to 18S rRNA,  $\beta$ -actin is a highly expressed gene and yields a strong amplification signal even from very small template amounts (Ullmannová and Haskovec, 2003). Therefore, the use of such a control may lead to a wrong interpretation of PCR assays if the investigated gene is expressed at a markedly lower level (Ullmannová and Haskovec, 2003).

$\beta$ 2-microglobulin is commonly used as a housekeeping gene for quantitative RT-PCR. However, its expression level varies considerably between tissues. For example, it has been found that the expression of  $\beta$ 2-microglobulin was 112-fold higher in leukocytes compared with fetal brain indicating large tissue specific variations (Oselin et al, 2003). Although it was suggested that  $\beta$ 2-microglobulin was a good target for normalization of leukocyte mRNA expression (Vandesompele et al, 2002), this study did not take into consideration the differential expression of  $\beta$ 2-microglobulin in leukocyte subtypes. Oselin et al (2003) have demonstrated that the mean  $\beta$ 2-microglobulin expression in CD19<sup>+</sup> cells was significantly lower than in CD4<sup>+</sup>, CD8<sup>+</sup>, and CD56<sup>+</sup> cells. They concluded that the use of  $\beta$ 2-microglobulin for normalization of the target mRNA expression in different subsets of PBMCs could lead to misinterpretation of the data.

The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) catalyzes the oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate during glycolysis as well as the reverse reaction in tissues involved in gluconeogenesis (Zainuddin et al, 2010). Its RNA is universally expressed and therefore it is one of the most commonly housekeeping genes used in comparisons of gene expression data (Barber et al, 2005). However, as with  $\beta$ 2-microglobulin, the expression of GAPDH varies considerably between tissues (Vandesompele et al, 2002; Barber et al, 2005). Moreover, Suzuki et al (2000) have shown that its expression may be up-regulated in proliferating cells and therefore its use in cancer studies could be criticized.

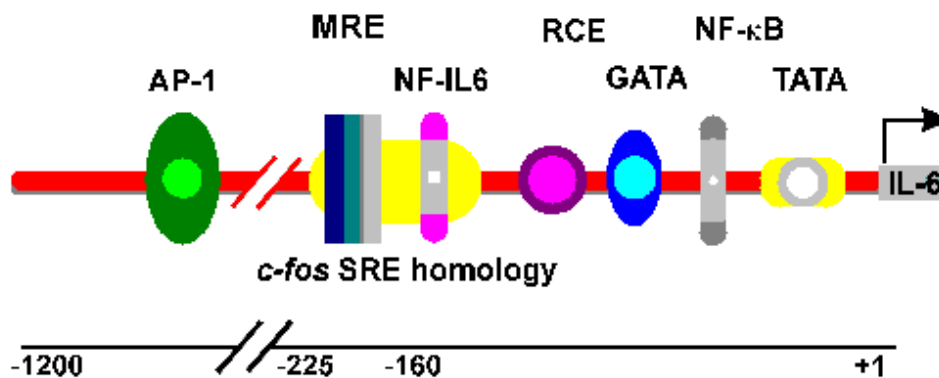
In addition to the variability in the level of expression of housekeeping genes among different tissues, it is also known that experimental treatment can also affect the expression of these genes. For example, the expression of  $\beta$ -actin increased up to nine-fold under all conditions of reverse transcription (Schmittgen and

Zakrajsek, 2000).  $\beta$ -actin expression also increased following treatment with insulin (Messina, 1992) but reduced after treatment with thyroid hormones (Savonet et al, 1997). Similarly, GAPDH expression is also influenced by treatment with insulin (Alexander et al, 1988), thyroid hormones and mitogenic agents such as epidermal growth factor (EGF) (Savonet et al, 1997). Moreover, the expression of 18S rRNA increased with serum-stimulation when the cDNA synthesized from non-normalized total RNA was assayed (Schmittgen and Zakrajsek, 2000).

There are no published papers about the validity of different housekeeping genes in NK cells. However, a search in the literature has shown that GAPDH was the most common housekeeping gene used to normalize real-time PCR data in NK cells. At least ten (but not all) recent publications used GAPDH as the housekeeping gene to normalize the real-time PCR data in PBNK cells (Takahashi et al ,2007 ; Duluc et al, 2009 ; Della Chiesa et al, 2010 ; Yamaguchi et al, 2010 ; De Maria et al, 2011; Textor et al, 2011 ; Negrini et al; 2011 ; Monaco-Shawver et al, 2011 ; Miah et al, 2011 ; Munk et al; 2011 , Vairo et al, 2011 ; Narni-Mancinelli et al, 2012 ; Durrenberger et al, 2012). Based on these publications it was decided to use GAPDH as internal reference gene (housekeeping gene) in this study. Furthermore, IL-6 mRNA expression by PBNK cells was studied at different time points and it has been found that GAPDH is the least affected housekeeping gene over time in comparison to other genes such as  $\beta$ -actin which shows a diurnal rhythm and its expression may increase up to five-fold after 12 hours (Bakkar and Timmer, 2004).

#### 4.4.3 IL-6 mRNA expression upon single receptor engagement

The results presented in this chapter showed that mAbs cross-linking of CD16, NKG2D, NKp46 and NKp30 receptors in isolation was sufficient for the induction of IL-6 mRNA expression by PBNK cells. This can be explained on the basis of IL-6 promoter activation. The human IL-6 promoter contains at least four regulatory elements (transcription factor binding sites) such as those binding the transcription factors belonging to activation protein-1 (AP-1), cAMP response element (CRE)-binding protein (CREB), nuclear factor for IL-6 expression (NF-IL6), and nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Figure 4.16).



**Figure 4.16: IL-6 promoter showing the transcription factor binding sites (Keller et al, 1996).**

The transcription factor AP-1 is a heterodimer constituting the oncoproteins resulting from *c-fos* and *c-jun* gene translation (Natoli et al, 1994). AP-1 may be activated by various cytokines including TNF- $\alpha$ , and via different types of protein tyrosine kinases and mitogen-activated protein (MAP) kinases (Karin et al, 1997) which subsequently activate a cascade of intracellular kinases, leading to the up-regulation of the IL-6 gene.

CREB regulates diverse cellular responses, including proliferation, survival and differentiation. It is induced by a variety of growth factors and inflammatory signals and subsequently mediates the transcription of genes containing a cAMP-responsive element (Wen et al, 2010). Several immune-related genes possess this cAMP-response element including IL-2, IL-6, IL-10, and TNF- $\alpha$  (Wen et al, 2010). In NK cells, it has been found that CREB activation has a prominent regulatory role in the IL-2-induced expression of functional c-fos and AP-1 (Ponti et al, 2002).

The nuclear factor- $\kappa$ B (NF- $\kappa$ B) family of transcription factors includes RelA (p65), c-Rel, RelB, NF- $\kappa$ B1 (p50 and p105) and NF- $\kappa$ B2 (p52 and p100) (Oeckinghaus and Ghosh, 2009). RelA, c-Rel, and RelB contain transcriptionally active domains in their C-terminal halves and mediate the majority of NF- $\kappa$ B-mediated gene transcription, whereas p50 and p52 do not contain transactivation domains but can modulate NF- $\kappa$ B activity by forming heterodimers with RelA, c-Rel, and RelB (Verma, 2004). Typical activators of NF- $\kappa$ B are Toll Like receptors (TLRs), which initiate a signalling cascade beginning with TLR adapter molecules MyD88 and TRIF, resulting in NF- $\kappa$ B pathway activation and the triggering of other important signalling cascades (Zhang et al, 2001). In addition to TLRs, the NF- $\kappa$ B pathway can also be activated by pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  (Lawrence et al, 2009). Activation of the NF- $\kappa$ B pathway promotes the transcription of genes involved in pro-inflammatory immune responses including IL-6.

Triggering of any of these regulatory elements via their transcription factors is sufficient for the activation of a cascade of intracellular kinases leading to the up-regulation of the IL-6 gene. Increasing DNA binding activity of IL-6 transcription factors via cross-linking of some of the activating NK cells receptors has been linked to the activation of IFN- $\gamma$  and TNF- $\alpha$  expression/or secretion and increase in the NK

cell cytotoxicity (Trotta et al, 1996; Chuang et al, 2001). Since the activation of these transcription factors also results in the up-regulation of IL-6 itself, therefore, it is not surprising that cross-linking of activating NK cells receptors leads to an increase in IL-6 transcripts as well. Thus a single regulatory element is functionally sufficient to confer responsiveness to a variety of inducers and hence functions as a multiple response element. This might explain why cross-linking of individual NK cell receptors was sufficient to induce IL-6 transcripts.

#### **4.4.4 IL-6 protein secretion upon single receptor engagement**

In contrast to IFN- $\gamma$ , which reportedly requires engagement of multiple different receptors, we demonstrated that the engagement of the activating receptors CD16, NKG2D, NKp46, NKp44 and NKp30 alone were sufficient for the rapid secretion of IL-6. The finding that NK cells are capable of IL-6 secretion upon single receptor engagement may be attributed to the relatively low expression of the SH2-containing 5' inositol phosphatase 1 (SHIP-1) enzyme (Ritz, 2005) and the high expression of the protein phosphatase inhibitor SET (Trotta et al, 2007) in NK cells, facilitating a lower activation threshold for IL-6 secretion. The engagement of multiple different receptors required for IFN- $\gamma$  induction suggests a tighter control and a higher activation threshold. Taken together these results may reveal different activation thresholds for distinct cytokine secretion by NK cells.

The SHIP-1 enzyme is a protein with an N-terminal SH2 domain, an inositol phosphatase domain, and two C-terminal protein interaction domains and the expression of this protein is restricted to haematopoietic cells (Giuriato et al, 2003) . Recruitment of SHIP-1 from the cytosol to plasma membrane is responsible for the inhibitory signals that modulate phosphatidylinositol 3-kinase (PI3K)-dependent

signalling pathways (Gloire et al, 2007). Overall, the protein functions as a negative regulator of cell proliferation and survival (Brooks et al, 2010). This adaptor protein was described to play an important role in the suppression of cytokine secretion by CD56<sup>bright</sup> NK cells (Ritz, 2005).

The SET (I2PP2A, IGAAD, and TAF1b) proteins are localized to the nucleus and cytoplasm and have a critical role in the regulation of normal and cancer signal transduction (Trotta et al, 2007). In fact, SET are potent inhibitors of protein phosphatase type 2A (PP2A) activity (a major cellular serine threonine phosphatase involved in the negative regulation of a variety of cellular processes and signal transduction pathways). The SET are highly expressed in human CD56<sup>bright</sup> cells and they are up-regulated upon monokine (IL-12, IL-15 and IL-18) stimulation of primary human NK cells (Trotta et al, 2007). Over-expression of SET significantly enhanced the IFN- $\gamma$  gene expression in these cells (Trotta et al, 2007). On other hand, RNAi-mediated suppression of SET expression renders NK cells inefficient in producing high levels of IFN- $\gamma$  in response to monokine co-stimulation (Trotta et al, 2007).

#### **4.4.5 Time course for IL-6 mRNA expression and protein secretion**

Despite some variation between receptors regarding the level of IL-6 protein production, cross-linking of PBNK cells with immobilized mAbs against CD16, NKG2D, NKp46, NKp44 and NKp30 resulted in increased IL-6 protein levels at all four time points studied. Compared to non-stimulated resting cells, IL-6 secretion in activated PBNK cells started to increase as early as 1 hour, increased further at 3 and 6 hours and reached its peak at 24 hours. This seems to be consistent in all donors with all receptors examined. The fact that IL-6 secretion became significantly detectable as early as 1 hour after incubation with immobilized mAbs is an important finding. It fits

with the role of NK cells as cells of innate immunity that appear at the inflammatory site at the early stages of an immune response. It also fits with the role of IL-6 as a cytokine that plays an important role in the induction of the acute phase response. Overall, these results support the concept that NK cells may promote resistance to infections and shape adaptive immune responses, at least in part through the secretion of IL-6. However, the observation that various receptors can induce IL-6 secretion suggests the involvement of different signalling pathways. As various signals can result in the activation of NK-cell function redundancy is apparent between receptors. For example, CD16 that signals through an ITAM containing subunit is sufficient to activate, but other NK cell receptors signalling through the same ITAM subunit (NKP46 for example) or through a different subunit (DAP10 associated) such as NKG2D can also activate (Bryceson et al, 2006). It appears that NK cells do not have a dominant activation receptor, unlike T-lymphocytes for example, where the TCRs are central for the activation process. This kind of redundancy forms the basis for the natural cytotoxicity observed with NK cells from mice with a double Syk/ZAP70 deficiency (signalling kinases for CD16) (Bryceson et al, 2006). These cells can still be activated and react with their targets using the NKG2D receptor signalling pathways. Moreover, apart from ITAM- and DAP10-based signals, additional combinations of signals are available and can result in the activation of cytotoxicity. Thus it is not unexpected to see a similar redundancy in the receptors that initiate cytokine secretion.

The detection of IL-6 protein within a relatively short time period following the initial signal may suggest that there is constitutive expression of the mRNA coding for this cytokine in NK cells and, therefore, IL-6 release happens almost without delay. However, our results with quantitating IL-6 transcripts clearly indicated that



this cytokine was also regulated at the transcriptional level. Apart from NKp44, stimulation via any receptor increased mRNA levels as early as 1 hour, and increased it further at 3 hours and caused a peak at 6 hours, in parallel with protein secretion. Whether NK cells regulate the IL-6 secretion at transcriptional or both transcriptional and translational levels needs to be further clarified by using earlier time points. Moreover, our study demonstrated that the IL-6 transcripts were declining at 24 hours in contrast to IL-6 protein which continued to increase. This means that IL-6 mRNA expression is not sustained for a long time even in presence of a continuous stimulus. This is probably due to the transient nature of AP-1, NF- $\kappa$ B and CREB (Adcock, 1997; Paludan, 2001; Mann et al, 2002). In addition, the stability of IL-6 mRNA could be affected via modulation of cytoplasmic factors binding the AU-rich sequences of the 3'untranslated region of IL-6 mRNA that control mRNA stability (Musso et al, 1992). Taking these facts into consideration, it is likely that the decline in IL-6 mRNA expression begins even earlier than 24 hours. In LPS stimulated human peripheral blood it has been shown that IL-6 mRNA levels showed rapid kinetics of induction, reaching a peak at 4 hours and returning to control levels at 8 hours (DeForge et al, 1991).

#### **4.4.6 The involvement of CD16**

CD16 (Fc $\gamma$  RIII) has been described as a receptor expressed on NK cells that facilitate lysis of virus-infected cells and tumour cells through antibody-dependent cellular cytotoxicity (Mandelboim et al, 1999). However, many studies have shown that CD16 has a broader function and is directly involved (alone or in-combination with other receptors) in cytokine and chemokine gene expression and protein secretion. One of the earliest studies on the role of CD16 in the induction of cytokine

gene expression and protein secretion by NK cells was published by Anegón et al in 1988. In this study the authors showed that cross-linking of CD16 by anti-CD16 antibodies induced the transcription of interleukin 2 receptor (CD25) and cytokines (IFN- $\gamma$  and TNF- $\alpha$ ) and the accumulation of their protein products in human natural killer cells. These findings were confirmed later by other studies (Aste-Amezaga et al, 1994; Mandelboim et al, 1998; Bryceson et al, 2006; Márquez et al, 2010). Moreover, CD16 stimulation is also known to stimulate cytokine secretion (IL-1 and TNF- $\alpha$ ) in other cells such as monocytes and monocyte-derived macrophages (Kramer et al, 2004). The present results extend these findings, showing that cross-linking of CD16 on the surface of PBNK cells is sufficient to stimulate IL-6 secretion as well. Thus, NK cells respond with IL-6 mRNA expression and protein production to activation through Fc $\gamma$ RIII, as it occurs upon recognition and killing of antibody sensitized target cells.

The CD16 receptor signals through immunoreceptor tyrosine-based activation motif (ITAM)-containing subunits (Lanier, 2003; Bryceson et al, 2006). In humans, NK cells express CD16 in association with homodimers or heterodimers of membrane-bound signalling adaptor CD3 $\zeta$  or Fc $\epsilon$ RI $\gamma$  molecules (Lanier, 2003). The preferred ligands for CD16 on NK cells are IgG1 and IgG3 (Wu et al, 1997). Cross-linking of CD16 on the surface of NK cells results in Ca<sup>2+</sup> flux followed by the phosphorylation of CD3 $\zeta$  or Fc $\epsilon$ RI $\gamma$  by Lymphocyte-specific protein tyrosine kinase (Lck) and the recruitment of SYK family kinases-tyrosine kinase ( Syk ) and Zeta-chain associated protein kinase 70kDa ( ZAP70 ) (Lanier, 2003) . The activation of these signalling cascades stimulates a series of downstream events including activation of nuclear factor of activated T cells (NFAT). NFAT activation results in transcription and expression of many cytokines including IFN- $\gamma$ , TNF- $\alpha$ , GM-CSF (Aramburu et al,

1995) and FasL (Furuke et al, 1999). This suggests that Fc $\gamma$ RIII (CD16)-dependent NK cell expression/or secretion of IL-6 can occur through molecular mechanisms in which Ca<sup>2+</sup> plays an important role.

The anti-FcR antibody used for the activation of NK cells in these experiments was clone 3G8. It is documented that the F(ab')<sub>2</sub> fragments of 3G8 are as active as the intact IgG in the induction of NK cell activation (Cassatella et al, 1989). The antibody molecules can mimic the effect of natural ligands on Fc $\gamma$ RIII (CD16) and this interaction is sufficient to transduce signals intracellularly. However, this effect might not be seen with other anti- FcR antibodies, for example anti-CDw32 antibody reportedly induces a [Ca<sup>2+</sup>] rise in granulocytes after cross-linking at the cell surface, but does not exert this effect on NK cells (Cassatella et al, 1989). This is obviously due to the fact that NK cells, unlike polymorphonuclear cells and monocytes, bear only one Fc $\gamma$ R type that is CD16, through which the NK cell activation is induced.

#### **4.4.7 The involvement of NKG2D**

The NKG2D is expressed as a disulphide-bonded homodimer on the surface of NK cells,  $\gamma\delta$  T cells and CD8<sup>+</sup> T cells (Ogasawara and Lanier, 2005). In the mouse, NKG2D is associated with both DAP10 and KARAP/DAP12 signalling subunits. The latter is an ITAM motif bearing subunit that activates Syk and ZAP70 kinases. In association with DAP12, the mouse NKG2D receptor has been shown to function as a primary recognition structure that can lead to the full activation of mouse NK cells, including NK cell cytotoxicity and cytokine secretion (Gilfillan et al, 2002; Diefenbach et al, 2002; André et al, 2004). In humans NKG2D is only associated with the DAP10 transmembrane adaptor that lacks any ITAM motif (Wu et al, 1999).

Instead, DAP10 bears an intracytoplasmic YxxM motif that upon ligand binding or antibodies cross-linking couples to and activates the phosphatidylinositol 3-kinase (PI3K) pathway (André et al, 2004). While the role of NKG2D in initiation of human NK cell cytotoxicity is well known, its role in stimulating cytokine secretion is less understood. Some studies argued that NKG2D in humans acts as a co-receptor rather than a primary receptor (Wu et al, 2000; Billadeau et al, 2003; André et al, 2004). This conclusion was reached based on their finding that cross-linking NKG2D receptors with immobilized mAbs did not stimulate cytokine secretion (IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF) and the observation that NKG2D can function as a co-stimulatory receptor for antigen-specific responses of CD8<sup>+</sup> T cells (Groh et al, 2001). This view was further supported by the fact that the PI3K-dependent pathway, the main signalling pathway for the NKG2D/DAP10 complexes, also mediates signalling of other co-stimulatory molecules, such as CD28, ICOS and CD19 (André et al, 2004). Nevertheless, other studies including that conducted by André et al clearly demonstrated that stimulation of human NK cells with soluble recombinant NKG2D ligands (i.e. ULBP-1 and MICA) induces GM-CSF and IFN $\gamma$  production by activated NK cells (Sutherland et al, 2001, Sutherland et al, 2002; André et al, 2004). In contrast to the above studies, the present results clearly demonstrated that cross-linking of NKG2D with an immobilized mAb was sufficient to induce IL-6 secretion at all four time points investigated. Moreover, the finding that NKG2D cross-linking produced more IL-6 transcripts and protein than CD16 cross-linking may suggest the relevant importance of DAP10 associated pathways in the initiation and triggering of IL-6 secretion by human NK cells. Considering the fact that IL-6 could be detected upon cross-linking of the NKG2D receptors with an immobilized mAb while other investigators failed to find this with the other cytokines they were studying, one can argue that the signalling

pathways which lead to IL-6 secretion upon NKG2D activation are distinct from those necessary for IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF production.

It is known that the CD16 is the most important receptor that induces IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF secretion by NK cells (Fauriat et al, 2010). No other activating receptor can induce such a response alone (although stimulating a combination other receptors can). CD16 cross linking on the surface of NK cells initiates a cascade of signalling events via Syk and ZAP70 kinases (Lanier, 2003). Thus, it is logical to assume that these kinases are the main signalling kinases that regulate the IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF secretion by NK cells. Since NKG2D cross-linking activates totally different kinases, so it not surprising that activation of NKG2D is unable to stimulate IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF secretion by NK cells. The situation might be different for IL-6. As described before, the activation of the NKG2D/DAP 10 complexes, whether by natural ligands or anti-NKG2D mAbs, leads to the activation of the phosphatidylinositol 3-kinase (PI3K) pathway. The activation of this pathway has been described to regulate IL-6 secretion in several other cells including B-cells (Venkataraman et al, 1999), Kupfer cells (Dahle et al, 2004) osteoblasts (Hanai et al, 2006), normal myeloid cells and cells that arose in myeloid leukaemia (Birkenkamp et al, 2000; Vesely et al, 2007) as well as cancer cells (Jee et al, 2000). Activation of the PI3K pathway was also reported to increase IL-6 mRNA expression in some cells lines (Dommisch et al, 2008). Moreover, it was found that the LPS dependent activation of IL-6 secretion in B-cells and the IL-1 dependent IL-6 mRNA expression and secretion by the Caco-2 cell line is mediated by PI3K pathway (Venkataraman et al, 1999; Cahill et al, 2008). However, the PI3K pathway might not be the only one activated upon NKG2D engagement in NK cells. Some studies also suggest the involvement of other activation pathways such as phospholipase-C  $\gamma$  and VAV

(Billadeau et al, 2003; Bryceson et al, 2006). Activation of these signalling pathways has been linked to IL-6 production in mast cells (Song et al, 1999). All these studies support the idea that the mechanism of IL-6 secretion and expression in NK cells is likely to be different from that involved in IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF secretion.

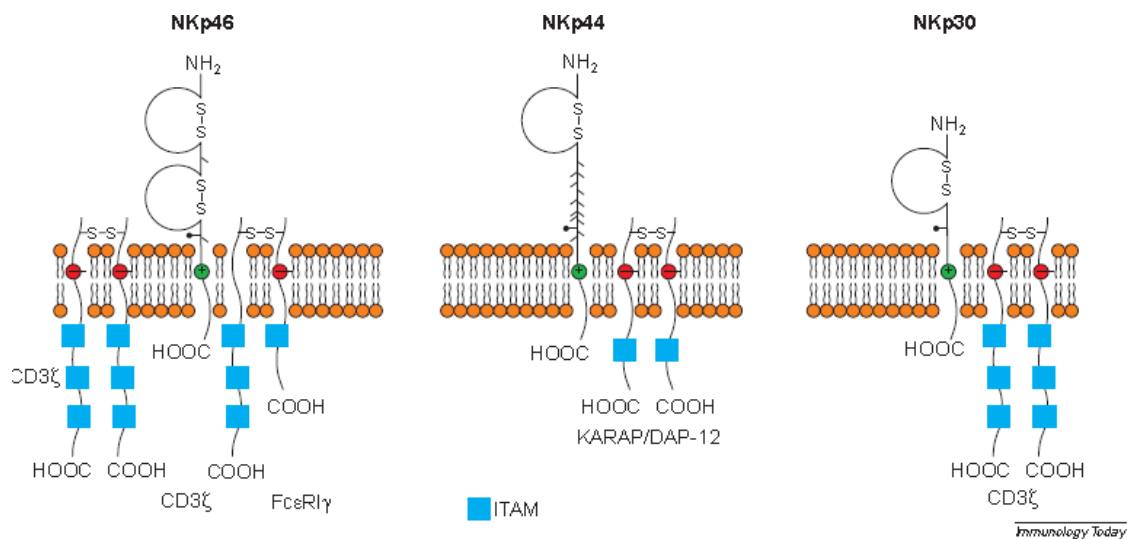
#### **4.4.8 The involvement of NCR receptors (NKp46, NKp44 and NKp30)**

Natural cytotoxicity receptors (NCRs) play a crucial role in MHC class I-independent recognition and killing of target cells by NK cells (Bottino et al, 2000). In humans NKp46 and NKp30 are expressed by both resting and activated NK cells (Sivori et al, 1997; Sivori et al, 1999; Pende et al, 1999), while the expression of NKp44 is restricted to IL-2 activated NK cells (Vitale et al, 1998). Cross-linking NKp46 on the surface of human or mouse NK cells with their specific mAbs elicit cytokine release as well as the de-granulation of cytotoxic effector molecules (Andre et al, 2004; Satoh-Takayama et al, 2009). Similar results have been obtained using anti-NKp30 antibodies, suggesting that the engagement of NCRs by their respective ligands or mAb can modulate both cytotoxicity and cytokine secretion (Andre et al, 2004; El - Costa et al, 2008; Satoh-Takayama et al, 2009).

Our results have shown that cross-linking NKp46 by immobilized mAb stimulated IL-6 cytokine secretion by PBNK cells. The pattern of secretion was similar to that found with CD16 and NKG2D and the amount of IL-6 produced was similar to that seen with NKG2D. NKp46 receptors are characterized by two immunoglobulin-type C2 domains in the extracellular portion (Figure 4.17). Their short intra-cytoplasmic portion lacks typical tyrosine-based activating motifs, while positively charged amino acids are present in the transmembrane region and allow association with ITAM-bearing polypeptides (Moretta et al, 2000). The latter for

NKp46 includes CD3 $\zeta$  and Fc $\epsilon$ RI $\gamma$ . As with CD16, the engagement of ITAM-dependent receptors initiates a signal transduction cascade that is Ca<sup>2+</sup> dependent. First, the members of src protein tyrosine kinase (PTK) family induce the phosphorylation of both ITAM tyrosine residues, allowing the consequent recruitment and activation of Syk and/or ZAP70. The finding that NK cells produce more IL-6 transcript and protein in response to NKp46 compared to CD16 is difficult to explain since both of these receptors use the same signalling pathways. One possible explanation might be a difference in the strength of signalling via the ITAM chains associated with NKp46 compared to those associated with CD16. Augugliaro et al (2003) has shown that mAb-mediated cross-linking of NKp46 resulted not only in tyrosine phosphorylation of the NKp46-associated CD3 $\zeta$  and Fc $\epsilon$ RI $\gamma$ , but also resulted in tyrosine phosphorylation of the NKp30-associated CD3 $\zeta$  and NKp44-associated DAP12 polypeptides at the same time. This “cross-phosphorylation” effect was strictly confined to NCR complexes, since cross-linking NKp46 never resulted in tyrosine phosphorylation on the CD3 $\zeta$  or DAP12 chains associated with CD16 and p50.3. Thus, the engagement of NKp46 appears to be able to activate the signalling cascades associated with the other NCRs, possibly resulting in the amplification of the activating signals. This suggests the existence of a functional cross-talk among NCR (Augugliaro et al, 2003) while a similar event does not happen with other CD3 $\zeta$  or DAP12-associated receptor molecules i.e. the engagement of CD16 or p50.3 receptors will not result in co-stimulation of other ITAM-bearing subunit receptors such as NKp46 and NKp30 or NKp44 respectively. This observation is supported by our finding that co-stimulation of CD16 and NKp46 together did not stimulate further IL-6 mRNA expression or protein production (data not shown). Moreover, other researchers have shown that IFN- $\gamma$  cytokine secretion by CD16-activated NK cells is

synergized by co-stimulation of other NK cell receptors (NKG2D, 2B4, CD2, or DNAM-1) but not with NKp46 (Bryceson et al, 2006), suggesting that CD16 is excluded from this interplay between NCRs despite the usage of similar signal transducing polypeptides.



**Figure 4.17: Structure of natural cytotoxicity receptors (NCRs) and their association with distinct signal-transducing molecules.** Reproduced from Moretta et al (2000)

The induction of IL-6 transcripts and protein secretion was also more prominent with NKp46 compared to that induced by NKp30. This observation gives rise to an important question whether this activity difference could be due to their downstream signalling through different adaptors. Biochemical analysis has revealed that NKp46 is associated with both CD3ζ and FcεRIγ adaptor proteins, which contain ITAMs, whereas NKp30 uses only CD3ζ (Figure 4.17). Does the association of NKp46 receptors with two different adaptor molecules further amplify their activating signals? siRNA silencing of CD3ζ and FcεRIγ in PBNK may help to further clarify this issue in the future.



IL-6 production by NKp44-activated PBNK cells was different from that seen with cells stimulated via the other receptors tested. After 1 and 3 hours of stimulation there was no significant increase in IL-6 transcripts levels compared to resting NK cells while cultures at 6 hours after mAb stimulation clearly demonstrated a decrease in IL-6 mRNA levels, followed by a further decrease at 24 hours. Surprisingly, in sharp contrast to mRNA expression, IL-6 protein secretion continued to increase at all 4 time points to levels even higher than those seen with the other receptors. It is essential to point out that the PBNK cells used in these experiments were pre-stimulated with IL-2 at a dose of 100 U/ml for 24 hours before NKp44 cross-linking with the immobilized mAb. As described in the previous chapter this dose of IL-2 in itself was insufficient for the induction of IL-6 production by NK cells. Similar observations were made in other cells such as monocytes in which the IL-2 induction of IL-6 protein secretion is a dose-dependent requiring a minimum of 250 U / ml of IL-2 for effective stimulation (Musso et al, 1992). We concluded that the down-regulation of IL-6 mRNA transcription was due to the transient nature of IL-6 transcription factors and to mRNA degradation as a result of prolonged NK cell activation. Nevertheless, how can continuous IL-6 protein secretion despite the down-regulation of IL-6 mRNA expression be explained? One could suggest that IL-2 induces the production of IL-6 mRNA and possibly even its translation into protein. However, in itself it may not be able to induce the secretion of the cytokine. If NKp44 cross-linking induced this secretory pathway this could explain the increased IL-6 protein secretion at all 4 times studied potentially even in the absence of IL-6 mRNA during the NKp44 stimulation phase of these experiments. However, given the constraints of time and resources we decided not to investigate this issue further.

# Chapter 5

## Production of Human NKG2D Ligands as Fc Fusion Proteins



2012

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## 5.1 Introduction

Experiments described in the previous chapter clearly demonstrated that cross-linking NKG2D and NKp46 with their corresponding antibodies on the surface of resting PBNK cells stimulated the highest IL-6 mRNA expression and protein production. Some investigators, however, argue that the mechanisms by which an anti-NKG2D mAb and the physiological NKG2D ligands activate NK cell proliferation and cytokine production are different (André et al, 2004). In these experiments human NK cells failed to produce IFN- $\gamma$  and GM-CSF in the presence of anti-NKG2D mAb whereas soluble recombinant NKG2D ligands such as MICA-Fc and ULBPs-Fc fusion proteins were able to elicit such responses (Sutherland et al, 2001; Sutherland et al, 2002, André et al, 2004). Moreover, NK cells did not undergo cell proliferation or increased expression of IL-2R $\alpha$  (CD25) in the presence of anti-NKG2D mAb, while stimulation with soluble recombinant NKG2D ligands induced both of these (André et al, 2004). Considering the highly heterogeneous structure of NKG2D ligands (Raulet, 2003), these observations suggest that NKG2D epitopes targeted by mAbs and the various natural ligands might be distinct and their stimulation could, therefore, lead to different signalling events (André et al, 2004). However, the finding that NKG2D cross-linking using a monoclonal antibody was sufficient to induce IL-6 mRNA expression and protein production contradicts this argument, at least as far as IL-6 production is concerned. Nevertheless, to test the hypothesis of specific epitope-dependent receptor triggering, it was decided to express the ligands of NKG2D, namely MICA, MICB, ULBP1, ULBP2 and ULBP3, as Fc fusion proteins to see if IL-6 production is affected by the nature of the NKG2D stimulation. Such experiments may also show if there was a preference for certain NKG2D ligands in inducing IL-6 secretion.

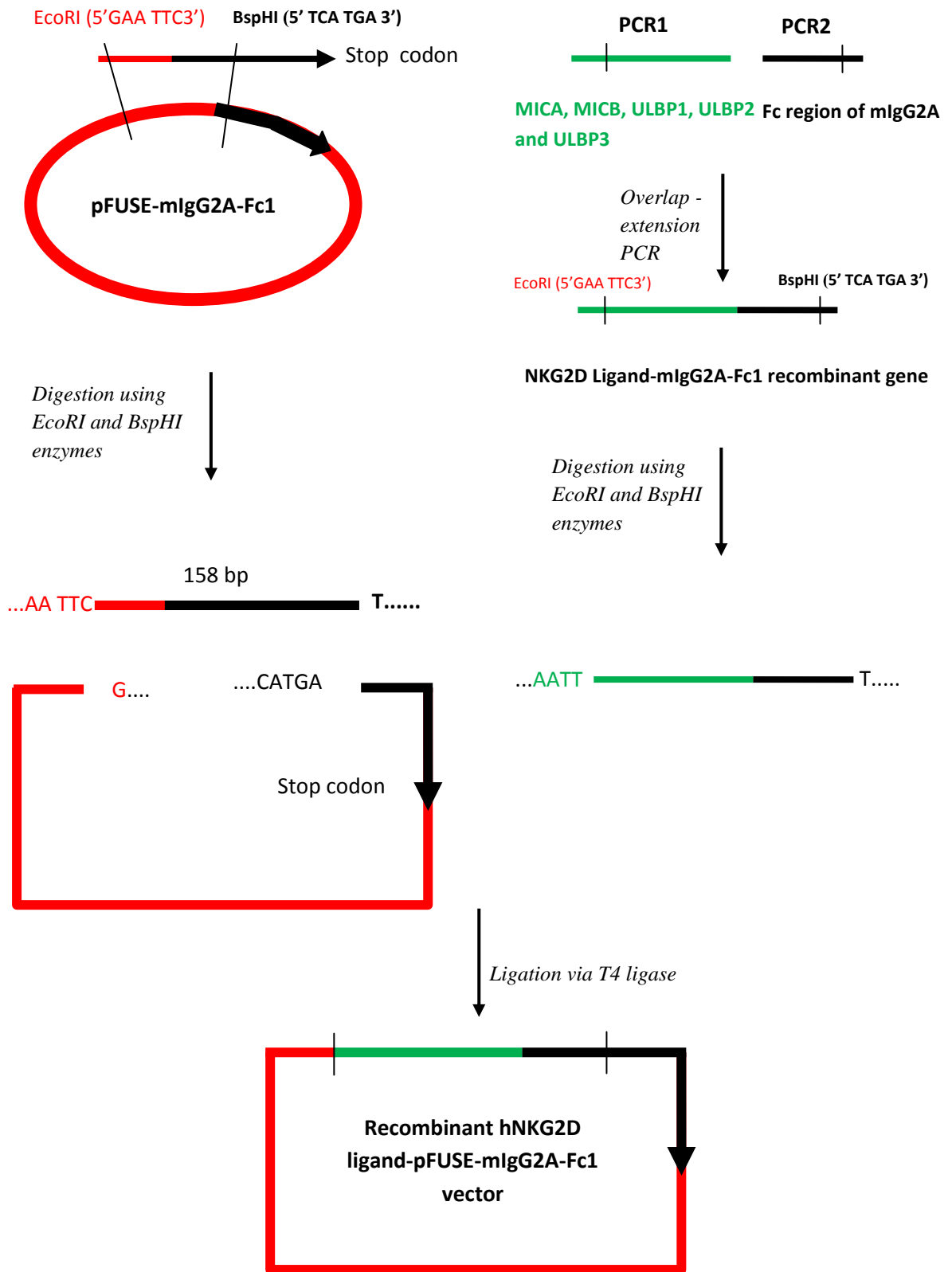
### **5.1.1 Aims of the chapter**

The aim of this chapter was to demonstrate the role of NKG2D signalling in the initiation of IL-6 by PBNK cells through the expression of hNKG2D ligands, namely MICA, MICB, ULBP1, ULBP2 and ULBP3 as Fc- fusion proteins. The production of these fusion proteins was planned to be achieved by fusing the sequence encoding for the extracellular portion of these ligands with the Fc sequence of mouse IgG (mIgG) either by overlap-extension PCR or by simple ligation. The plasmids containing these recombinant genes were then attempted to be expressed as fusion proteins via transfection into CHO or HEK-293 cells.

## **5.2 Approach 1 For Expression of hNKG2D Ligand - mFc Fusion Proteins by Overlap-Extension PCR Using the pFUSE-mIgG2A-Fc1 Vector**

### **5.2.1 Strategy used for cloning of hNKG2D ligand - mFc recombinant genes**

The C-terminal ends of the extracellular region of hNKG2D ligands, namely MICA, MICB, ULBP1, ULBP2 and ULBP3 proteins were planned to be fused to the N-terminus of the Fc-region of mouse IgG2a. To combine the two proteins, fragments of their genes were generated in separate PCRs using specific primers designed to create products which contain complementary sequences at their 3' ends. These intermediate PCR products overlapped partially and an extension of the matching sequence produced a recombinant gene in which the original sequences were 'fused' together using overlap-extension PCR. The resulting recombinant hNKG2D ligand - mFc gene was then double digested with restriction enzymes and orientationally cloned into the pFUSE mIgG2A-Fc1 expression vector digested using the same restriction enzymes (Figure. 5.1).

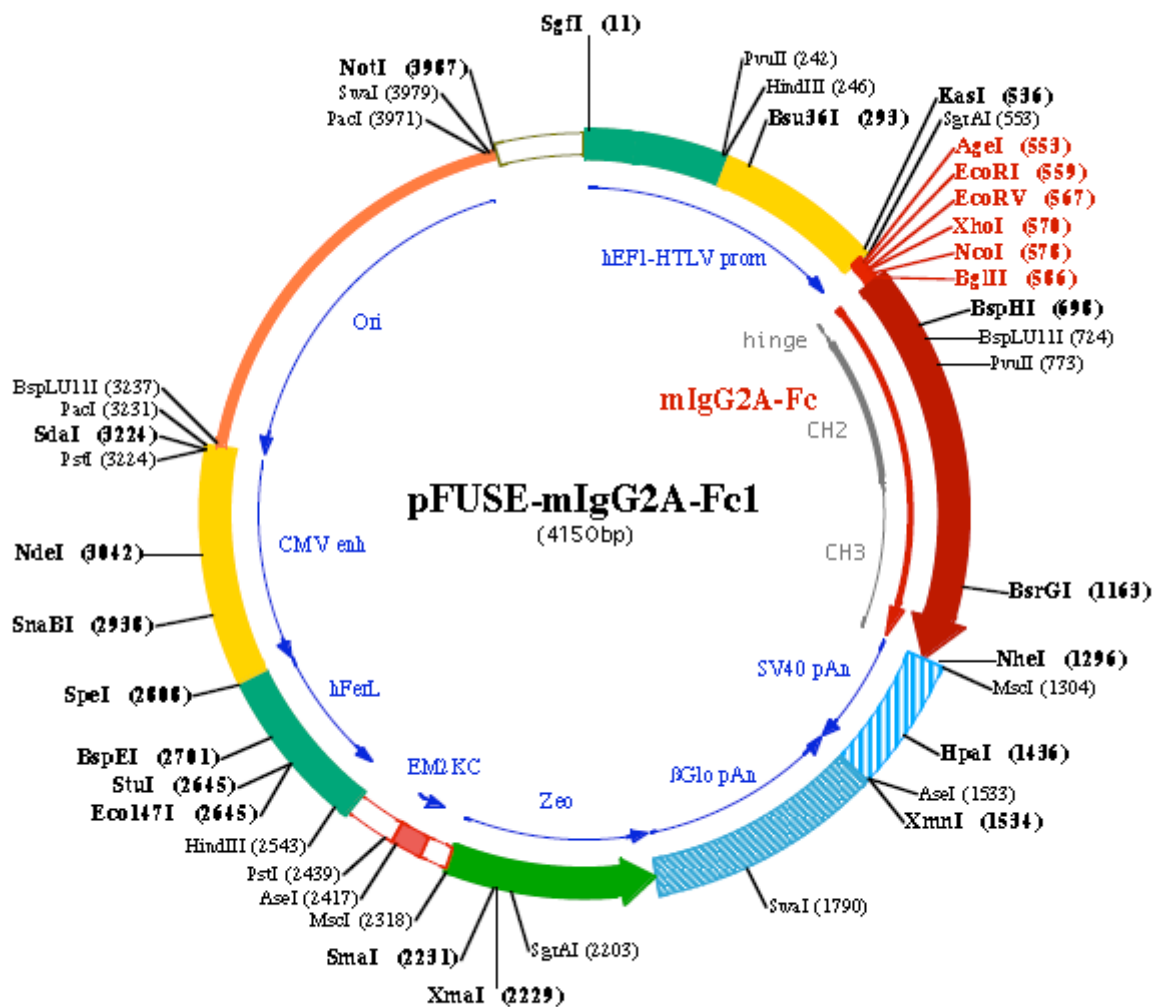




**Figure 5.1: Overall strategy used for the cloning of hNKG2D ligand -mFc recombinant gene into pFUSE-mIgG2A-Fc1 expression vector.** The hNKG2D ligand - mFc recombinant gene was generated by three-round PCRs. The PCR1 amplified the gene sequence encoding for extracellular portions of hNKG2D ligands started from the start codon (ATG). The EcoRI restriction site was incorporated upstream of forward primer of PCR1. The PCR2 amplified a relatively short nucleotide sequence (169 bp) started from position 1 to 169 of the Fc portion of mIgG2a (from beginning of mIgG2a sequence to downstream of the BspHI restriction site). The primers were designed so that the end of PCR1 overlaps the beginning of PCR 2. The two genes were fused together through a third-round PCR (PCR3) and the hNKG2D ligand-mFc recombinant gene was generated. Both the recombinant gene and the vector were then double digested via EcoRI and BspHI restriction endonucleases. The fused gene was then introduced *in frame* into the digested pFUSE-mIgG2A-Fc1 vector.

### 5.2.2 pFUSE-mIgG2A-Fc1 vector

pFUSE - Fc vectors (Invivogen, USA) are a family of plasmid vectors developed to facilitate the construction of Fc-fusion proteins by fusing the extracellular region of a protein to the Fc fragment of an immunoglobulin G (IgG). Given that the pFUSE-mIgG2A-Fc1 vector used for the cloning of hNKG2D ligand-mFc genes is an unusual commercial design its details are summarised here (Figure 5.2). The plasmid has two composite promoters: the hEF1-HTLV promoter and the CMV enh/hFerL promoter. The hEF1-HTLV promoter drives the expression of the Fc fusion protein. It is comprised of the Elongation Factor-1 $\alpha$  (EF-1 $\alpha$ ) core promoter ([www.invivogen.com](http://www.invivogen.com); Kim et al, 1990) and the R segment and part of the U5 sequence (R-U5') of the Human T-Cell Leukaemia Virus (HTLV) Type 1 Long Terminal Repeat ([www.invivogen.com](http://www.invivogen.com); Takebe et al, 1988). The EF-1 $\alpha$  promoter exhibits a strong activity and yields lasting expression of the gene of interest *in vivo* ([www.invivogen.com](http://www.invivogen.com)). The R-U5' has been coupled to the EF-1 $\alpha$  core promoter to enhance the stability of the resulting mRNA. The CMV enh/hFerL promoter combines the human cytomegalovirus immediate-early gene 1 enhancer and the core promoter of the human ferritin light chain gene. This ubiquitous promoter drives the expression of the Zeocin resistance gene in mammalian cells. The pFUSE vector has also the Simian Virus 40 late polyadenylation (SV40 pAn) signal which enables efficient cleavage and polyadenylation reactions resulting in high levels of steady-state mRNA. Unique restriction enzyme sites within the multi-cloning segment (MCS) are shown in red bold print while the unique restriction enzyme sites within the whole plasmid are shown in black bold print. The EcoRI site within the MCS and a unique BspHI site within the mIgG2A-Fc sequence were utilised for the orientational cloning of the inserts.



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**Figure 5.2: pFUSE-mIgG2A-Fc1 vector map (4150 bp)** showing the MCS (multiple cloning site), hEF1-HTLV promoter driving the Fc fusion and CMV enh / hFerL promoter driving the Zeocin resistance in mammalian cells. Reproduced from [www.Invivogen.com/cloning and expression/Fc fusion/pFUSE-mIgG2A-Fc1](http://www.Invivogen.com/cloning_and_expression/Fc_fusion/pFUSE-mIgG2A-Fc1).

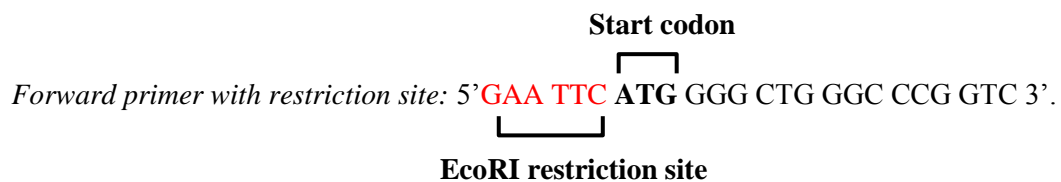
### 5.2.3 NKG2D ligands- mFc1 sequences

The MICA, MICB, ULBP1, ULBP2 and ULBP3 nucleotide and protein sequences together with their recombinant Fc sequences and their homology to published NKG2D sequences were presented in Chapter 9 (Appendix chapter). The gene sequences encoding for the extracellular portion of NKG2D ligands were amplified and fused to the gene encoding for Fc portion of mIgG2a.

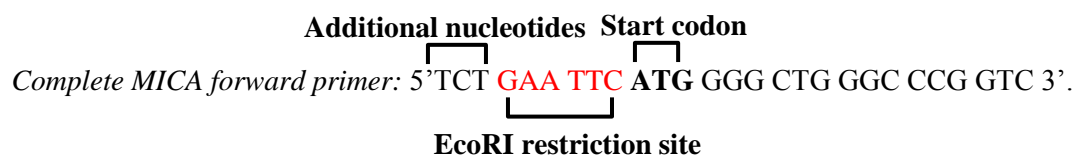
### 5.2.4 Details of primer design

#### 1. Forward or sense primer for PCR 1 (primer a)

The open reading frame of the MICA gene starts with the sequence 5'ATG GGG CTG GGC CCG GTC 3' (Chapter 9). Since it was planned to insert the MICA-Fc1 recombinant gene into the pFUSE-mIgG2A-Fc1 vector at the EcoRI and BspHI restriction sites, therefore an EcoRI restriction site (5'GAA TTC3') was introduced upstream of the 5' end of the primer:



To ensure that the restriction site at the end of the amplified segment is optimally cleaved by the enzyme three additional nucleotides were placed upstream of the restriction site:



The total length of this oligonucleotide is 27 bp. The forward primers for MICB, ULBP1, ULBP2 and ULBP3 were designed using the same principles. The sequences are as follows:

*Complete MICB forward primer: 5' TCT GAA TTC ATG GGG CTG GGC CGG GTC 3'.*

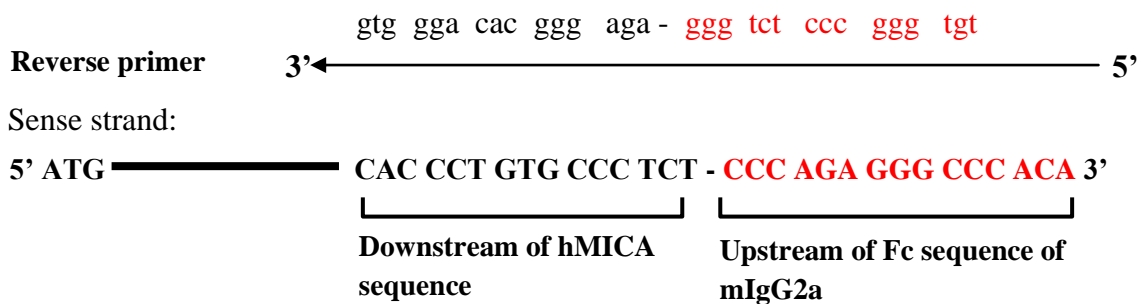
*Complete ULBP1 forward primer: 5' TCT GAA TTC ATG GCA GCG GCC GCC AGC 3'.*

*Complete ULBP2 forward primer: 5' TCT GAA TTC ATG GCA GCA GCC GCC GCT 3'.*

*Complete ULBP3 forward primer: 5' TCT GAA TTC ATG GCA GCG GCC AGC CCC 3'.*

## 2. Reverse or anti-sense primer for PCR 1 (primer b)

The reverse primer was designed so that it would anneal to the end of the extracellular portion of the MICA gene followed by an overlap extension to the beginning of the mIgG2A-Fc sequence. The 3' end of the MICA sequence encoding for the extracellular portion of MICA protein is 5' CAC CCT GTG CCC TCT 3' (Bahram et al, 1996) and the beginning of Fc sequence of mIgG2a is 5' CCC AGA GGG CCC ACA 3':



Since all primers should be in 5' to 3' orientation (for purchasing purposes), so the final reverse primer for PCR1 is as follows (reverse complement):

*MICA reverse (pFUSE overlap) primer for PCR1: 5' TGT GGG CCC TCT GGG AGA GGG CAC AGG GTG 3'.*

In the same manner, the reverse primers for MICB, MICB, ULBP1, ULBP2 and ULBP3 were:

*MICB reverse (pFUSE overlap) primer: 5' TGT GGG CCC TCT GGG – AGG GTG AGT GCC GTG 3'*

*ULBP1 reverse (pFUSE overlap) primer: 5' TGT GGG CCC TCT GGG – TTG TTC CCA GTA CAT 3'*

*ULBP2 reverse (pFUSE overlap) primer: 5' TGT GGG CCC TCT GGG – GCT GTC CAT GCC CAT 3'*

*ULBP3 reverse (pFUSE overlap) primer: 5' TGT GGG CCC TCT GGG – CTT CTT CCT GTG CAT 3'*

### 3. Forward primer for PCR 2 (primer c)

The forward primer for PCR2, encoding the Fc portion of the fusion protein, is the beginning of Fc sequence of mIgG2a (5' CCC AGA GGG CCC ACA 3') with an overlap extension to the 3' end of MICA sequence (5' CAC CCT GTG CCC TCT 3'). Thus, the final forward primer for PCR2 is:

*pFUSE forward (MICA overlap) primer for PCR2*      5' CAC CCT GTG CCC TCT **CCC AGA GGG CCC ACA** 3'



Using the same principle, the following forward primers were used for PCR2:

*pFUSE forward (MICB overlap) primer: 5' CAC GGC ACT CAC CCT- **CCC AGA GGG CCC ACA** 3'*

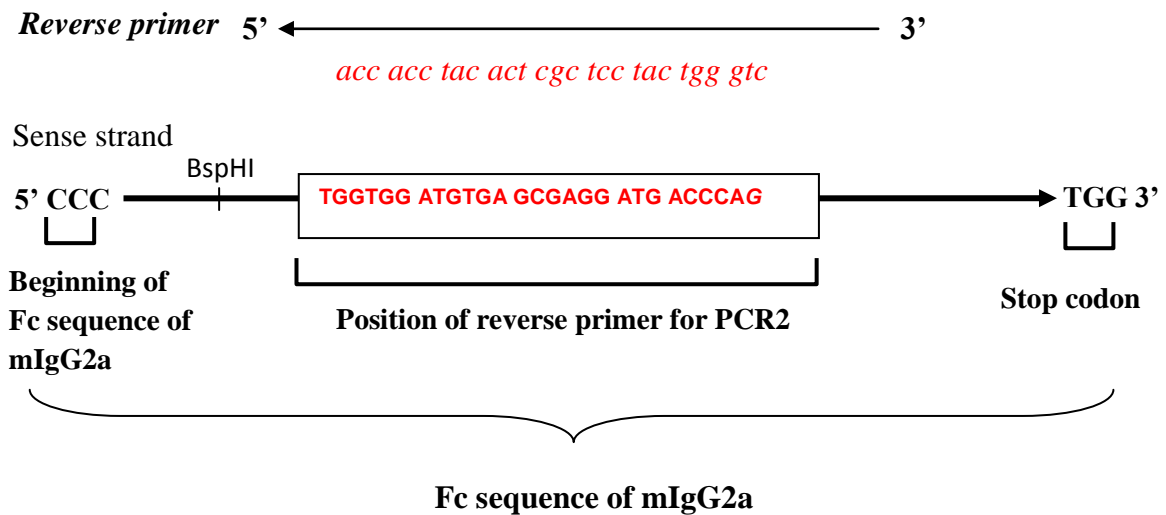
*pFUSE forward (ULBP1 overlap) primer: 5' ATG TAC TGG GAA CAA - **CCC AGA GGG CCC ACA** 3'*

*pFUSE forward (ULBP2 overlap) primer: 5' ATG GGC ATG GAC AGC – CCC AGA GGG  
CCC ACA 3'*

*pFUSE forward (ULBP3 overlap) primer: 5' ATG CAC AGG AAG AAG - CCC AGA GGG  
CCC ACA 3'*

#### 4. Reverse primer for PCR2 (primer d)

This reverse primer should ideally anneal to the 3' end of the mIgG2a-Fc sequence. However, since this end of the PCR product was planned to be incorporated in to the pFUSE-mIgG2A vector using the BspHI restriction site, therefore this primer could be placed anywhere in the mIgG2a-Fc sequence downstream of the BspHI restriction site:



The final reverse primer for PCR2 was as follows (5' to 3' orientation):

*PFUSE reverse primer for PCR2: 5' CTG GGT CAT CCT CGC TCA CAT CCA CCA 3'.*

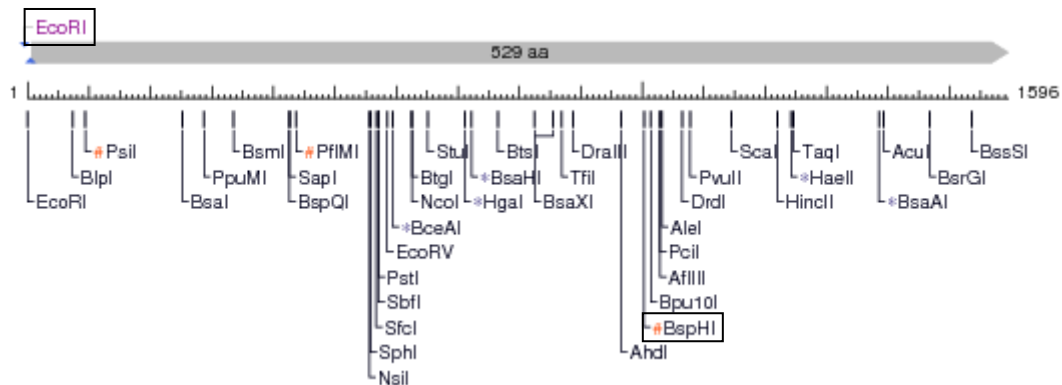
As the amplified mFc fragment is the same for all the constructs, only a single primer was necessary for all the reactions.

### 5.2.5 Checking the recombinant MICA-, MICB-, ULBP1-, ULBP2- and ULBP3-mFc1 gene sequences for EcoRI and BspHI restriction sites

Since the recombinant hMICA-, hMICB-, hULBP1-, hULBP2- and hULBP3-mFc1 genes were to be cloned into the pFUSE-mIgG2A-Fc1 vector at the EcoRI and BspHI restriction sites, it was necessary to check both the vector and the recombinant gene sequences for these restriction sites. This was done using an online tool called NEB cutter V.2.0 that produces a comprehensive report of the restriction sites within the input sequence (Vincze et al, 2003). Sequences for all the designed molecules only contained a single EcoRI and a single BspHI site, so the planned cloning strategy was feasible (Figure 5.3).

#### hMICA – mFc1 sequence

*Single cutter enzymes*



**Figure 5.3: Restriction map of the hMICA-mFc1 sequence.** Enzymes with a single cutting site are shown. Note that the EcoRI restriction site was added to the beginning of the sequences by the primers while the BspHI restriction site was part of the mFc sequence. The # symbol indicated that these enzymes are methylation sensitive and their cutting here is blocked by DNA methylation.

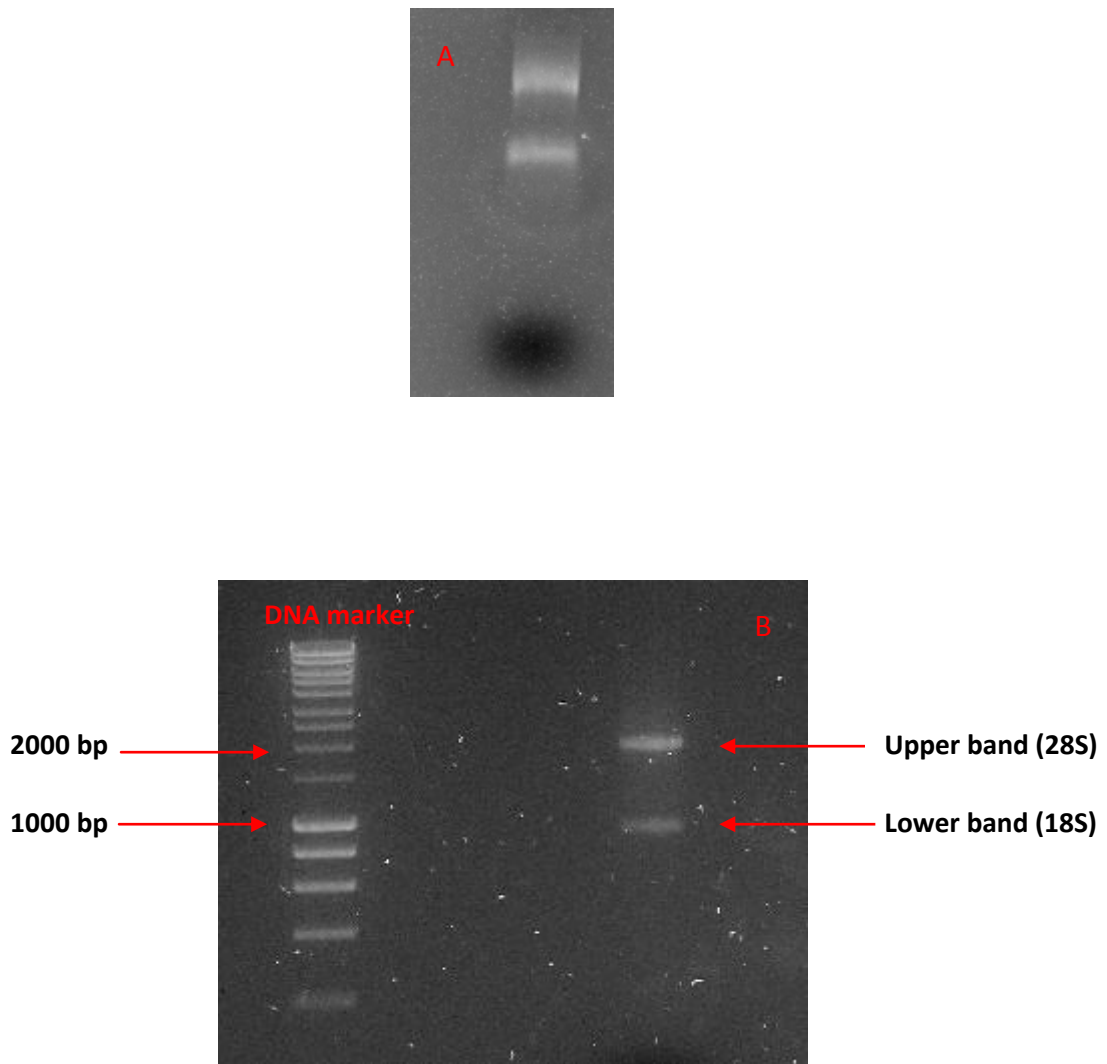


## 5.2.6 Results 1

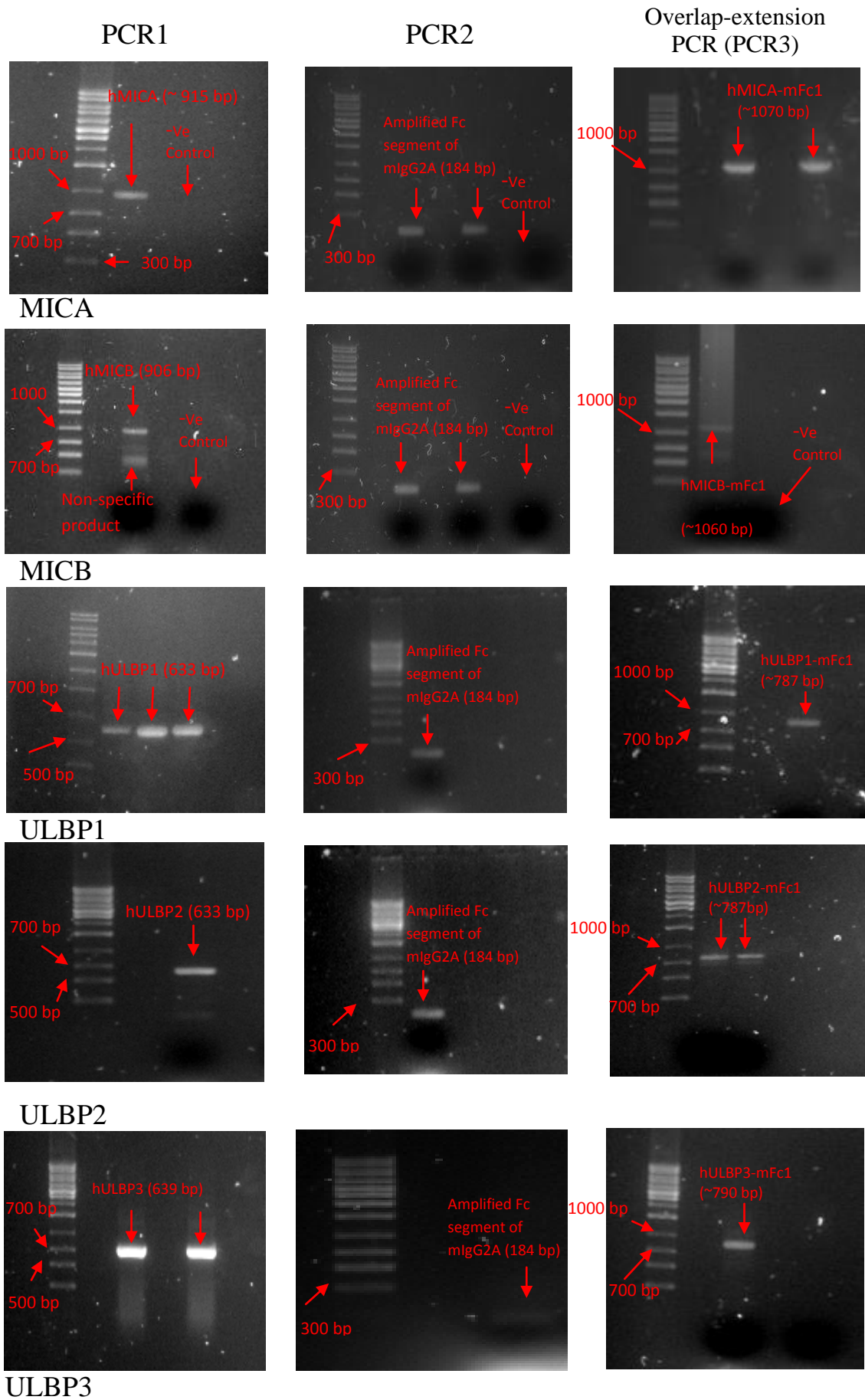
### 5.2.6.1 Overlap - extension PCR

As several studies have shown that HeLa cells express MICA (Groh et al, 1999; Zhang et al, 2001; Butler et al, 2009) cDNA derived from these cells was used for amplification of the extracellular portion of MICA. Caco-2 cDNA was used to amplify the extracellular regions of MICB, ULBP2 and ULBP3 as these cells are known to express these transcripts (Butler et al, 2009). ULBP1 proved difficult to amplify; amplification of this molecule from HeLa, Caco-2 and Jurkat cDNAs failed. Therefore, the extracellular fragment of ULBP1 was amplified from cDNA derived from ULBP1 transfected CHO Cells (Gift from Dr. Hugh Reyburn, Spain). In brief, the total RNA was first extracted from these cell lines using QiaAmp RNA Blood Mini Kit. Total RNA concentration and integrity were checked by spectrophotometer and agarose gel respectively (Figure 5.4) and then converted into cDNA using AMV reverse transcription system. The cDNAs were stored at -20 °C until use. The overlap-extension PCR reactions were carried out as described in Chapter 2. The amplicons generated during the various stages of the overlap-extension PCR process together with their approximate sizes are shown in Figure 5.5. The recombinant (fused) PCR products were gel purified using PureLink™ Gel Purification Kit and then digested with EcoRI and BspHI. The sizes of the digested inserts were 1003, 994, 721, 721 and 724 bp for MICA-, MICB-, ULBP1-, ULBP2- and ULBP3-Fc1 respectively (Figure 5.6 A). However, the double digest of the pFUSE-mIgG2A-Fc1 vector proved problematic. 0.5-1µg of empty pFUSE-mIgG2A-Fc1 vector obtained from minipreps of the plasmid propagated in DH-5 α E.coli was checked for single EcoRI and BspHI digests. While the EcoRI digest was complete after two hours, digestion with BspHI resulted in the formation of two bands (Figure 5.6 B). This was unlikely due to cutting

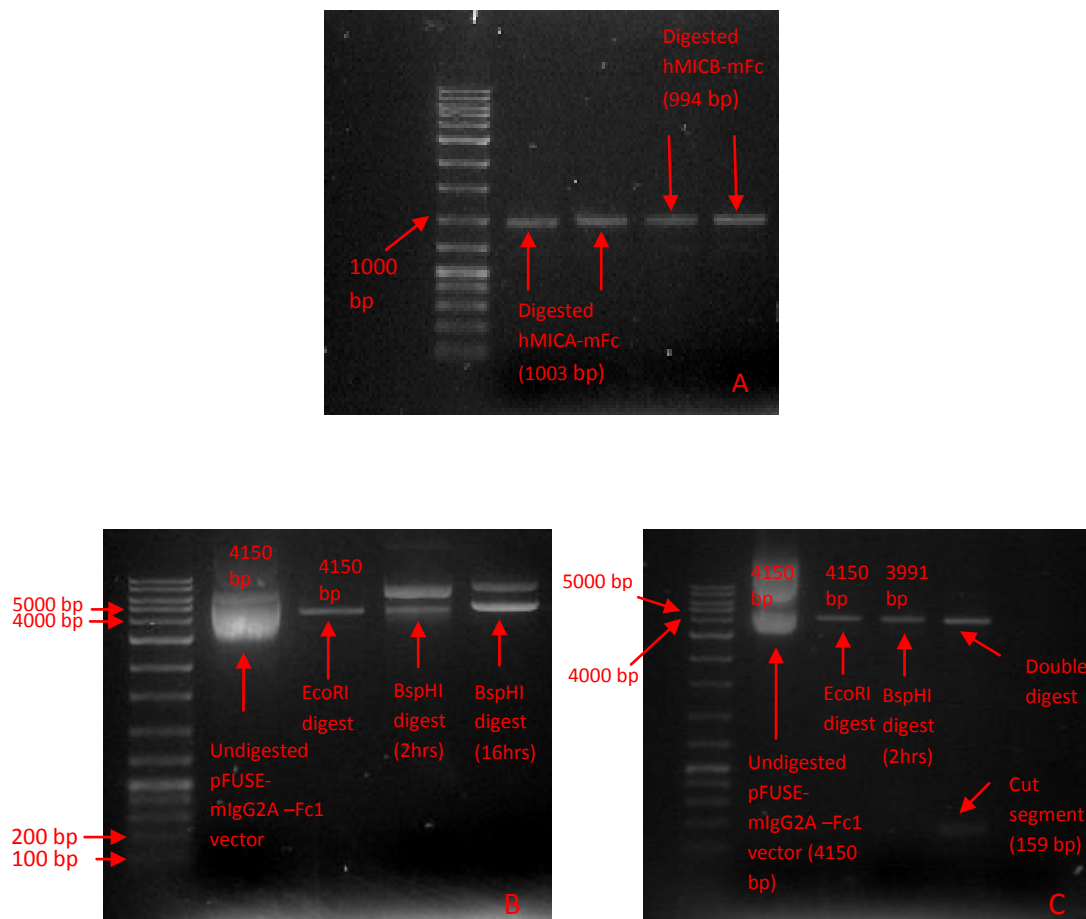
at two different sites since there is only one documented BspHI restriction site in the entire plasmid sequence. It was also unlikely due to star activity (the enzyme cutting at non-specific sites during extended digests) as, according to the manufacturer, the enzyme shows no star activity and it is suitable for extended or overnight digest (NEB website). Overnight digestion did not improve results (Figure 5.6 B) and subsequent ligation and transformation failed. The problem with BspHI digestion was that this restriction enzyme is methylation sensitive; i.e. the digestive activity of this enzyme is blocked by overlapping *dam* methylation. For this enzyme to work the BspHI restriction site should be non-methylated. This was achieved by propagating the pFUSE-mIgG2A-Fc1 vector in the *dam*<sup>-</sup> strain of E. coli such as C2925I strain. As illustrated in Figure 5.6 C using this bacterial strain resolved the problem. The digested plasmid was then gel purified before proceeding to ligation to ensure that the vector would not re-ligate to its digested fragment during the ligation step.



**Figure 5.4:** A) RNA agarose-formaldehyde gel stained with ethidium bromide showing 18 S and 28 S ribosomal RNA bands. The total RNA was extracted from  $1 \times 10^6$  HeLa cells using the Qiagen QIAamp RNA Blood Mini Kit. 1  $\mu$ l was loaded on the gel. B) Regular agarose gel. 5  $\mu$ l of DNA marker and 1  $\mu$ l of HeLa RNA were loaded on 1% molecular biology grade agarose in 1X TAE buffer stained with ethidium bromide. The left lane shows the 200-10000 bp HyperLadder (BioLine, UK) while the right lane shows the RNA bands. Note the position of the 28S and 18S ribosomal RNA bands appear at approximately 2000 and 1000 bp and respectively.



**Figure 5.5: Generation of recombinant hMICA-, hMICB-, hULBP1-, hULBP2- and hULBP3 – mFc1 genes by overlap – extension PCR.** In PCR1 (column 1) the genes encoding for extracellular portions of hMICA, hMICB and hULBPs (including the leading sequences) were amplified. In PCR (column 2) the genes encoding for part of the Fc sequence of mIgG2a were amplified. In PCR3 (column 3) the PCR1 and PCR2 products were fused together to generate the recombinant (chimeric) genes. All PCR products were run on 1% agarose gels. Lane 1 contained a 300-10000 bp DNA ladder.

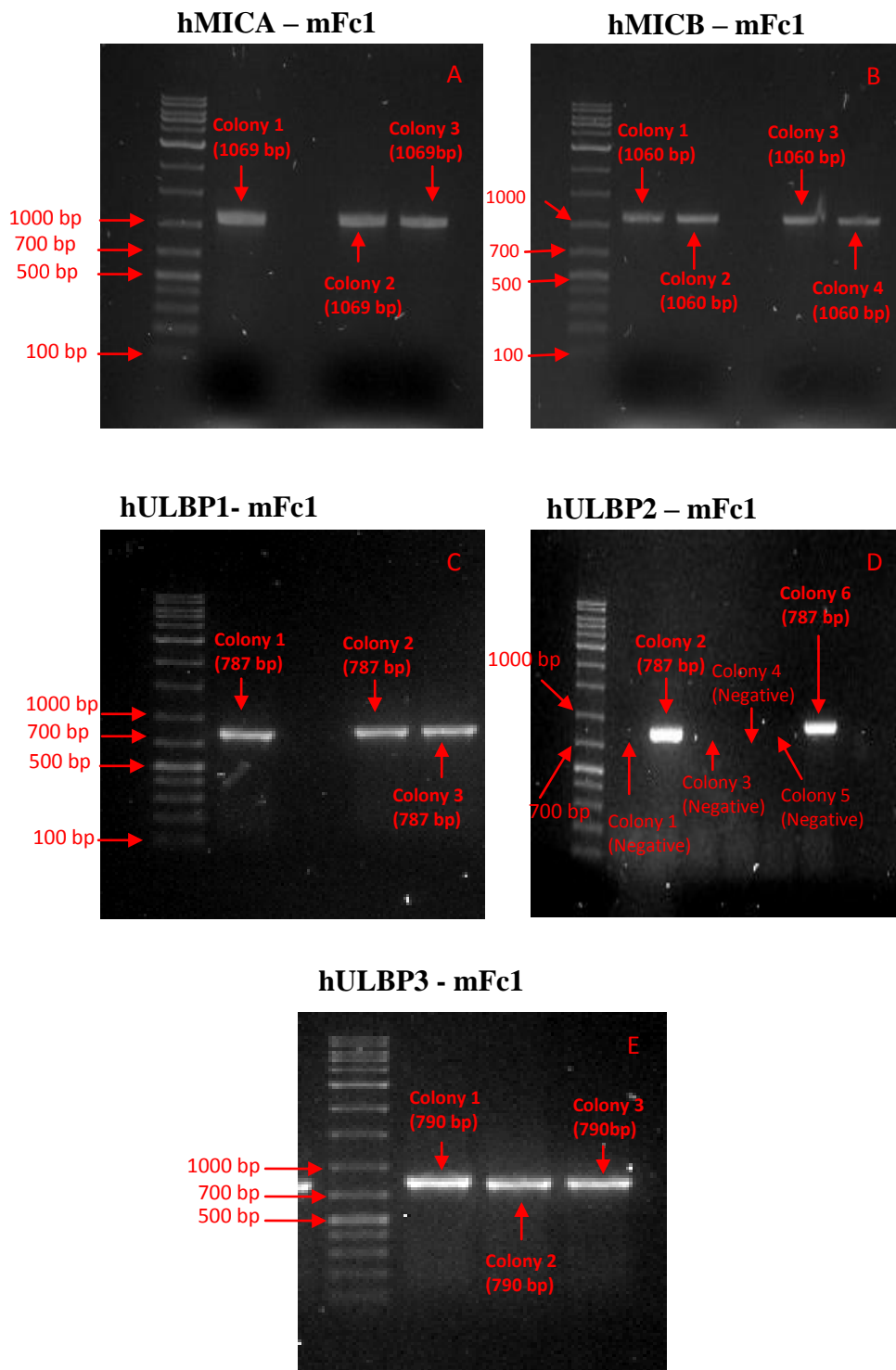


**Figure 5.6: Digestion of pFUSE-mIgG2A-Fc1 vector and recombinant PCR products with EcoRI and BspHI.** **A)** Double digest of hMICA-mFc1 and hMICB-mFc1 overlap PCR products. **B)** Incomplete digestion of the pFUSE-mIgG2A-Fc1 vector (obtained from minipreps after transformation into DH-5  $\alpha$  E.coli) by BspHI enzyme at 2 and 16 hrs due to overlapping *dam* methylation. **C)** Complete digestion of the pFUSE-mIgG2A-Fc1 vector (obtained from minipreps after transformation into  $dam^-$  E.coli) by BspHI enzyme at 2 hrs. Note that there was no visible difference between the single (EcoRI or BspHI) and double (EcoRI + BspHI) digest on the agarose gel. However the presence of another band (~159 bp) in lane 5 confirmed the double digest. Lane 1 is the 100-10000 bp DNA ladder.

### **5.2.6.2 Ligation of the digested overlap PCR products into the digested pFUSE-mIgG2A vector, and transformation into competent E.coli**

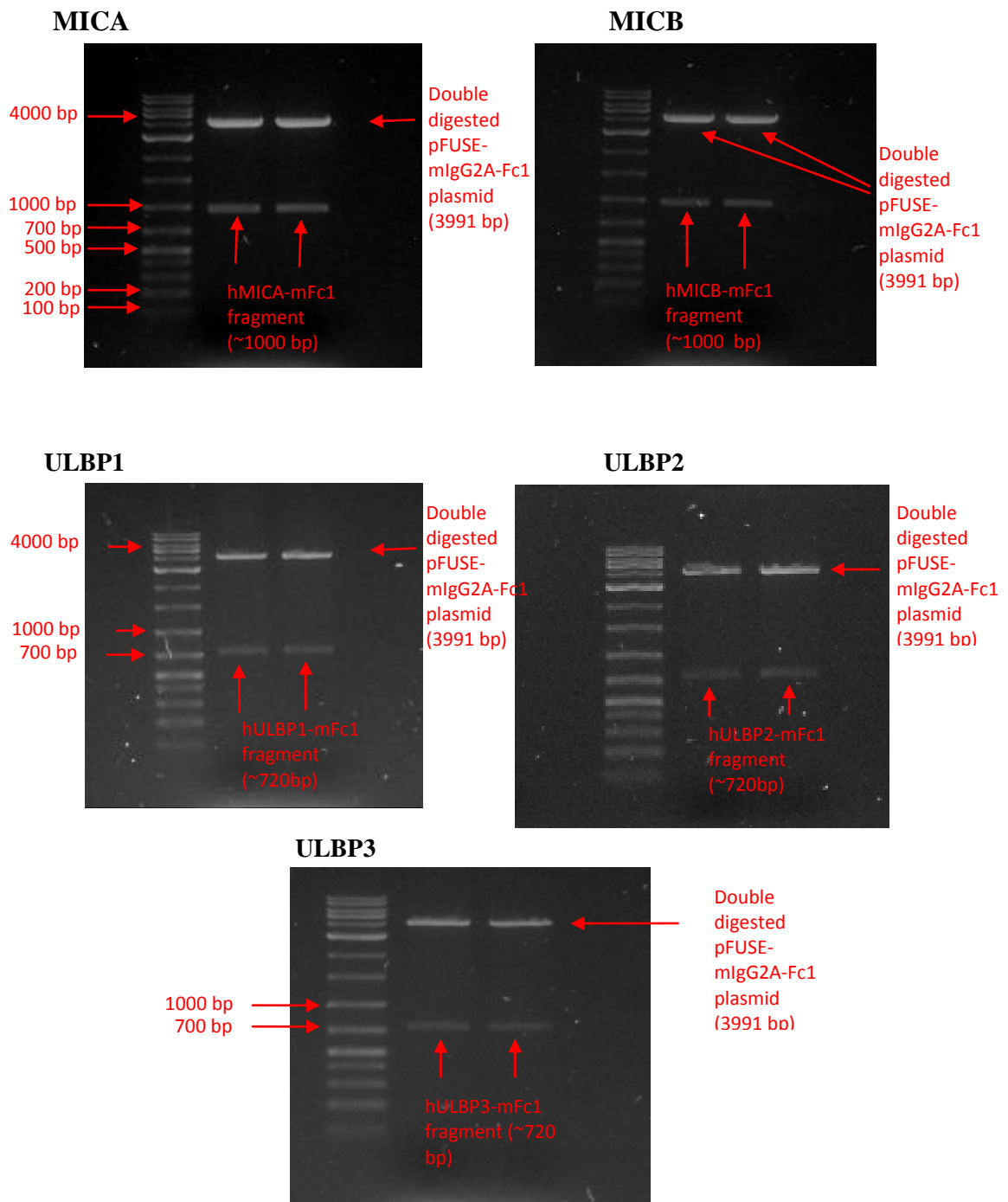
The digested purified vector and recombinant overlap PCR products were then ligated together using T4 Ligase enzyme according to manufacturer's instructions. The ligation mix was left overnight at 4 °C. The next day all the ligation mixture was transformed into competent *dam*<sup>-</sup> E.coli using the method described by Chung et al (1989). The E.coli were then plated onto LB agar with Zeocin and allowed to grow overnight at 37°C with vigorous shaking. The next day bacterial colonies were screened by colony PCR using the forward primer of PCR1 and the reverse primer of PCR2 (Chapter 2). The resulting PCR products were then visualized on 1% agarose gel/ 1X TAE buffer stained with 1 µl ethidium bromide (Figure 5.7).

Positive colonies on colony PCR were allowed to grow in 6 ml LB broth + Zeocin over night at 37°C with vigorous shaking. Plasmid DNA was purified using Qiagen Plasmid Miniprep Kit and digested with EcoRI and BspHI. The resulting DNA fragments were separated on a 1 % agarose gel/ 1X TAE buffer stained with 1 µl ethidium bromide (Figure 5.8) to confirm that the cloning of hNKG2D ligands-mFc1 recombinant genes into pFUSE-mIgG2A-Fc1 vector was successful. The insert sequences of the plasmid clones were confirmed by DNA sequencing that was carried out by Source BioScience LifeSciences (Cambridge, UK). Figure 5.9 shows an example chromatogram of the ULBP 2-mIgGaFc1 plasmid. Translating the obtained DNA sequences resulted in the expected protein sequences, although occasional single nucleotide differences from the published sequences were observed. Cultures containing the correct inserts were used for the preparation of DNA plasmids for transfection using Qiagen Plasmid Maxiprep Kit.

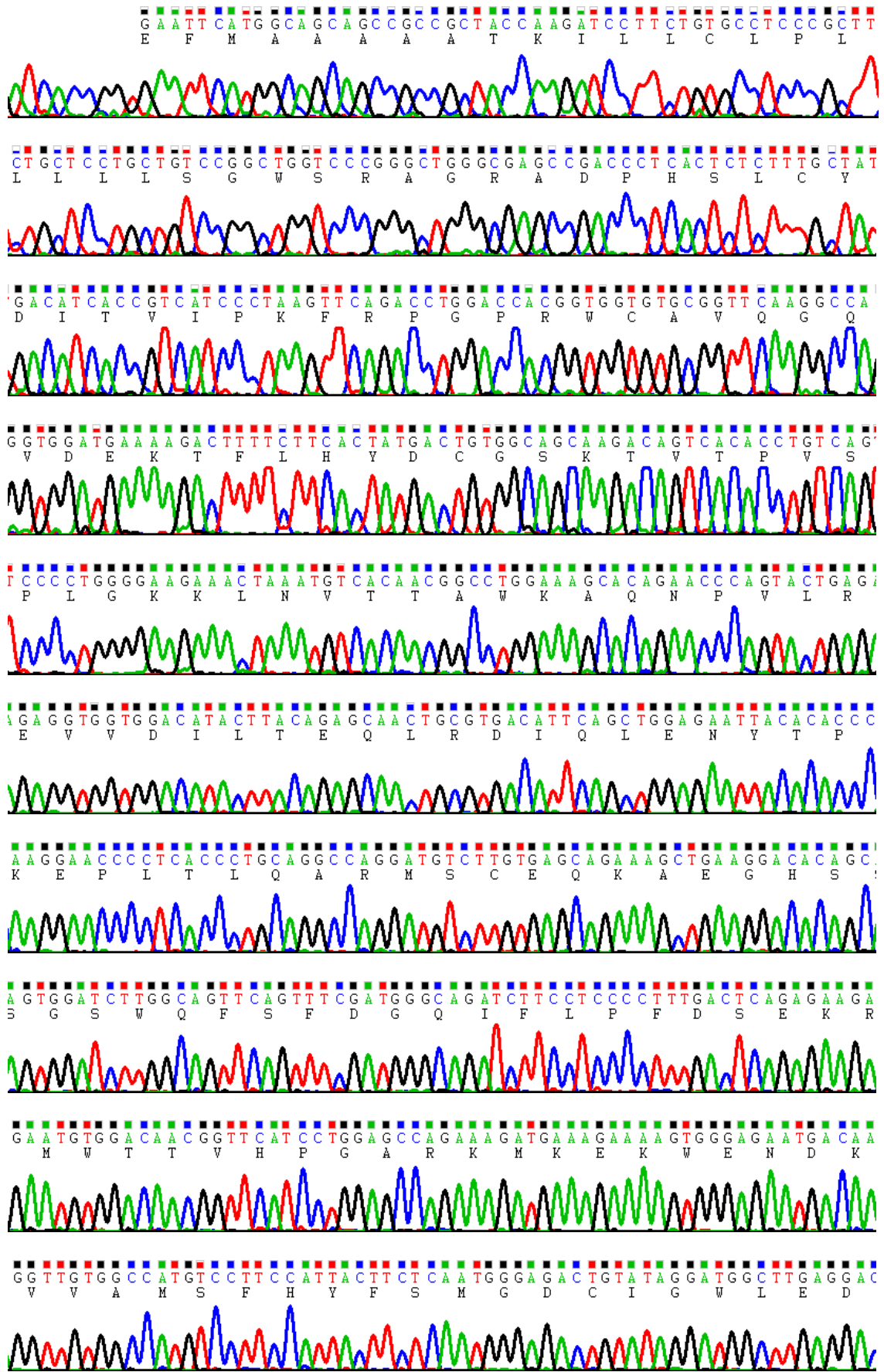


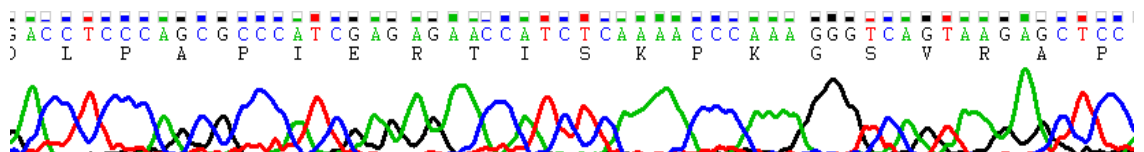
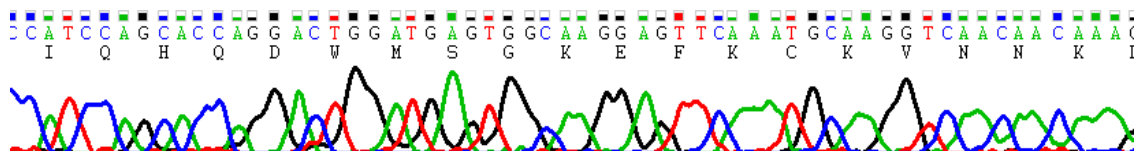
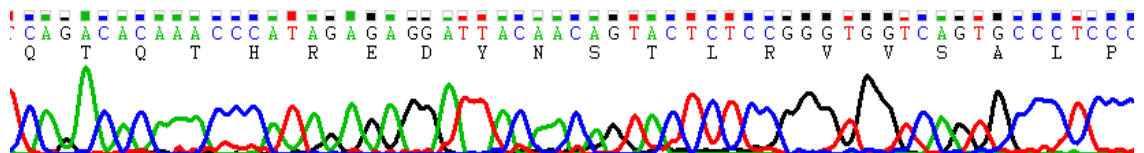
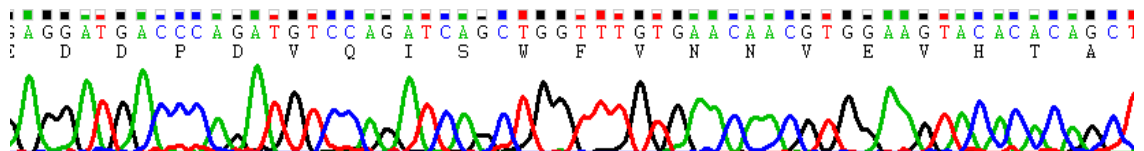
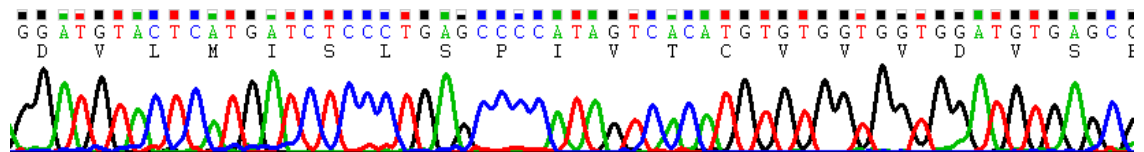
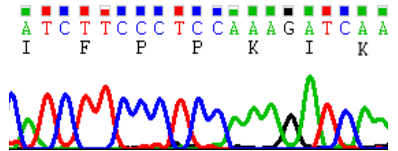
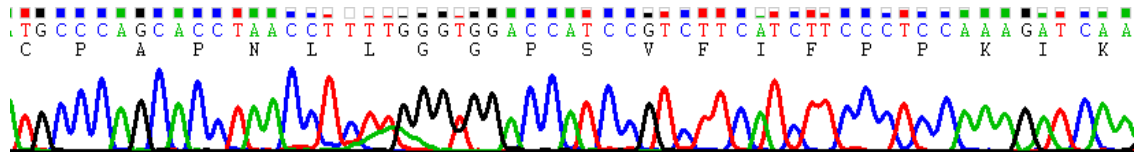
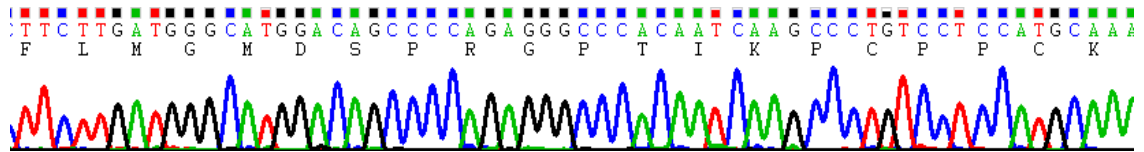
**Figure 5.7: Results of Colony-PCR.** The colonies of *dam*<sup>-</sup> *E.coli* were picked from the Zeocin LB plates after transformation with recombinant plasmid constructs. The PCR reaction was carried out using the forward primer of PCR1 and the reverse primer of PCR2 as described in Chapter 2. Thereafter, 5  $\mu$ l of resulting PCR products were loaded and visualized on 1% agarose gel/ 1X TAE buffer stained with 1  $\mu$ l ethidium bromide. In most cases, more than one colony needed to be checked for the recombinant inserts.





**Figure 5.8: Double digest of NKG2D ligands-mIgG2A-Fc1 minipreps.** The positive colonies on colony PCR were grown in LB broth + Zeocin over night. Plasmid DNA was purified using Qiagen Miniprep Kit and digested with EcoRI and BspHI enzymes. The resulting DNA fragments were separated on a 1 % agarose gel/ 1X TAE buffer stained with 1  $\mu$ l ethidium bromide. Note the presence of two bands on double digest, one corresponding to the size of digested plasmid and the other corresponding to the size of the recombinant insert.





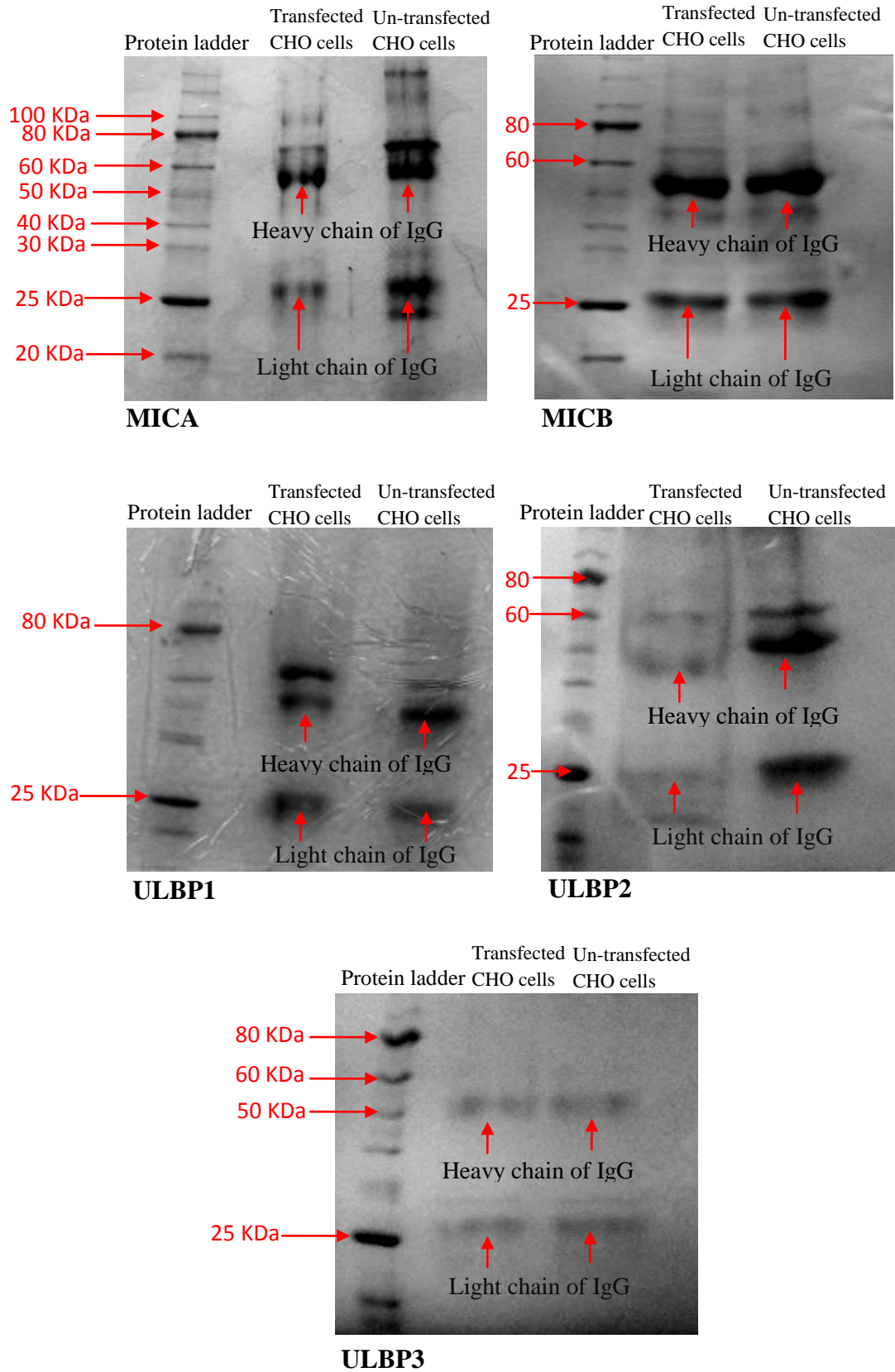
GAATTCATGGCAGCAGCCGCCGCTACCAAGATCCTTCTGTGCCTCCGCTTCTGTCTCTGCTGTCCGGCTGGTCCC  
 GCTGGGCGAGCCGACCTCACTCTTTGCTATGACATCACCGTCAATCCCTAAGTTCAGACCTGGACCACGGTGGTGT  
 GCGGTTCAAGGCCAGGTGGATGAAAAGACTTTTCTCACTATGACTGTGGCA CAAGACAGTCACACCTGTCACTCCC  
 CTGGGAAGAACTAAATGTCACAACCGCCTGGAAAGCAGAACCCAGTACTGAGAGAGGTGGTGGACATACTTACA  
 GAGCAACTGCGTGCATTTCAGCTGGAGAATTACACACCCAAGGAACCCCTCACCCCTGCAGGCCAGGATGTCTTGTGAG  
 CAGAAAAGCTGAAGGACACAGCAGTGGATCTTGGCAGTTCAGTTTCGATGGGCAGATCTTCC CCTCTTTGACTCAGAG  
 AAGAGAATGTGGACAACGGTTCATCTGGAGCCAGAAAAGATGAAAGAAAAGTGGGAGAATGACAAGTTGTGGCCATG  
 TCCTTCCATTACTTCTCAATGGGAGACTGTATAGGATGGCTTGAGGACTTCTTGATGGGCATGGACAGCCCAGAGGG  
 CCCACAATCAAGCCCTGTCTCCATGCAAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCT  
 CCAAAGATCAAGGATGTAC TCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGAC  
 CCAGATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAAACCCATAGAGAGATTAC  
 AACAGTACTCTCCGGTGGTCACTGCCCTCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCAAAGATGCAAG  
 GTCAACAACAAAGACCTCCAGCGCCATCGAGAGAACCATCTCAAAAACCAAGGGTCAGTAAGAGCTCC (1007  
 bp)

**Figure 5.9: A representative example of the NKG2D ligand-mIgG2A-Fc1 sequencing chromatogram.** The ULBP2- mIgG2AFc1 vector construct was purified by Qiagen miniprep kit, quantified by spectrophotometry and sent to Source BioScience LifeSciences (Cambridge, UK) for DNA sequencing. The hULBP2 sequence is highlighted in black and the mFc1 sequence highlighted in blue. The hULBP2-mFc1 insert (727 bp) is underlined. 5' **GAATTC**3' is the EcoRI restriction site while 5' **TCA TGA**3' is the BspHI restriction site. The total length of the sequenced segment is 1007 bp. Chromas 2.33 software (Technelysium Pty Ltd, Australia) was used to draw the chromatogram, copy the nucleotide sequence and analyse the protein translation.

### 5.2.6.3 Transfection

After confirming the results of DNA sequencing and preparation of recombinant plasmid constructs, the expression of the recombinant hMICA-, hMICB-, hUBP1-, hULBP2- and hULBP3-mFc1 clones was attempted by transient transfection into Chinese Hamster Ovary (CHO) cells. To insert the recombinant plasmids into these cells, jetPRIME transfection reagent was used. jetPRIME is a non liposomal transfection reagent with claimed transfection efficiencies between 70-90% on a wide variety of adherent cell lines ([www.polyplus.com](http://www.polyplus.com)). Transient transfection was used before proceeding with the generation of stable cell lines to permit preliminary assessment of the recombinant products. As described in Chapter 2, 20 ml of supernatants were harvested 27-96 hours after the transfection, stored at -20°C until purified by affinity chromatography using protein A/G cartridges. The purified supernatants together with the purified supernatants from un-transfected CHO cells (negative controls) were reduced and run on denaturing 10% SDS-PAGE acrylamide gels followed by Coomassie Blue staining. The expected sizes of the recombinant proteins were around 60 kDa for MICA/B and 50 kDa for ULBPs although the

predicted and actual molecular weights might not be identical due to post-translational modifications. Search in the literature revealed that the actual molecular weight of recombinant MICA/B- Fc fusion proteins in reducing conditions is between 90-100 kDa and that for ULBPs-Fc fusions proteins is between 55-60 kDa. Unfortunately, in these experiments, the production of the fusion proteins was unsuccessful in the supernatants collected from all transiently transfected CHO cells (Figure 5.10). Since the choice of transfection technology can influence transfection efficiency therefore other methods, including electroporation and combination of both cell-type specific solutions (nucleofactor<sup>®</sup> Solution) and eletroporation were also attempted (Chapter 2). However, the expected Fc fusion proteins remained undetectable. Using another cell lines such as HEK-293 was also tried but without any improvement of results.



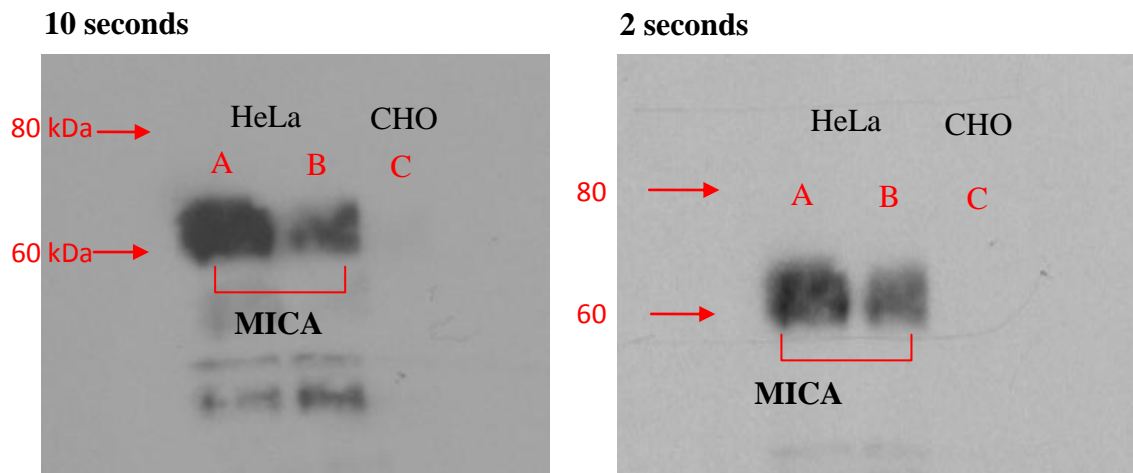
**Figure 5.10: SDS-PAGE analysis of recombinant hNKG2D ligands – mFc1 proteins.** CHO cells were transiently transfected with the recombinant pFUSE-

mIgG2A-Fc1 constructs containing the recombinant MICA-, MICB-, ULBP1-, ULBP2- and ULBP3-mFc1 genes using the jetPRIME transfection reagent. 20 ml of supernatants from the transfected as well as from un-transfected CHO cells were collected, purified by affinity chromatography using protein A/G cartridges and analyzed on 10% SDS-PAGE gels stained with Coomassie Blue. Lanes: 1, protein standards; 2, transfected CHO cells; 3 un-transfected CHO cells. Note that the heavy chain (~55 kDa) and light chain (~25kDa) of IgG were purified from the fetal calf serum present in the tissue culture medium.

#### **5.2.6.4 Western blot**

Failure of detection of recombinant fusion proteins by Coomassie Blue staining did not necessarily mean that the fusion proteins were not produced and secreted. This could be due to the relatively low sensitivity of Coomassie Blue staining. The approximate sensitivity of this dye is ~ 100-150 ng protein / spot (Dunn, 1999) and it depends largely on the visual inspection of the gel. Therefore, to verify the secretion of NKG2D ligands fusion proteins, Western blot analysis was employed. This method has the additional advantage of not detecting the heavy and light chains of IgG and other contaminating proteins present in the tissue culture medium. Using an anti-MICA/B mAbs (Clone F6, Santa Cruz biotechnology, USA), a preliminary experiment was carried out first on HeLa and Caco-2 cell lysates to check for the antibody and to optimize the Western blot protocol. This preliminary experiment detected a MICA band around 62 kDa (Figure 5.11). After this experiment, the secretion of recombinant MICA fusion proteins by transfected CHO cells was tested. Unfortunately, no protein was detected in the supernatant. This was also confirmed in the supernatants collected from the hMICA- mFc1 transfected CHO cells by electroporation, and the nucleofactor<sup>®</sup> Solution and electroporation. Based on the

results of both Coomassie Blue staining and Western blotting, it was concluded that no fusion proteins were secreted by the transfected cells.



**Figure 5.11: Western blot analysis of MICA expression in HeLa and CHO cell lysates.** The cells were cultured for 4-5 days in complete DMEM medium (for HeLa cells) or Ham F-12 medium (for CHO cells) without changing the media. Thereafter, the cells were trypsinized, centrifuged and re-suspended in 1X SDS sample buffer at a concentration of  $\sim 1 \times 10^6$  cells / 100  $\mu$ l. The cell suspensions were then boiled for 5-10 minutes and sonicated 3-4 times. 10  $\mu$ l (100,000 cells) of total cell lysate were loaded on 10% SDS-PAGE under reducing conditions and analyzed for MICA expression by Western blot, using anti-MICA antibody as a primary antibody and biotinylated goat anti-mouse HRP-labelled monoclonal antibody as a secondary antibody. **A & B** are HeLa cell lysate while **C** is CHO cell lysate. Positions of molecular weight markers (kDa) are indicated.



### 5.2.6.5 Stable transfection

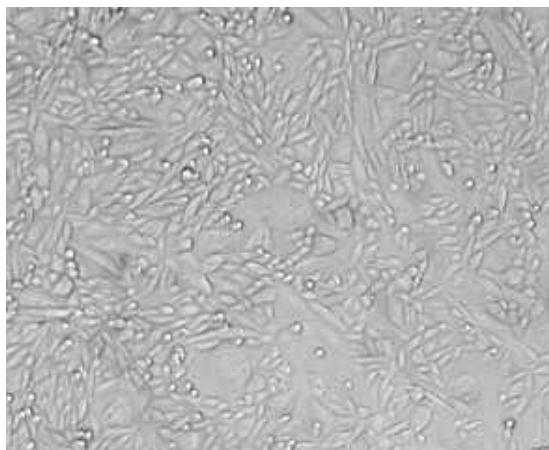
Although the secretion of recombinant fusion proteins by transient transfection failed, it was decided to try stable transfection for the following reasons:

- 1) Stable transfection often involves the integration of the gene of interest into the target cell chromosome and hence the gene of interest can be analyzed in a more natural chromatin configuration and at more natural copy number than when analyzed by transient transfection (Carey and Smale, 2001). These features allows the gene of interest to mimic its normal function more accurately than in transient transfection and therefore stable transfection is useful for studying a control region that is inactive or does not exhibit its expected activity in transient transfection (Carey and Smale, 2001).
- 2) The expansion of antibiotic resistant cell clones gives an idea about the efficiency of the transfection protocol.

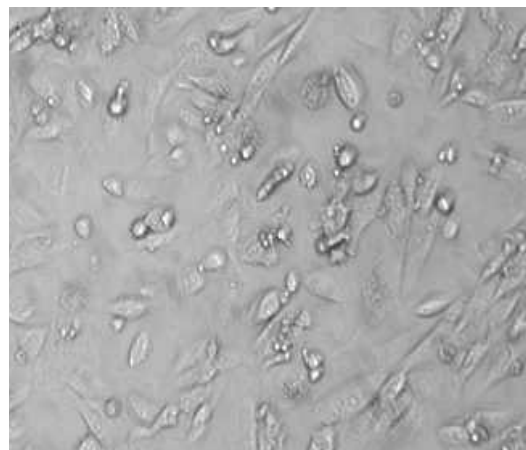
Before using Zeocin for selection purposes, the sensitivity of the untransfected CHO was determined using Zeocin concentrations of 100, 250, 400, 800 and 1000 µg/ml for up to 3 weeks. Fresh medium and antibiotic were added every 2-3 days. 400 µg/ml Zeocin killed most cells by days 14-15 (Figure 5.12). No spontaneously resistant colonies appeared at this concentration after 3 weeks. After establishing the Zeocin kill curve, the pFUSE-mIgG2A-Fc1 vectors containing the recombinant MICA-, MICB-, ULBP1-, ULBP2- and ULBP3-mFc1 genes were linearized by digestion at the NotI restriction site and purified by phenol extraction and ethanol precipitation. Thereafter, CHO cells were transfected in 12-well tissue culture plate using jetPRIME, electroporation or a combination of electroporation and nucleofactor<sup>®</sup> Solution according to standard protocols described in Chapter 2. Twenty four hours later the cells were exposed to Zeocin at a dose of 400 µg / ml to establish the recombinant CHO- Zeo<sup>R</sup> cells. Fresh medium and antibiotic were added to the

cells every 2-3 days as described previously. Resistant clones started to appear 2-3 weeks later. They were isolated, expanded in 25 cm<sup>2</sup> then in 75 cm<sup>2</sup> tissue culture flasks while maintaining continuous antibiotic selection. The supernatants were collected, purified using protein A/G cartridges and analysed for the secretion of fusion proteins by SDS-PAGE gel. While colonies resistant to the selective medium were generated with all 3 methods, the production of the fusion protein was still undetectable during all these experiments.

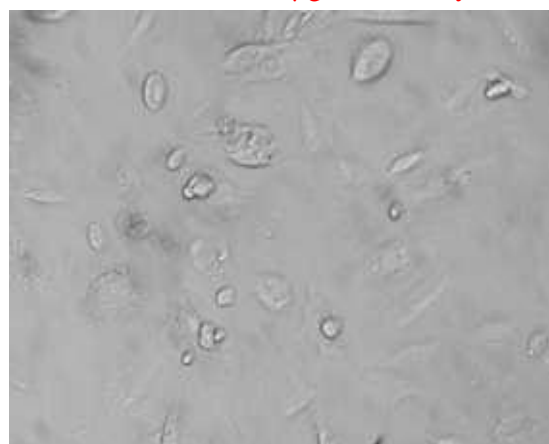
Un-transfected CHO Cells, un-selected, 15



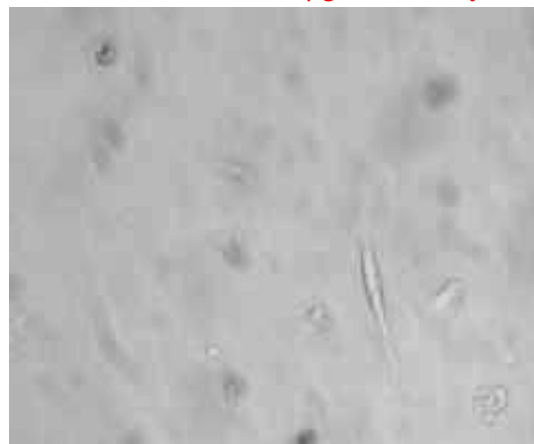
Zeocin selection, 100  $\mu\text{g} / \text{ml}$ , 15 days



Zeocin selection, 250  $\mu\text{g} / \text{ml}$ , 15 days



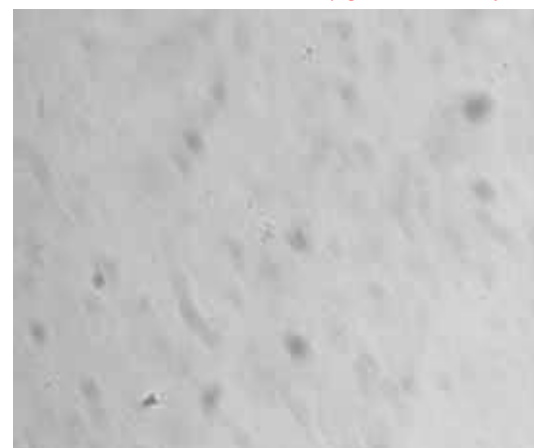
Zeocin selection, 400  $\mu\text{g} / \text{ml}$ , 15 days



Zeocin selection, 800  $\mu\text{g} / \text{ml}$ , 15 days



Zeocin selection, 1000  $\mu\text{g} / \text{ml}$ , 15 days

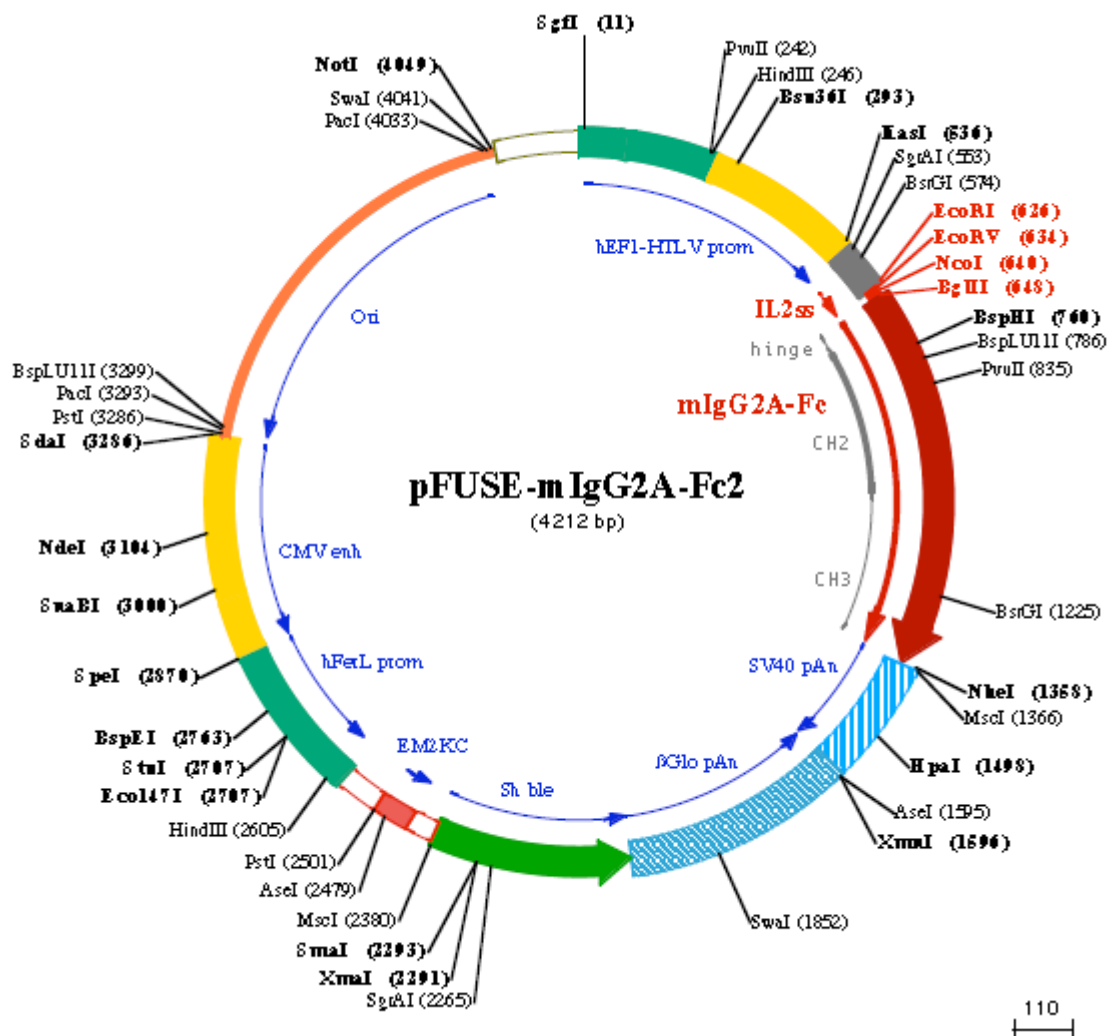


**Figure 5.12: Zeocin kill curve for CHO cells.** The CHO cells were allowed to grow in complete Ham-F12 culture medium in 12-well plate until reached  $\sim 60\text{-}80\%$  confluency. Thereafter Zeocin were added to the cells at concentrations of 100, 250, 400, 800 and 1000  $\mu\text{g} / \text{ml}$  for up to three weeks. Fresh medium and antibiotic were added every 2-3 days. The minimum concentration needed to kill most of CHO cells by 2 weeks was 400  $\mu\text{g} / \text{ml}$ .

### **5.3 Approach 2 For Expression of hNKG2D Ligand - mFc Fusion Proteins by Overlap-Extension PCR Using pFUSE-mIgG2A-Fc2 Vector**

#### **5.3.1 Introduction**

Despite significant amount of time and effort spent at cloning the recombinant genes into pFUSE-mIgG2A-Fc1 fusion vector, the production of soluble NKG2D ligands – mFc fusion proteins was unsuccessful. Following this, another pFUSE vector called pFUSE-mIgG2A-Fc2 was used in the hope of producing the desired fusion proteins. The main difference between pFUSE-mIgG2A-Fc1 and Fc2 vectors is that the Fc2 series vectors contain the human interleukin-2 signal sequence (hIL-2ss) that facilitates the secretion of Fc-fusion proteins by mammalian cells. Since the hNKG2D ligands are not naturally secreted proteins (MICA/B are transmembrane proteins while the ULBPs are GPI anchor proteins), therefore there was a possibility that their natural internal signal peptides directed translocation into the secretory pathways, but they were not cleaved when the proteins passed through the ER and hence the proteins were not processed as expected. To test this hypothesis, the sequences encoding for these signal peptides were removed from the NKG2D ligands and replaced with the hIL-2ss using the pFUSE-mIgG2A-Fc2 vector. Moreover, the Fc2 series of pFUSE vectors also contain a Kozak sequence immediately upstream of the IL-2ss. These structural differences in the vector backbone should, at least theoretically, improve the expression and secretion of the recombinant proteins that are not physiologically secreted by eukaryotic cells. Figure 5.13 shows the map of the pFUSE-mIgG2A-Fc2 vector used in these experiments.



**Figure 5.13: The pFUSE-mIgG2A-Fc2 vector map (4212 bp) showing the hEF1-HTLV promoter driving the Fc fusion, hIL-2ss, MCS (multiple cloning site), mIgG2a-Fc region and CMV enh / hFerL promoter driving the Zeocin resistance in mammalian cells. [Reproduced from www.Invivogen.com/cloning and expression / Fc fusion/pFUSE-mIgG2A-Fc2](http://www.Invivogen.com/cloning_and_expression/Fc_fusion/pFUSE-mIgG2A-Fc2).**



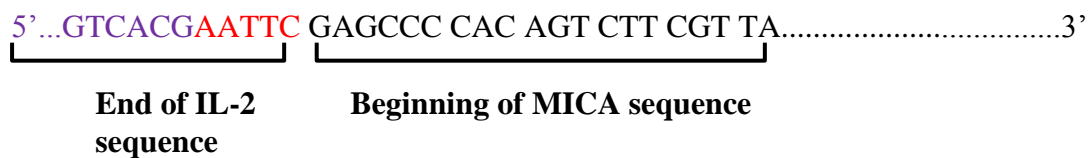
Designing a primer starting with EcoRI restriction site and annealing at the beginning of the MICA nucleotide sequence (without internal leader sequence) results in:



Digestion of the EcoRI restriction site in 5' to 3' orientation leads to:



Ligation of the two sequences after digestion with EcoRI enzyme results in:



However, this would lead to the loss of a G nucleotide at the end of the IL-2ss sequence and so if the above primer was used, this would result in a frame shift. To keep the sequence *in frame* a G nucleotide needs to be added after the C nucleotide at the end of the IL-2 sequence. Therefore the forward primer (primer a) for PCR1 should be as follows:



Finally 2-4 nucleotides are needed to be added to the beginning of this primer to facilitate digestion of the amplified gene segment. Therefore the complete forward primer for PCR1 (primer a) will be as follows:

*Complete MICA forward primer: 5' TCT GAA TTC GGA GCC CCA CAG TCT TCG TTA3'*

Similarly, the final forward primers for MICB, ULBP1, ULBP2 and ULBP3 are as follows:

*Complete MICB forward primer: 5' TCT GAA TTC GGA GCC CCA CAG TCT TCG TTA3'*

*Complete ULBP1 forward primer: 5' TCT GAA TTC GTG TGA AGT TCA AGG CCT GGT 3'*

*Complete ULBP2 forward primer: 5' TCT GAA TTC GTG TGC GGT TCA AGG CCA GGT 3'*

*Complete ULBP3 forward primer: 5' TCT GAA TTC GTG TGA GGT CCA GAG CCA GGT 3'*

#### **5.3.4 Cloning strategy**

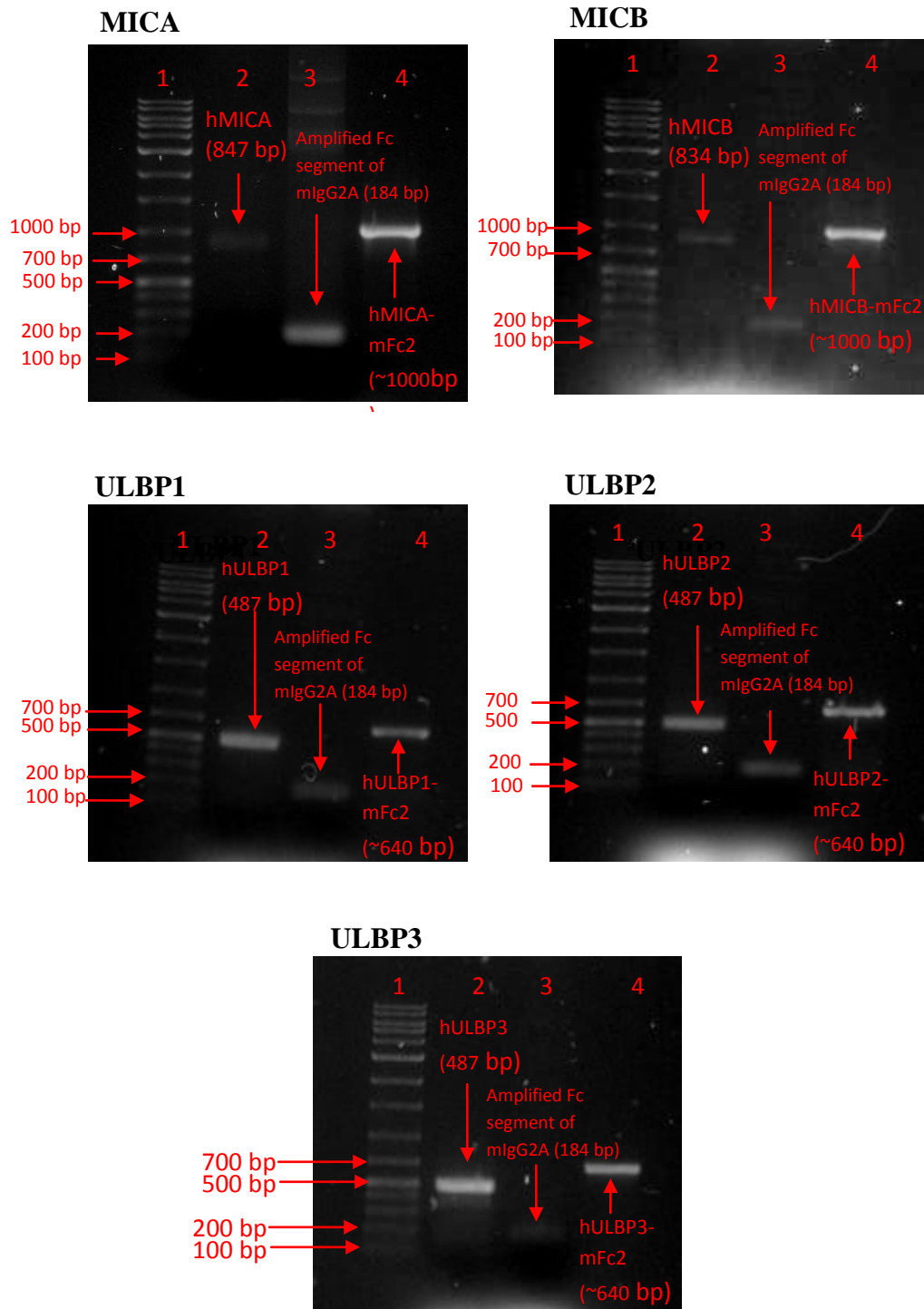
The strategy used for the cloning of the recombinant hMICA-, hMICB-, hULBP1-, hULBP2- and ULBP3-mFc2 genes into pFUSE-mIgG2A-Fc2 vector was similar to that used for the cloning of Fc1 recombinant genes into pFUSE-mIgG2A-Fc1 vector (Figure 5.1). This involved the generation of the chimeric genes by overlap extension PCR and then inserting these genes *in frame* into pFUSE-mIgG2A-Fc2 vector at the EcoRI and BspHI restriction sites.



### **5.3.5 Results 2**

#### **5.3.5.1 Generation of hMICA-, hMICB-, hULBP1-, hULBP2- and hULBP3 - mFc2 genes by overlap - extension PCR**

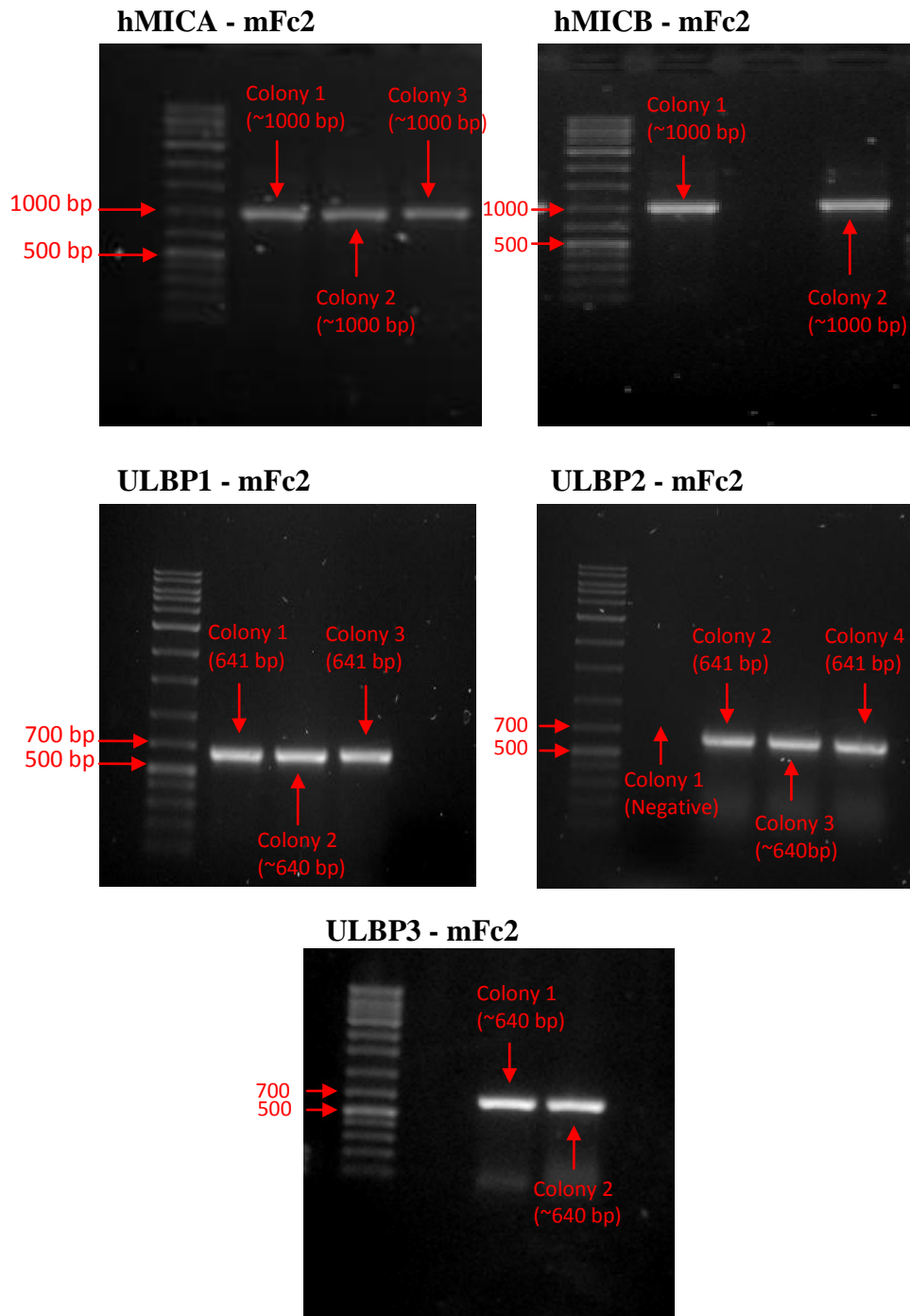
The recombinant hNKG2D ligand- mFc2 genes were constructed by overlap extension PCR using the gene specific forward primers described above (Section 5.3.3) in combination with the gene specific reverse primers for PCR1 and the forward and reverse primers used in the first cloning attempt (primers b, c and d, section 5.2.4). The resulting amplified PCR fragments were 846 bp for MICA, 834 bp for MICB, 487 bp for ULBP1, ULBP2 and ULBP3 respectively, while the Fc sequence of mIgG2a including the overlap area is 184 bp (Figure 5.14). The sizes of the recombinant (fused) PCR products were ~ 1000 bp for MICA/B-mFc2 and ~ 640 bp for ULBP1-, ULBP2- and ULBP3-mFc2 (Figure 5.14).



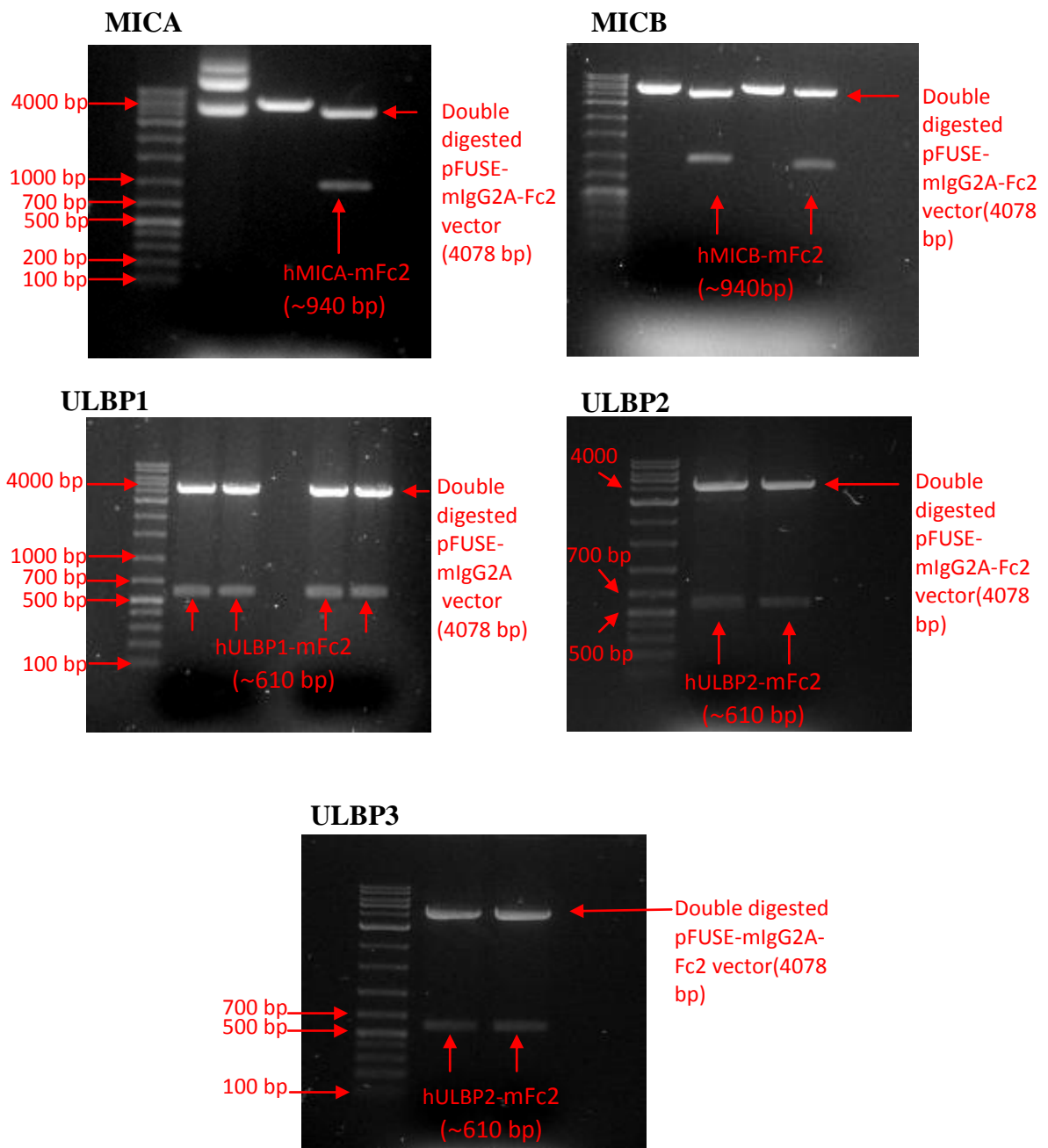
**Figure 5.14: Generation of the recombinant MICA-, MICB-, ULBP1-, ULBP2-, and ULBP3-mFc2 genes by overlap-extension PCR.** Lane 1 is the 100-10000 bp DNA ladder; Lane 2 is the PCR1 in which the genes encoding for extracellular region of MICA, MICB, ULBP1, ULBP2, and ULBP3 (without internal leading sequence) were amplified; Lane 3 is the PCR2 in which part of the sequence encoding for Fc sequence mIgG2A was amplified; Lane 4 is the overlap PCR products (fusion of PCR1 and PCR2).

### 5.3.5.2 Cloning of hNKG2D ligands – mFc2 recombinant genes

Double digestion of the pFUSE-mIgG2A-Fc2 vector with the EcoRI and BspHI enzymes lead to release of two linear fragments, one large fragment of ~ 4078 bp and one small fragment of ~ 134 bp. Similarly, the MICA-, MICB-, ULBP1-, ULBP2- and ULBP3 - mFc2 recombinant genes were double digested by these two enzymes. The rest of the cloning process was identical to that described earlier for the pFUSE-mIg2A-Fc1 constructs. In brief the digested purified recombinant genes were ligated to purified pFUSE-mIgG2A-Fc2 vector using T4 ligase and the plasmid constructs were then transformed into competent *dam*<sup>-</sup> *E. coli* cells. DNA from Zeocin resistant colonies was purified using standard DNA minipreps before the plasmid was tested for the presence of inserts by colony PCR (Figure 5.15), confirmed by endonuclease digestion of minipreps (Figure 5.16) and sequencing. When the sequencing of the pFUSE-mIgG2A vector confirmed the presence of the recombinant genes, maxiprep freezing stocks were made and stored at -80 °C to be used for transfection. Transient expression of the generated plasmids was carried out as described above (Section 5.2.6.3) and detailed in Chapter 2. Again, no resolution of a single band that approximates the predicted or glycosylated molecular weight was detected in the supernatant collected from all transfected CHO cells or HEK293 as determined by SDS-PAGE gel followed by Coomassie Blue staining. This was confirmed by Western blotting for MICA.



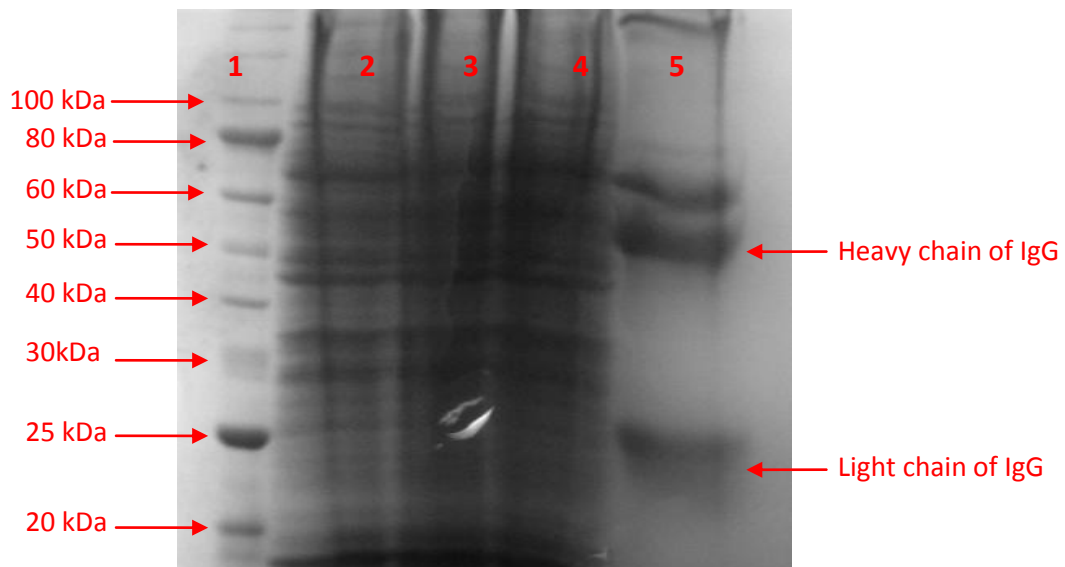
**Figure 5.15: Results of colony – PCR.** The hMICA-, hMICB-, hULBP1-, hULBP2- and hULBP3- mFc2 plasmid constructs were transformed into *dam*<sup>-</sup> E.coli. Some of the Zeocin resistant colonies were picked up and tested for the presence of the recombinant DNA inserts by colony- PCR using the forward primer for PCR1 and reverse primer for PCR2. The resulting DNA fragments were separated on a 1 % agarose gel/ 1X TAE buffer stained with 1  $\mu$ l ethidium bromide.



**Figure 5.16: Endonuclease double digestion of hMICA-, hMICB-, hULBP1-, hULBP2-, hULBP3 - mFc2 plasmid constructs.** The recombinant plasmids containing the recombinant NKG2D ligands were double digested by EcoRI and BspHI. This resulted in release of two fragments as shown on 1% agarose gel/TAE buffer stained with ethidium bromide. Upper bands corresponded to the size of the digested plasmid while the lower bands corresponded to the size of the recombinant DNA insert.

### 5.3.5.3 Analysis of transfected CHO lysate for the recombinant protein production

Failure of detection of the recombinant NKG2D ligands in the supernatant collected from the transfected CHO cells raised the possibility that these proteins were produced but not cleaved and secreted by the cells. To check for this possibility, cell lysates from the hMICA-mFc1, hMICA-mFc2 and un-transfected CHO cells respectively were prepared using the method described in section 5.2.6.4. These cell lysates were then run on 10% acrylamide SDS-PAGE gel followed by Coomassie Blue staining and analysed for recombinant MICA proteins (Figure 5.17). No differences were found between the cell lysates collected from the MICA-Fc1, MICA-Fc2 and un-transfected CHO cells indicating that no recombinant proteins were produced.



**Figure 5.17: Whole CHO Cell Lysate - Coomassie Blue staining.** 10  $\mu$ l (100,000 cells) of total cell lysate were loaded on 10% SDS-PAGE under reducing conditions and analyzed for MICA expression by Coomassie Blue stain. Lane 1 is the protein ladder; lane 2 is the MICA-mFc1 transfected CHO cells; lane 3 is the MICA-mFc2 transfected CHO cells; lane 4 is the un-transfected CHO cells; lane 5 is supernatant collected from un-transfected CHO cells and purified by affinity chromatography. No visible differences were found between the transfected and un-transfected cell lysates.

## **5.4 Approach 3 For the Production of hNKG2D Ligand - mFc Fusion Proteins by Simple Ligation Using pFUSE-mIgG2A-Fc2 Vector**

### **5.4.1 Introduction**

As the overlap - extension PCR based cloning strategies failed, a simpler approach for the construction of the Fc-fusion proteins was tried. This is based on a simple ligation of the genes encoding for extracellular regions of hNKG2D ligands into the MCS of the pFUSE – mIgG2A-Fc2 vector. This approach, which was described by Flanagan et al (2007), leaves a few extra nucleotides between hNKG2D gene sequence and Fc-fragment, thus extending the hinge segment between the gene of interest and the immunoglobulin fusion partner. This strategy was carried out on MICA only as a prototype hNKG2D ligand.

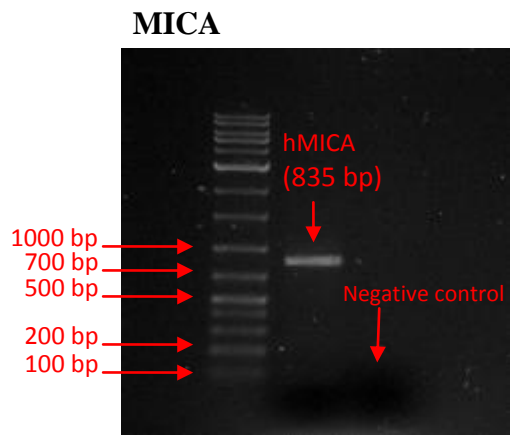
### **5.4.2 Cloning strategy**

The multiple cloning site of the pFUSE-mIgG2A-Fc2 vector is a short nucleotide sequence (21 bp in length) containing only three restriction sites for EcoRV, NcoI and BglII (Figure 5.13). Since the hMICA sequence contains restriction sites for both EcoRV and NcoI (analysed by NEB cutter), these restriction sites could not be used for inserting the hMICA genes into the pFUSE-mIgG2A-Fc2 vector. Instead the genes encoding for the extracellular region of hMICA was planned to be inserted between the EcoRI restriction site at the end of the IL-2ss and BglII restriction site in 5' to 3' orientation respectively. Here, unlike the overlap-extension PCR, the BglII restriction site separates the hMICA sequence from the mFc sequence (for complete sequence see Chapter 9).

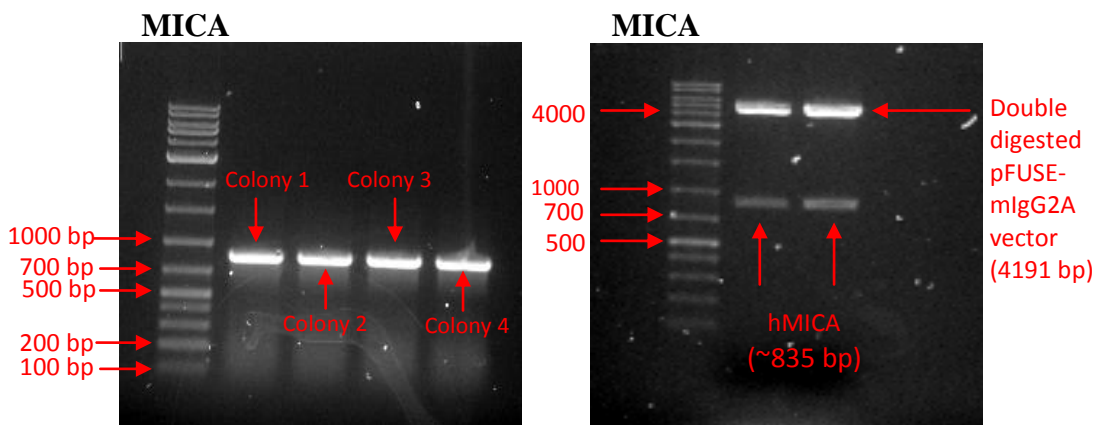




were tested for the presence of the hMICA by colony-PCR (Figure 5.19 A). Plasmid DNA was purified from positive colonies using a Qiagen Miniprep Kit and confirmed positive by endonuclease digestion (Figure 5.19 B). When the sequencing of the pFUSE-mIgG2A vector confirmed the presence of the hMICA gene, freezing stocks from maxipreps were made and stored  $-80\text{ }^{\circ}\text{C}$  for transfection. CHO cells were then transfected with the pFUSE-mIgG2A vector containing the recombinant hMICA gene. Transient transfection with the jetPRIME transfection reagent was used and the supernatant collected after 72 hours. After collection, supernatants were purified by protein A/G cartridges and tested for the secretion of hMICA by running on SDS-PAGE gel stained with Coomassie Blue staining. Unfortunately, no secretion of protein was detected. This failure of secretion was confirmed by Western blotting using anti human MICA antibody.



**Figure 5.18: Agarose gel electrophoresis of amplified hMICA gene.** The HeLa cDNA was used a template for amplification of the genes encoding for the extracellular portion of hMICA. The size of the amplified segment including the flanking EcoRI and BglII restriction sites is 835 bp.



**Figure 5.19: A) Colony – PCR of the hMICA.** All the 4 colonies were positive for hMICA. **B) Results of EcoRI and BglII digestion of hMICA-mFc2 plasmid minipreps.**

## **5.5 Discussion**

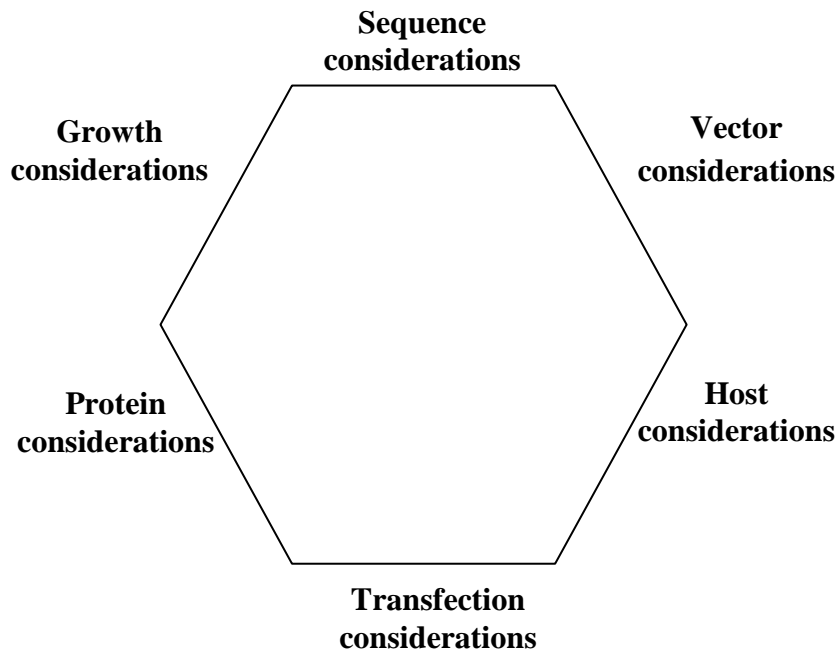
### **5.5.1 Expression of NKG2D ligands as Fc fusion proteins**

Results presented in the previous chapter did not support the idea that signals initiated from cross-linking of NKG2D receptors by their mAbs are different from that initiated by NKG2D ligands since it was found that cross-linking NKG2D with an antibody on the surface of NK cells was sufficient to induce IL-6 mRNA expression and protein production. However, it was decided to express NKG2D ligands as soluble Fc fusion proteins for the following reasons: **1)** To see if the stimulation of NKG2D receptors via their soluble ligands induces IL-6 mRNA expression and protein secretion profiles distinct from those produced by antibody cross-linking. **2)** To see if there was a preference between various NKG2D ligands in the induction of IL-6 secretion. **3)** To test the hypothesis of epitope – dependent receptor triggering. André et al (2004) suggest that NKG2D epitopes targeted by mAb and ligands might be distinct. Since the structure of the NKG2D ligands is highly heterogeneous, therefore NKG2D engagement may lead to the initiation of distinct signalling events (André et al, 2004). As no documented ligands are still known for the other activating receptors (NKp46, NKp44 and NKp30) these cannot be utilised for similar studies.

### **5.5.2 Failure of Fc- protein production: possible implications**

The results presented in this chapter showed that the MICA-, MICB-, ULBP1-, ULBP2- and ULBP3- mFc genes were successfully cloned into pFUSE-mIgG2A-Fc1 and Fc2 vectors whether by overlap extension PCR or by simple ligation. This was demonstrated by colony-PCR, restriction digestion of the recombinant plasmids and by DNA sequencing of the plasmids. However, in all these

experiments the production and secretion of the recombinant hNKG2D ligand- mFc fusion proteins failed irrespective of the methods used. The reason for this is difficult to ascertain, but there are a number of possible factors that can be involved. When considering these results, it is logical to find an explanation for the expression problems at the levels of gene, vector, host, protein and induction conditions (see below).



**Figure 5.20: Possible explanations for the failure of recombinant MICA-, MICB-, ULBP1-, ULBP2- and ULBP3 - mFc fusion protein production and secretion.** Different parameters which might affect the protein expression were considered at levels of gene, vector, host, protein and induction conditions.

### 5.5.2.1 Sequence parameters that can affect protein expression

#### 1. Sequence translation and open reading frame

The most critical process in construction of an immunoglobulin fusion gene is the actual joining of the sequence encoding the target protein and the immunoglobulin sequence (Hollenbaugh and Aruffo, 2002). Any mistakes that result in an out-of-frame fusion, which sometimes arise inadvertently during PCR amplification of the target gene, will result in no fusion protein being produced (Hollenbaugh and Aruffo, 2002). Moreover, mutations during PCR amplification of the target gene can result in the creation of stop codons and result in severely truncated and rapidly degraded proteins. Although this might be the most important explanation for failure of protein production and secretion by the host cells, it was unlikely to be the case in this study for several reasons. First, all the primers used throughout this study were designed to ensure the accurate amplification of the target genes and the proper overlap of the of the hNKG2D ligand sequences to mFc sequence. Second, all the recombinant fusion sequences were checked to ensure that they run in *open reading* frame and hence they do not contain premature stop codons. Third, all the PCR experiments were carried out using the high fidelity Phusion® DNA polymerase that has a low reported error rate (approximately 50-fold lower than that of *Thermus aquaticus* DNA polymerase, and 6-fold lower than that of *Pyrococcus furiosus* DNA polymerase). Finally, all recombinant plasmid constructs containing the recombinant MICA-, MICB-, ULBP1-, ULBP2- and ULBP3-mFc inserts were sent for plasmid DNA sequencing and the presence of the expected sequence was confirmed before transfection into the mammalian cells was done.

## 2. Initiation of translation: role of Kozak sequence

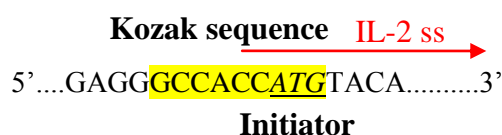
Gene expression is regulated at multiple levels; including the transcriptional level in which the gene is copied to produce mRNA (a primary transcript) with essentially the same sequence as the gene; and the translational level where the mRNA is translated into protein (Day and Tuite, 1998). Compared to transcriptional regulation, translational control of mRNAs allows for more rapid changes in cellular concentration of the encoded proteins (Sonenberg and Hinnebusch, 2009). The process of translation can be divided into initiation, elongation, termination, and ribosome recycling (Merrick, 2009). Most regulation is exerted at the first stage, where the AUG start codon is identified and decoded by the methionyl tRNA (Met-tRNA<sub>i</sub>) (Sonenberg and Hinnebusch, 2009). Initiation of translation proceeds via recognition of the m<sup>7</sup>G cap at the 5' terminus of the mRNA followed by scanning of the ribosome to the initiation codon, which is identified by proximity to the 5' -end and sequence context (Kozak, 1986*a*; Kozak, 1999; Welch et al, 2011). Kozak (1986*b*) found that the presence of an A/GCCACC sequence upstream of the start codon (AUG) followed by another G plays a major role in the initiation of the translation process.

### Eukaryotic (Kozak sequence)



#### Initiator

The absence of the Kozak sequence might, at least partially, explain the failure of the recombinant NKG2D ligand-mFc1 protein production in the experiments detailed in Section 5.3 of this chapter. However, it is unlikely to explain the failure of recombinant protein production in the experiments described in Sections 5.3 and 5.4. In these experiments a pFUSE-mIgG2A-Fc2 vector was used that has an internal Kozak sequence just upstream of the IL-2ss as shown below.



### **3. mRNA instability and mRNA secondary structures: Role of poly (A) tail and cis – elements**

Studies have demonstrated that the modulation of mRNA stability plays an important role in regulating gene expression (Guhaniyogi and Brewer 2001; Hollams et al, 2002). The stability of eukaryotic mRNA transcripts is determined by multiple factors including the presence of the m<sup>7</sup>G cap at the 5' end and the poly (A) tail at the 3' end of mRNA molecule (Hollams et al, 2002). Both the cap and poly (A) tail function to prevent mRNA degradation and improve translation efficiency (Shimotohno et al, 1977; Preiss and Hentze, 1998). The presence of a poly (A) tail at the 3' end of the cloned sequences was not a problem since all the pFUSE vectors used have the Simian Virus 40 late polyadenylation (SV40 pAn) signal directly downstream of the Fc sequence (Figures. 5.2 & 5.13). The presence of this sequence enables efficient cleavage and polyadenylation reactions resulting in high levels of steady-state mRNA (Carswell and Alwine, 1989). Similarly the presence of the UR5' segment of HTLV1 virus (part of the vector promoter) at the 5' end of the fusion sequence further increases the stability of mRNA (Attal et al, 2000).

Among the other factors that determine the stability of a given mRNA transcript is the presence of sequences within the mRNA known as cis-elements (Hollams et al, 2002; Cheneval et al, 2010). Cis elements are RNA (or DNA) sequence-specific regions which can be bound by trans-acting RNA (or DNA)-binding proteins to inhibit or enhance mRNA decay (or gene expression) (Strachan and Read, 2004). The presence of negative cis- elements within the mRNA transcripts may shorten their half- life and enhance their degradation. In addition, some sequences in the mRNA molecule might be complementary with the ribosome binding site and/or translation start site. This leads to the formation of RNA secondary structures which

further inhibit protein translation. Research was attempted to determine the mRNA structure and to predict the cis-elements and mRNA secondary structures. Approaches include both experimental and computational methods (Welch et al, 2011).

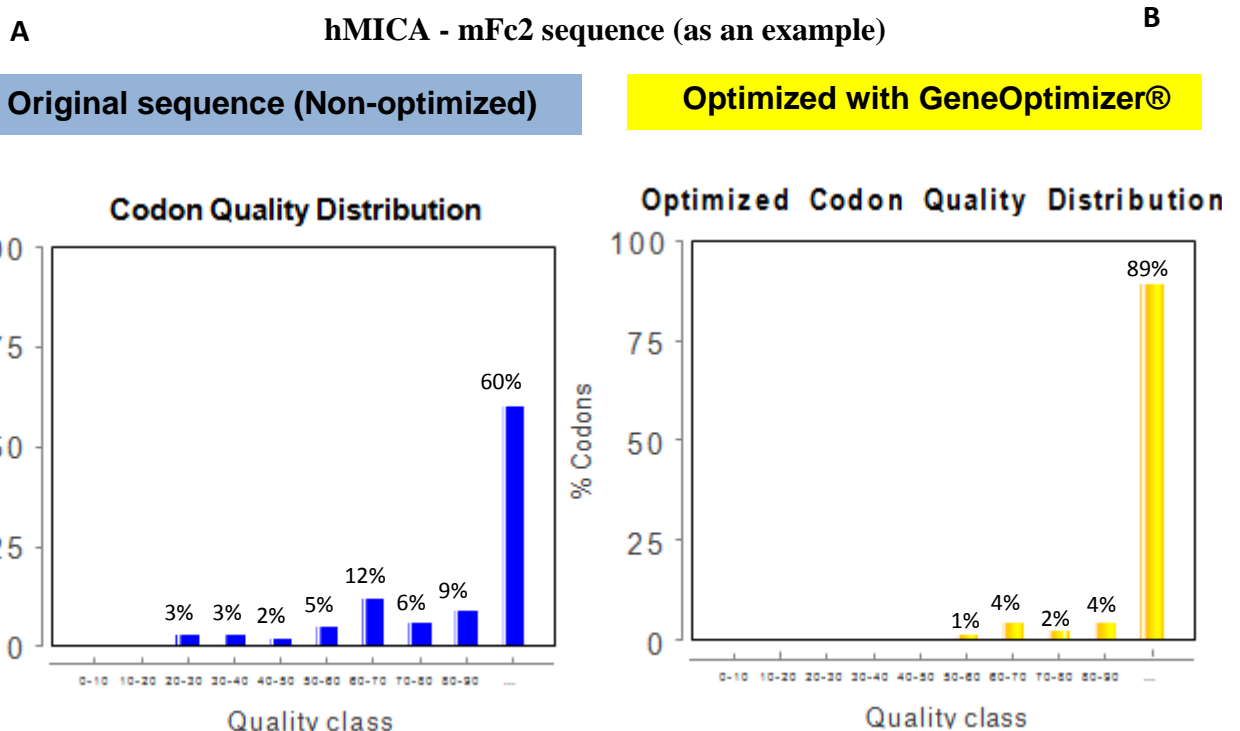
#### **4. Codon bias and GC content of the gene**

Each amino acid is encoded from one (methionine and tryptophan) to as many as six different codons (arginine, leucine, and serine) in the canonical genetic code (Welch et al, 2011). Different organisms use synonymous codons with different apparent preferences (Welch et al, 2011). Several studies have proven the immense impact of codon choice on gene expression in mammalian cells (Graf et al, 2004; Gustafsson et al, 2004; Fath et al, 2011). These studies concluded that proteins which are difficult to express outside their original context might contain codons that are rarely used in the desired host. This is particularly true when expression of a non-mammalian gene is attempted in a mammalian host (Fath et al, 2011). However, besides inter-species variations, evidence accumulated in the last decade suggesting codon usage even differs among different tissues of the same host (Plotkin et al, 2004; Fath et al, 2011). Recently, differences in tissue-specific expression of individual tRNA species and the relative abundance of tRNA- isoacceptors were described to strongly correlate with the codon usage of genes highly expressed in specific tissues (Fath et al, 2011). Studies on the more commonly expressed mammalian housekeeping genes indicate that they are usually associated with higher GC-content than low-expressing genes (Kudla et al, 2006; Fath et al, 2011).

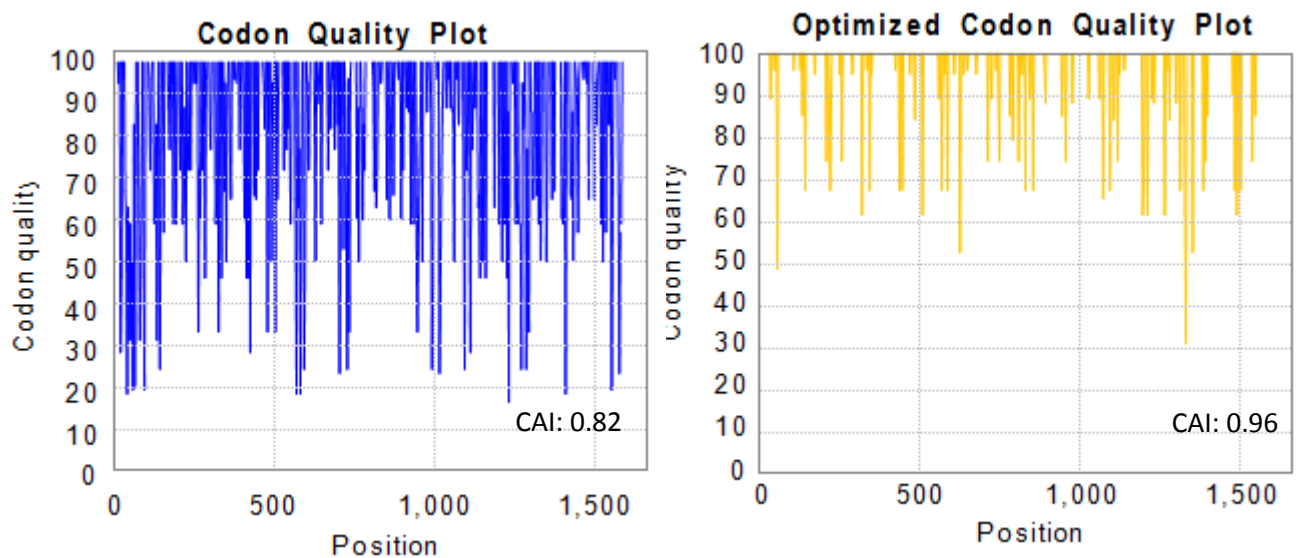


### 5.5.2.1.1 Suggested solution for the sequence problems

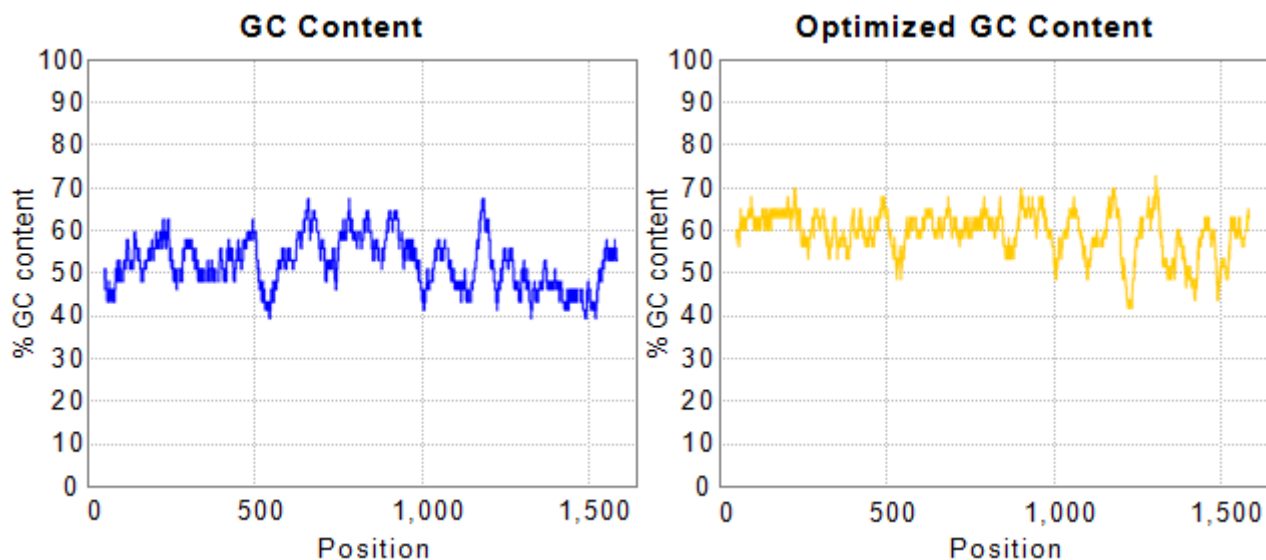
Obviously some of these sequence parameters which negatively affect the expression of proteins such as mRNA instability, negative cis elements and codon bias are internally inherited and cannot be controlled experimentally. However, the above findings strongly necessitate a comprehensive optimization strategy that involves a simultaneous modulation of multiple sequence parameters to enhance an optimal performance of human genes in autologous expression systems. This can be achieved by using a gene optimization technology that can alter both the naturally occurring and recombinant gene sequences to achieve the highest possible levels of productivity in any given expression system. This technology (OptimumGene™ from GeneScript or GeneOptimizer® from Life Technologies) takes into consideration a variety of critical factors involved in different stages of protein expression, such as codon adaptability, mRNA structure, and various *cis*-elements in transcription and translation. Using codon optimization tools, the chances of recombinant NKG2D ligand - mFc proteins to be expressed in CHO or HEK-293 cells were not very high and the gene sequences needed to be optimized (see below).



**Figure 5.21: The histograms show the percentage of distribution of hMICA-mFc2 codons which fall into a certain quality class.** The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the CHO mammalian expression organism. Codons with values lower than 30 are likely to hamper the expression efficiency. **A)** Original hMICA-mFc2 sequence before optimization. This non-optimized gene can reduce the efficiency of translation or even disengage the translational machinery. **B)** hMICA-mFc2 after optimization using geneOptimizer® from Life Technologies. The codon usage was adapted to the codon bias of *Cricetulus griseus* (CHO) genes.



**Figure 5.22: The plots show the quality of the used codon at the indicated codon position to be expressed in CHO cells.** The possibility of high protein expression level correlates with the value of Codon Adaptation Index (CAI). A CAI of 1.0 is considered to be ideal while a CAI of > 0.9 is rated as high chance for expression in the desired expression organism. The lower the number, the higher the chance the gene would be expressed poorly. **A)** Original hMICA-mFc2 sequence before optimization. CAI: 0.82. **B)** hMICA-mFc2 after optimization using geneOptimizer® from Life Technologies. CAI after optimization: 0.96.



**Figure 5.23: The average GC content.** The ideal percentage range of GC content is between 30 to 70%. Any peaks outside of this range will adversely affect transcriptional and translational efficiency. **A)** Original hMICA-mFc2 sequence before optimization. Average GC content: 52.98%. **B)** hMICA-mFc2 after optimization using geneOptimizer® from Life Technologies. Average GC content: 61%.

The advantages of this optimization process were that it automatically allowed the following cis-acting sequence motifs, which negatively inhibit protein secretion, to be avoided (where applicable in the hNKG2D ligand- mFc sequences): **1)** internal TATA-boxes, chi-sites and ribosomal entry sites. **2)** AT-rich or GC-rich sequence stretches **3)** RNA instability motifs **4)** repeat sequences and RNA secondary structures. **5)** Cryptic splice donor and acceptor sites in higher eukaryotes. In addition, regions of very high (> 80 %) or very low (< 30 %) GC content have been avoided where possible.

This optimized hMICA-mFc2 sequence generated by the software was highlighted in yellow and aligned to the non-optimized sequence below.

```

Query 1      ATGTACCGGATGCACTGCTGTCTGTATGCCCCTCTCTCTGGCCCTCGTACCAAATCC 60
Sbjct 1      ATGTACAGGATGCACTCCTGTCTTGCATGCACTAAGTCTTGCCTTGTACGAAATCC 60
Query 61     GAGCCTCACTCCCTCGGTAAACCTGACGTGCTGTCTTGGGACGGCTCCGTGCAGTCT 120
Sbjct 61     GAGCCACACAGTCTCGTTAAACCTCACGTGCTGTCTTGGGATGGATCTGTGCAGTCA 120
Query 121    GGCTTTCTTGGCCGAAAGTGCACCTGGACGGCAGCCCTTCTCTGAGATACGACGGCAGAAC 180
Sbjct 121    GGCTTTCTTGTCTGAGGTACACTCTGGATGGCAGCCCTTCTCTGCGTATGACAGGCAGAAA 180
Query 181    TGCCGGGC AAGCC CAGGGACAGTGGGC GAAGATGTCTGGG AACCAAGACCTGGGAC 240
Sbjct 181    TGCCGGGC AAGCC CAGGGACAGTGGGC GAAGATGTCTGGG AATCAAGACATGGGAC 240
Query 241    AGAGAGACAAGGGACTGACCGGCAACGG AAGGACCTGAGATGACCCTGGCCCAATC 300
Sbjct 241    AGAGAGACAAGGGACTGACCGGCAACGG AAGGACCTGAGATGACCCTGGCTCAATC 300
Query 301    AAGGACCAGAAAGAAGGCC TGCACAGCCTCAGGA AATCAGAGTGTGCGAGATCCAAGAG 360
Sbjct 301    AAGGACCAGAAAGAAGGCTTGCATTCCTCAGGA AATAGGTTGTGAGATCCAAGAA 360
Query 361    GACAACCTCCACCCGGTCTCTCCAGCACCTTCTACTACGACGGCGAGCTGTTCTCTGAGCCAG 420
Sbjct 361    GACAACAGCACCAAGGACTCTCCAGCACTTCTACTACGATGGCGAGCTTCTCTCTCCAA 420
Query 421    AACCTGGAACCGGAGGAATGGACGTGCCCCAGTCTCCAGAGCCAGACCTGGCCATG 480
Sbjct 421    AACCTGGAACCTGAGGAATGGACGTGCCCCAGTCTCCAGAGCCAGACCTGGCCATG 480
Query 481    AACGTGCGGAACTTCTGAAAGAGGACGCCATGAAGACCAAGACC ACTA CACGCATG 540
Sbjct 481    AACGTGAGGAACTTCTGAAAGAGGATGCCATGAAGACCAAGACC ACTA CACGCATG 540
Query 541    CAGCCGACTGTCTGCAGGAAGTCCGGAGATACCTGGAATCCGGCGTGGTCTGCGGAGA 600
Sbjct 541    CATGCAAGACTGTCTGCAGGAAGTCCGGAGATACCTGGAATCCGGCGTGGTCTGAGGAGA 600
Query 601    ACCGTGCCCTCTATGGTCAAGTACCAGATCCGAGGCCAGCGAGGGCAACATCACCCTG 660
Sbjct 601    ACCGTGCCCTCTATGGTCAAGTACCAGATCCGAGGCCAGCGAGGGCAACATCACCCTG 660
Query 661    ACCGTGCAGAGCCTCCCTCTTCTACCCCCGGAATATCATCTGACATGGCGCAGGATGGC 720
Sbjct 661    ACCGTGCAGAGCCTCCCTCTTCTATCCCCGGAATATCATCTGACATGGCGCAGGATGGC 720
Query 721    GTGTCCTGTCTCAGGATAC CAGCAGTGGGGGAGGTGCTGCCTGACGGCAATGGAC 780
Sbjct 721    GTATCTTTGAGCCACGACAC CAGCAGTGGGGGAGGTGCTGCCTGATGGCAATGGACC 780
Query 781    TATCAGACCTGGGTGGCCACAGGATCTGCGAGGGAGAGGA CAGCGGTTACCTGTAC 840
Sbjct 781    TACAGACCTGGGTGGCCACAGGATCTGCGAGGGAGAGGA CAGCGGTTACCTGTAC 840
Query 841    ATGGAACACACCGGAAACCACTCCACCACCCTGTGCCCTTCTCCAGGGGCTTACATC 900
Sbjct 841    ATGGAACACACCGGAAACCACTCCACCACCCTGTGCCCTTCTCCAGGGGCTTACATC 900
Query 901    AAGCCTTGCCCCCTTGCAAATGCCCTGCTGCTGGGGGAGACCCTCCGTCTTC 960
Sbjct 901    AAGCCTTGCCCCCTTGCAAATGCCCTGCTGCTGGGGGAGACCCTCCGTCTTC 960
Query 961    ATCTTCCCACCAAGATCAAGGAGTGTCTATGATCTCCCTGTCCCCCATGTGACCTGT 1020
Sbjct 961    ATCTTCCCACCAAGATCAAGGAGTGTCTATGATCTCCCTGTCCCCCATGTGACCTGT 1020

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Query 1021 GTGGTGGTGGACGTGTCGAGGACGACCCGATGTGCAGATCAGTTGGTTGTGAACAAC 1080
Sbjct 1021 GTGGTGGTGGATGTGAGCGAGGATGACCCGATGTGCAGATCAGTTGGTTGTGAACAAC 1080

Query 1081 GTGGAAGTGCACACGGCTCAGACCCAGACACACAGAGAGGATACAACAGTACCTCCGG 1140
Sbjct 1081 GTGGAAGTGCACACGGCTCAGACCAACACATAGAGAGGATACAACAGTACTCTCCGG 1140

Query 1141 GTGGTGTCCGCCCTCCCTATCCAGCACCAGGACTGGATGTCCGGCAAAGAATCAAGTGC 1200
Sbjct 1141 GTGGTCAAGTCCGCCCTCCCTATCCAGCACCAGGACTGGATGAGTGGCAAGGAATCAAGTGC 1200

Query 1201 AAAGTGAACAACAAGATCTGCCAGCCCCATCGAGCGGACCATCTCCAACTTAAAGGC 1260
Sbjct 1201 AAAGTGAACAACAAGACTCTGCCAGCCCCATCGAGGAGACCATCTCCAACTTAAAGGC 1260

Query 1261 AGCGTGCGGGCTCCGAGGTGTACTGTGTGCCACCTCCTGAGGAAGAGATGACCAAGAAA 1320
Sbjct 1261 TCAGTAAAGAGCTCCGAGGTGTACTGTGTGCCACCTCCTGAGGAAGAGATGACTAAGAAA 1320

Query 1321 CAAGTGACCTGACCTGCATGGTCAACGACTTCATGCCGAGGACATCTACGTGGAATGG 1380
Sbjct 1321 CAGGTGACTCTGACCTGCATGGTCAACGACTTCATGCCGAGGACATCTACGTGGAATGG 1380

Query 1381 ACAACAATGGCAACACCGAGCTAACTACAAGAACACCGAGCCCGTGTCTGGACTCCGAC 1440
Sbjct 1381 ACCAACAATGGCAACACAGAGCTAACTACAAGAACACTGAAACCGTGTCTGGACTCCGAT 1440

Query 1441 GGCTCCCTACTTCATGTACTCCAAAGCTGCGGTGGAAAAGAACTGGGTGGAAACGGAAAC 1500
Sbjct 1441 GGTTCCTACTTCATGTACTCCAAAGCTGAGGTGGAAAAGAACTGGGTGGAAAGAAAT 1500

Query 1501 TCCTACTCCTGCTCCGTGGTGCACGAGGGCTGCACAATCACCACACCACCAAGTCTTC 1560
Sbjct 1501 AGCTACTCCTGTTCCTGTGGTGCACGAGGGCTGCACAATCACCACACCACCAAGTCTTC 1560

Query 1561 TCCCGGACCCCGCAAGTGA 1581
Sbjct 1561 TCCCGGACCCCGCAAGTGA 1581

```

**Figure 5.24: Alignment of optimized hMICA-mFc2 sequence with non optimized sequence.** Although there were many nucleotide differences between the two sequences, both of them encode for the same hMICA-mFc2 fusion protein. The optimized gene, which can be made synthetically, should therefore allow high and stable expression rates in CHO cells.

### 5.5.2.2 Leader sequence (Signal sequence)

Successful protein secretion requires effective translocation of the protein across the endoplasmic reticulum (ER) before being transport into Golgi complex (Tan et al, 2002; Stern et al, 2007). A signal peptide at the N-terminal end of the nascent polypeptide directs the molecule into the ER lumen. This is cleaved off by signal peptidase once it has served its purpose of targeting the protein to, and importing it

into the ER (Stern et al, 2007). Thus the signal peptide serves as a “stamp” that direct the protein towards its destination pathway. In the experiments presented in Section 5.2, the native signal peptide sequences of hMICA, hMICB, hULBP1, hULBP2 and hULBP3 were used. The use of these signal peptide sequences was based on the assumption that many secreted eukaryotic proteins are efficiently processed in mammalian expression host via their native signal sequences (Tan et al, 2002). Since both membrane and secreted proteins are synthesized on ER membrane bound ribosomes and enter the ER to be moved and matured through the same secretory pathway (Lodish et al, 2000), it was decided to keep these signal sequences without change despite the fact that these proteins are not naturally secreted. However, failure of secretion of the fusion proteins with these native signal sequences led to a change of strategy. It was hoped that removing the native leader sequences of hMICA, hMICB, hULBP1, hULBP2 and hULBP3 and replacing them with the leader sequence of the secreted IL-2 protein (hIL-2ss) may improve the secretion. Nevertheless, the new strategy also failed, and whether this was due to the low efficiency of the hIL-2ss as a secretion signal needs further clarification. Stern et al (2007) found that the IL-2 signal peptide is inferior to other signal peptides in secreting the Gaussia luciferase (Gluc) recombinant protein in CHO cells. They concluded that the choice of the signal peptide may have a major impact on the synthesis/secretion of the recombinant proteins in mammalian expression system. The different efficiencies of signal peptides with which a nascent polypeptide enters the secretory pathway may be attributed to the diversity of their primary sequences. Although all the commonly available leader sequences possess a positively charged N-terminal region, a central hydrophobic region and a C-terminal cleavage region (Nothwehr and Gordon, 1990), they are rarely conserved in their primary structures (Tan et al, 2002). Moreover, alterations or

modifications in the basic N-terminal and/or the central hydrophobic domains of the same signal peptide can alter the secretion of mature protein. Zhang et al (2005) found that by increasing the basicity of the N-terminal and hydrophobicity of the classic IL-2 signal peptide increased the secretion of two different proteins by 3 fold.

### **5.5.2.3 Vector considerations**

The most important vector parameter to be considered is the vector promoter driving the expression of the recombinant fusion proteins. A promoter is a specific DNA sequence usually located upstream of the gene (towards the 5' region) and facilitate its transcription (DuBois, 1990). In the experiments presented in this chapter, the pFUSE-mIgG2A-Fc1 and pFUSE-mIgG2A-Fc2 vectors were used. Both of them have the same hybrid promoter known as hEF1-HTLV. This promoter consists of two parts: **1)** The human Elongation Factor 1 $\alpha$  (EF-1 $\alpha$ ) core promoter which exhibits a strong activity and long lasting expression of a transgene *in vivo* (Kim et al, 1990). **2)** The RU5' segment of human T-Cell Leukemia Virus (HTLV) -1 Long Terminal Repeat which enhances the stability of mRNA (Attal et al, 2000). This is thought to be the strongest promoter ever found because it combines the unique properties of both the EF-1 $\alpha$  promoter and the RU5' segment of HTLV-1. Several studies have shown that EF-1 $\alpha$  is a stronger and more efficient promoter than the more commonly used cytomegalovirus (CMV) promoter for the generation of both transient and stable cell clones in different cell lines. Chung et al (2002) found that the EF-1 $\alpha$  promoter robustly drove reporter gene expression in transiently transfected mouse embryonic stem cell lines, while the CMV promoter was inactive. Moreover, they also demonstrated that the EF-1 $\alpha$  promoter effectively drove gene expression at different stages of cell development while the CMV promoter had only significant activity at

the later stage of cell differentiation. Tokushige et al (1997) found that the generation of stable human hepatocellular carcinoma (HCC) and murine myeloma cell lines expressing the Hepatitis C virus (HCV) core protein were only established using constructs driven by the EF-1  $\alpha$  promoter and not by constructs driven by the CMV promoter. Similarly, Teschendorf et al (2002) compared the efficacy of the EF-1  $\alpha$  promoter to the promoter of CMV for the generation of stable colon carcinoma cell (HT-29) transfectants using green fluorescent protein (GFP) expression cassettes both *in vitro* by FACS analysis and *in vivo* after HT-29 clones were grown as xenografts in nude mice. They found that >97% of all cells homogeneously expressing GFP were generated with the EF-1  $\alpha$  promoter compared to only up to 60% of the cells were GFP-positive using the CMV promoter. *In vivo* tumours carrying the EF-1  $\alpha$  promoter were homogeneously GFP-positive, whereas the CMV promoter gave rise to a scattered pattern of GFP expression. Based on the results of these studies, it was concluded that the failure of fusion protein production and/or secretion was unlikely to be due to vector issues. This conclusion was supported by two other observations from these experiments. Firstly, the hMICA-mFc2 fusion protein failed to be expressed in the pcDNA3 vector which is a CMV driven vector (data not shown). Secondly, stable CHO cell lines expressing the Zeocin resistant marker with all recombinant hMICA-, hMICB-, hULBP1-, hULBP2- and hULBP3-mFc plasmid constructs were successfully generated, suggesting that the failure of expression of these genes could not be attributed to other vector causes such as vector loss or instability.



#### **5.5.2.4 Host considerations**

##### **1. Prokaryotic vs. eukaryotic expression systems**

To achieve the goal of this chapter, it was necessary not only to be successful in secreting these fusion proteins, but also to produce biologically and functionally active proteins. Although the prokaryotic expression system is the easiest system to express the recombinant Fc-fusion proteins, the use of this system didn't fulfil the aim of this study since several factors limited its use in these experiments. When eukaryotic proteins are produced in prokaryotes they usually lack posttranslational modifications (PTMs) including phosphorylation, glycosylation and acetylation that are often necessary for their function (Stern et al, 2007). In addition, recombinant protein expression is usually under the control of strong inducible promoters from plasmids which results in unusually high transcription rates (Gasser et al, 2008). Such over-production of recombinant proteins is stressful for the bacterial host and has a profound negative impact on protein quality (Gasser et al, 2008). Finally, recombinant proteins produced by prokaryotic cells very often fail to fold and reach their native conformation due to diverse events such as improper codon usage and lack of disulfide-bond formation (Gasser et al, 2008; Stern et al, 2007). Mis-folded protein species usually deposit as amorphous masses of insoluble material called inclusion bodies. Refolding recombinant proteins from inclusion bodies can be challenging, taking a lot of time, and the yields of correctly folded proteins can be very low (Tao et al, 2010). Obviously the most effective system to express a recombinant protein with full biological functions that are comparable to those of their natural counterparts is the use of eukaryotic mammalian cells. Taking all these factors in considerations, it was decided to use the eukaryotic expression system because this system provides the best chances of obtaining fully functional proteins.

## **2. The use of CHO cells: Advantages and disadvantages**

CHO cells were used in this study because they are the most commonly used cells for recombinant protein production (Andersen and Krummen, 2002). Despite the availability of many cell lines, nearly 70% of all therapeutic recombinant proteins produced today are made in CHO cells (Jayapal et al, 2007). Beside this, CHO cells have a lot of other advantages that make them a logical first choice to express recombinant fusion proteins. First, although these cells are not of human origin, they have been proven to produce proteins with glycoforms that are both compatible and bioactive in humans (Jayapal et al, 2007). Second, these cells have been demonstrated to be the safest host for synthesis of biologic therapeutic agents (Jayapal et al, 2007). Third, purified CHO cell products have been proven to contain no more than picogram levels of contaminating CHO DNA per dose of the product (Wurm, 2005). Lastly, CHO cells are very easy to transfect using different transfection methods. However, the use of CHO cells may not be without problems. A quality issue that was observed with recombinant Fc- molecules is the fragmentation of the Fc moiety after purification by affinity chromatography (Robert et al, 2009). This degradation was first attributed to many causes that can be related to the protein sequence and its conformations, or to physico-chemical environment such as storage, temperature, pH and buffers (Liu et al., 2006; Cohen et al, 2007; Gaza-Bulsecu and Liu, 2008). However, accumulating evidence suggests that the cause for this degradation may be biological rather than a physico- chemical action and was attributed to the activity of proteases released by cultured cells (Robert et al, 2009). Many cell lines are known to produce and release such proteases such as NS0 myeloma cells (Spens and Häggström, 2005), hybridoma cells (Karl et al, 1990), and CHO cells (Satoh et al,1990; Elliott et al, 2003; Robert et al, 2009). Although the

extracellular proteolytic degradation of recombinant Fc-fusion proteins could be an explanation for the experienced expression problems, two factors make this possibility less likely. **1)** In all experiments complete culture medium supplemented with serum was used for the expression of Fc-fusion proteins. It is known that serum consists of up to 10% protease inhibitors (Cook and Chen, 1988), particularly of serine proteases (McGilligan and Thomas, 1991). The protein components of this supplemented medium are also substrates for extracellular proteases and may compete with the recombinant proteins for degradation by these proteolytic enzymes, thus decreasing the overall proteolysis of recombinant proteins. **2)** Because the expression of the recombinant NKG2D ligand - mFc fusion proteins in CHO cells failed, so it was worth testing another cell line (HEK-293) which are not known to produce extracellular proteases. However, similarly to CHO cells, production and secretion of the recombinant proteins in HEK-293 also failed.

#### **5.5.2.5 Transfection considerations: The choice of transfection method**

Several methods for transfection of nucleic acids have been developed over the years. These include chemical reagents such as DEAE-dextran and calcium phosphate, physical methods such as electroporation, viral carriers such as adenoviral vectors, and lipid methods such as liposomal and non-liposomal lipids. The choice of the most appropriate transfection technology among these different methods may be difficult. Ideally, the transfection method should be fast and easy to perform, give high efficiencies and reproducible results, cause minimal cytotoxicity and preferably be compatible with serum and antibiotics. A non-liposomal lipid transfection reagent, jetPRIME, was used to perform transfections. It is a fast and easy to perform and extremely efficient on a wide variety of cell lines even in the presence of serum and

antibiotics, with reported transfection efficiencies between 70-90%. It also requires only low amounts of nucleic acid per transfection; hence resulting in very low cytotoxicity. However, since the choice of transfection technology can strongly influence the transfection efficiency, therefore two additional methods based on electroporation and a combination of both cell-type specific solutions (nucleofactor<sup>®</sup> Solution) and electroporation were also introduced. The failure to detect Fc fusion proteins was unlikely to be due to transfection since it was able to generate stable CHO clones that were resistant to Zeocin in all experiments indicating that the transfection process itself was working, ensuring the entry of the plasmid into the host cells.

#### **5.5.2.6 Protein considerations**

##### **1. Protein mis-folding**

Quite often the target protein itself, due to properties of its structure or its activity, is a strong determinant of expression yield (Welch et al, 2011). In fact protein stability has been identified as an important determinant of the probability that a protein will be retained in the ER (Ellgaard and Helenius, 2003; Hegde and Ploegh, 2010). The protein may be particularly unstable in the host, especially if it is poorly folded due to inherent instability (Privalov, 1996), mistranslation (Lee et al, 2006; Welch et al, 2011), or improper post-translational modifications (Dobson, 2003) resulting in diverse non-native conformations. Mis-folded proteins formed in the ER are transported back to the cytosol where they are degraded by cytosolic proteasomes (Tsai et al, 2002). Protein folding problems are one of the major challenges in large scale protein production. Although it is known that a given sequence of amino acids almost always folds into a 3D structure with certain functions, it is impossible to

predict, with high precision, the exact folding pattern. A computational approach has been introduced to help scientists to predict the 3 D structure of the protein depending on the amino acid sequence (Dill et al, 2007). This approach greatly improved the prediction of 3D structure of simple proteins consisting of a single polypeptide chain (Dill et al, 2007). However, for complex proteins containing multiple structural domains or for proteins consisting of multiple subunits, prediction of their 3D structures can be challenging (Dill et al, 2007). Since the Fc-fusion proteins are complex proteins consisting of multiple subunits therefore, prediction of their folding and their 3 D structure is impossible. Protein mis-folding could be one of the most likely explanations for the failure of NKG2D ligand-mFc protein expression. Indeed the theory of Fc-fusion proteins mis-folding in these experiments is supported by the following facts **1)** Searching the web for the commercially available recombinant MICA proteins revealed that the manufacturers used sequences with 9 amino acids longer than the used MICA sequence. These 9 amino acids (GKVLVLQSH) are actually stalk amino acids and not part of the extracellular portion of the MICA protein according to the sequence described by Bahram et al (1996a). This is also observed with other proteins namely, MICB, ULBP1, ULBP2 and ULBP3 where there were also additional amino acids at the C-terminal ends of their extracellular portions. These observations give rise to an important question whether these additional amino acids are essential for proper folding and hence proper expression of these proteins. **2)** We could not detect any of the recombinant NKG2D ligand-mFc fusion proteins neither in the supernatant nor in the cell lysate of transfected CHO cells. This indicated that these proteins are either not produced or being degraded inside the cells. Degradation of the proteins inside the cells favours the possibility that these proteins are mis-folded since the mis-folded proteins are rapidly removed by the cells (Tsai et

al, 2002; Ellgaard and Helenius, 2003). **3) Protein mis-folding in Fc-fusion proteins** was observed by others who suggested that the Fc-fusion proteins are in fact artificial constructs that do not appear in nature, and *some* constructs may therefore be susceptible to problems with folding and/or secretion (Lee et al, 2007).

## **2. Protein toxicity**

Protein toxicity is another possibility for the failure of Fc-fusion expression and secretion. Although MICA, MICB and ULBPs proteins are naturally occurring proteins, it is not known whether their fusion with mFc protein renders them toxic to host cells. Protein toxicity often leads to host suppression of protein synthesis and expression (Welch et al, 2011).

### **5.5.2.7 Growth and induction parameters**

Several factors related to the expression environments have been observed to influence the expression level. Among these factors is the cell culture temperature. Some studies suggested that shifting of the cell culture temperature from 37 °C to a lower temperature (28 to 33 °C) may improve the expression of recombinant proteins in mammalian cells. The effect ranged from moderate improvement (Kaufmann et al, 1999; Yoon et al, 2003) to significant improvement (Lee et al, 2007). The principle is to allow an initial phase of rapid and maximum cell growth at 37 °C followed by a stationary phase (growth arrest phase) at a lower temperature where cell viability is extended and the cellular energies are conserved towards the production of recombinant proteins (Kumar et al, 2008). Indeed, this parameter was not tested since all these studies presumed at least some degree of protein production and they are

aimed to improve this production; while in experiments presented in this chapter there was no detectable expression at all.

The failure to produce the desired NKG2D ligand-Fc fusion proteins was a major disappointment. Many of the above considerations could have been tried. However, due to the time intensive nature of any further troubleshooting of the protein expression process meant that work on the production of these proteins was not feasible.

# Chapter 6

## Involvement of Different NKG2D Ligands in NKG2D-Induced IL-6 Secretion by NK Cells



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## **6.1 Introduction**

As discussed in the previous chapter a significant amount of time and effort was spent at cloning NKG2D ligands into Fc fusion vectors and expressing these constructs. The aim was to provide further evidence of the role of NKG2D signalling pathways in the initiation of IL-6 production by NK cells. Since this approach was unsuccessful an alternative strategy to study the role of NKG2D ligands in inducing IL-6 secretion was pursued. Here stably transfected CHO cell lines expressing the full length human MICA, MICB, ULBP1, ULBP2 and ULBP3 ligands on the cell surface were co-cultured with human PBNK cells. These cells were a gift from Dr. Hugh Reyburn (Spain). The work could provide valuable data on the affinity of the various ligands for this receptor for the following reasons: **1)** The CHO cells are non-human, therefore any detection of human IL-6 in the supernatant after their co-culture with human NK cells can be attributed directly to the NKG2D ligand-NKG2D receptor interaction since no other factors such as adhesion molecules or NCR ligands are present. **2)** In contrast to other human cell lines that express NKG2D ligands and secrete human IL-6 at the same time (such as HeLa cells), these cells are non human and therefore their secretion of IL-6 (if any) cannot be detected using a human IL-6 ELISA kits.

## **6.2 Approach used for the detection of human IL-6 from co-cultures**

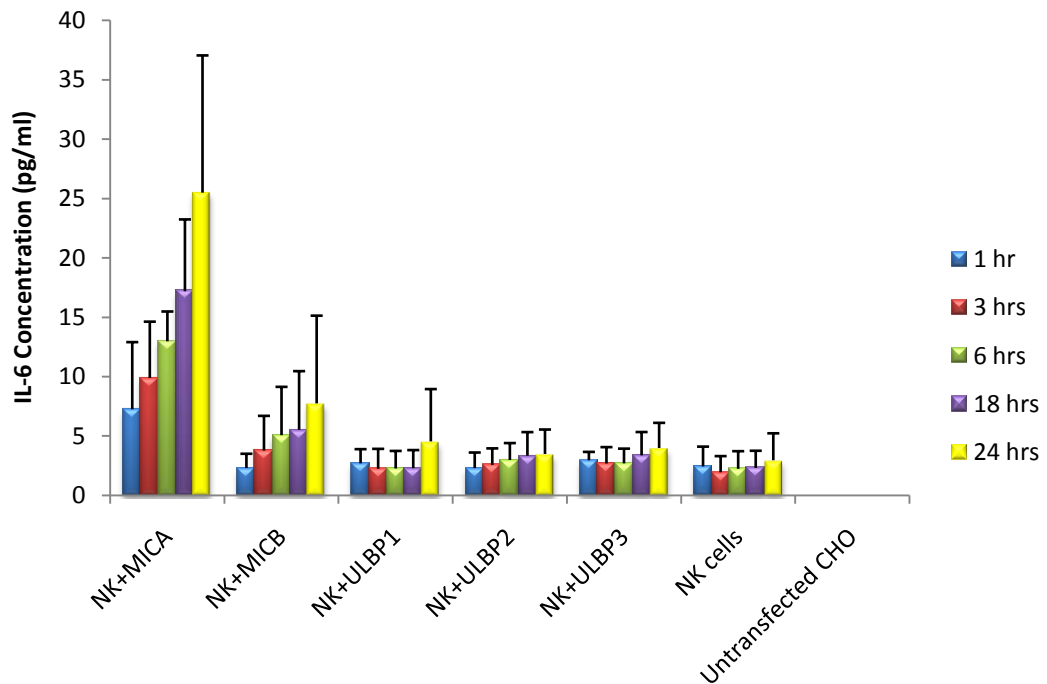
Human NK cells were purified from short term- frozen PBMCs by immunomagnetic negative selection using the method described in Chapter 2. The purity of the cells was confirmed to be more than 95% by flow cytometry. After isolation, aliquots of  $1 \times 10^5$  NK cells were co-cultured with similar concentrations of MICA-, MICB-, ULBP1-, ULBP2- and ULBP3- transfected CHO cells in 96-well

round bottom tissue culture plate in the presence of equal volumes of complete RPMI and Ham-F12 media. The cells were incubated in a humidified incubator at 37 °C with 5% CO<sub>2</sub>. Supernatants were collected from wells at 1, 3, 6, 18 and 24 hours and stored at -20 °C until analysed for IL-6 secretion using the human IL-6 Ready Set – Go<sup>®</sup> ELISA kit. Supernatants from the purified NK cells and un-transfected CHO cells in same concentrations as co-cultures were also collected and used as negative controls.

## **6.3 Results**

### **6.3.1 MICA (and to lesser extent MICB) were involved in the NKG2D-induced secretion of IL-6 by NK cells**

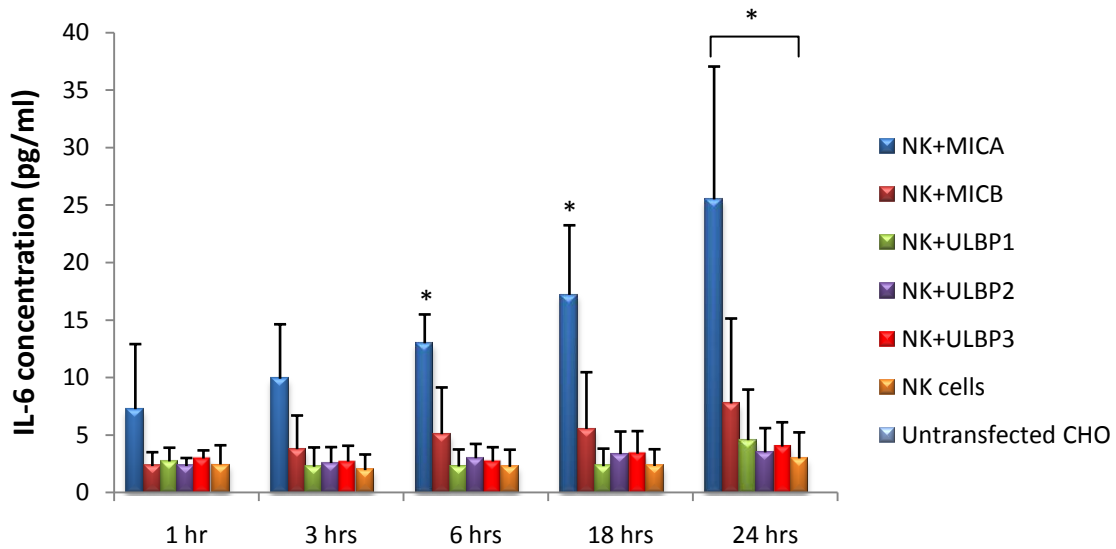
All the co-culture experiments were carried out in duplicates. The results of these experiments are shown in Figure 6.1. These results are representative of 4 independent experiments and are presented as mean  $\pm$  SD. In MICA co-cultures IL-6 started to increase as early as 1 hr (7.25 pg/ml  $\pm$  5.66) in comparison to (2.44 pg/ml  $\pm$  1.67) in resting NK cells. Continuous steady increase in the IL-6 concentration was observed at 3 hours (9.89  $\pm$  4.74), 6 hours (12.98  $\pm$  2.5) and 18 hours (17.19  $\pm$  6.05) respectively. The maximum concentration was found at 24 hours where the IL-6 in the supernatant was 25.49 pg/ml  $\pm$  11.55. No significant difference in the IL-6 concentration was found between 1 and 24 hours ( $p=0.068$ ). The supernatants collected from the co-cultures of NK cells with MICB-transfected CHO cells produced less IL-6 at all time points in comparison to MICA. At 1 hour, the IL-6 concentration was 2.3  $\pm$  1.20. It increased slightly thereafter at 3 hours (3.77  $\pm$  2.9); 6 hours (5.07  $\pm$  4.07); 18 hours (5.52  $\pm$  ) and 24 hours (7.75  $\pm$  7.4). However, these concentration were higher (although not statistically significant) than those obtained from NK + ULBP co-cultures.



**Figure 6.1: IL-6 production by NK cells following activation with NKG2D ligands.** Aliquots of  $1 \times 10^5$  purified NK cells isolated from 4 healthy donors were cultured with the same number of MICA-, MICB-, ULBP1-, and ULBP2- and ULBP3-transfected CHO cells in 96 well plates. Supernatants were harvested at 1, 3, 6, 18 and 24 hrs and analyzed for IL-6 production by ELISA. Co-cultures of PBNK + MICA-transfected CHO cells produced the highest IL-6 concentration at all time points in comparison to MICB, ULBPs co-cultures and negative controls. Supernatants collected from co-cultures of PBNK + ULBP –transfected CHO cells did not stimulate further IL-6 production over those obtained from NK cells alone. All measurements were performed in duplicates and presented as the mean of 4 independent experiments. Statistical analysis was performed using a Wilcoxon signed rank test. The error bars denote the SD.

### **6.3.2 MICA involvement induced significant amounts of IL-6 in comparison to other NKG2D ligands at 6, 18 and 24 hours post activation**

For the next analysis IL-6 concentrations were compared in the supernatants taken from co-cultures of purified PBNK and MICA-transfected CHO cells with those obtained from co-cultures of purified PBNK cells and MICB-, ULBP1-, ULBP2- and ULBP3-transfected CHO cells at each time point. The results are presented in Figure 6.2. 1 hr post activation, the IL-6 concentration in the PBNK+ MICA co-cultures ( $7.25 \text{ pg/ml} \pm 5.66$ ) was higher, but not significantly different, from those obtained from PBNK + MICB ( $2.3 \pm 1.2$ ,  $p > 0.05$ ), PBNK + ULBP1 ( $2.7 \pm 1.2$ ,  $p > 0.05$ ), PBNK + ULBP2 ( $2.3 \pm 1.3$ ,  $p > 0.05$ ), and PBNK + ULBP3 ( $2.96 \pm 0.7$ ,  $p > 0.05$ ) co-cultures respectively. The concentration of IL-6 continued to increase insignificantly in PBNK + MICA co-cultures at 3 hours ( $9.89 \pm 4.7$ ) in comparison to those obtained from PBNK + MICB ( $3.8 \pm 2.9$ ), PBNK + ULBP1 ( $2.23 \pm 1.7$ ), PBNK + ULBP2 ( $2.6 \pm 1.4$ ) and PBNK + ULBP3 ( $2.7 \pm 1.3$ ) co-cultures respectively. The significant increase in the IL-6 concentrations in PBNK + MICA co-cultures over ULBP co-cultures became apparent at 6 hrs ( $p = 0.029$ ). However, this concentration was still not significantly different from that obtained from MICB co-culture ( $p = 0.057$ ). At 24 hrs following activation the IL-6 production in PBNK+ MICA co-cultures was  $25.5 \text{ pg/ml} \pm 11.6$ . This concentration was significantly higher than that observed with PBNK+ MICB ( $7.7 \pm 7.3$ ,  $p < 0.05$ ), PBNK + ULBP1 ( $4.55 \pm 4.4$ ,  $p < 0.05$ ), PBNK + ULBP2 ( $3.5 \pm 2.06$ ,  $p < 0.05$ ) and PBNK + ULBP3 ( $3.99 \pm 2.11$ ,  $p < 0.05$ ) co-cultures respectively.



**Figure 6.2: Comparison of IL-6 secretion by NK cells in different NKG2D ligand-CHO co-cultures.** At all time points, the concentrations of IL-6 were higher in the supernatant collected from NK cells + MICA- transfected CHO cells. Significant difference in IL-6 production in NK + MICA co-cultures over NK+ULBPs co-cultures were noted as early as 6 hours post stimulation. However, a longer time period (24 hours) was needed for the secretion to be significantly different over NK+ MICB co-cultures. Statistical analysis was performed using Wilcoxon signed rank test. The error bars denote the SD; \*,  $p < 0.05$ .

## **6.4 Discussion**

### **6.4.1 The advantages of using non-human cell lines transfected with human NKG2D ligands.**

To clarify the role of NKG2D ligand- receptor interaction in the initiation of IL-6 secretion by NK cells, NKG2D ligand-Fc fusion proteins were first proposed as an experimental model (Chapter 5). However, failure to produce these fusion proteins made the use of alternative approaches necessary. One of the possible alternatives was to transfect human cell lines (known to be NKG2D ligand negative) with full length NKG2D ligands. These cell lines would then be co-cultured with NK cells to test how the IL-6 secretion would be affected. Another approach was to transfect non-human cell lines with the human NKG2D ligands and then to test if these induce IL-6 secretion by NK cells. The latter approach was more valuable since any detection of IL-6 in the supernatant is a strong indication of direct involvement of the NKG2D signalling pathway without interference from other signalling pathways. It also allowed indirect study of the role of other factors such as co-stimulation or adhesion molecules that contributed to this secretion. Another major advantage of using non-human cell lines was that any secretion of IL-6 by these cell lines would not be detectable using a human ELISA kit.

### **6.4.2 NKG2D-transfected CHO co-cultures produced less IL-6 than HeLa cell co-cultures**

In the experiments presented in this chapter, purified PBNK cells were co-cultured with CHO cells transfected with full length MICA, MICB, ULBP1, ULBP2 and ULBP3. The supernatants were collected over 1, 3, 6, 18 and 24 hours and analysed for IL-6 by ELISA. In general, the level of IL-6 detected in these co-culture

experiments was very low in comparison to that obtained when NK cells were co-cultured with NKG2D expressing human cell lines such as HeLa cells (Chapter 3) at all time points studied (Table 6.1).

**Table 6.1: Comparison of IL-6 levels in the supernatants collected from PBNK cells + HeLa with those obtained from PBNK cells + MICA- transfected CHO cells**

	<b>PBNK + HeLa (pg/ml ± SD)</b>	<b>PBNK + MICA-transfected CHO cells (pg/ml ± SD)</b>
1 hr	79.8 ± 12.26	7.25 ± 5.66
3 hrs	139.78 ± 13.29	9.89 ± 4.7
6 hrs	189.27 ± 13.7	12.98 ± 2.51
18 hrs	270 ± 14.8	17.19 ± 6.04
24 hrs	390 ± 22.77	25.49 ± 11.55

These results might point to the significance of other factors such as co-stimulation or the adhesion molecules in the secretion of IL-6 by PBNK cells when co-cultured with target cells. Some studies demonstrated that HeLa cells are not only NKG2D ligand positive, but they are also NCR ligand positive (Moretta et al, 2001). These studies showed that lysis of HeLa cells when co-cultured with NCR<sup>bright</sup> NK cells was completely inhibited by masking both NCR and NKG2D receptors, while only partial inhibition was noted when the NCR or NKG2D receptors were blocked individually. Does co-engagement of both the NKG2D and NCR receptors further amplified the IL-6 secretion by NK cells when co-cultured with HeLa cells?



Unfortunately, NCR receptor ligands remain poorly characterized, so they could not be used to answer this question directly.

Adhesion molecules play a central role in NK cell-target cell interactions that are critical in the generation of effective immune responses (Robertson et al, 1990; Stewart et al, 2006). Resting NK cells are known to express a number of adhesion molecules. These include lymphocyte function-associated antigen (LFA-1 or CD11a/CD18), LFA-3 (CD58) and neural cell adhesion molecule (NCAM or CD56) (Robertson et al, 1990). Surface expression of these adhesion molecules increases four to six fold after treatment with IL-2 (Robertson et al, 1990). Increases in the expression of these molecules have been linked to enhanced NK cell-mediated killing of sensitive targets and the induction of cytotoxicity against cells that are otherwise resistant to NK cell-mediated lysis (Robertson et al, 1990). Recent evidence indicated that some of these adhesion molecules were not only involved in the regulation of NK cell cytotoxicity but also in the regulation of cytokine secretion by these cells. Fauriat et al (2010) demonstrated that the co-engagement of LFA-1 augmented the secretion of cytokines and chemokines by NKG2D and 2B4 receptors in *Drosophila* cells. While the role of LFA-1 is only synergistic for NKG2D and 2B4 in the secretion of TNF- $\alpha$ , MIP-1  $\alpha$ , MIP-1  $\beta$  and RANTES, the secretion of IFN- $\gamma$  by NKG2D and 2B4 is particularly LFA-1 dependent (Fauriat et al, 2010). Overall these findings addressed not only the significance of LFA-1-mediated adhesion, but also indicate the signalling pathways involved. Since HeLa cells express high level of ICAM-1 (Jakiela et al, 2008) which is a ligand for the LFA-1 receptor, therefore, one explanation for high IL-6 secretion in HeLa co-cultures could be the involvement of different signalling pathways including different NK cell receptors (NKG2D and NCRs) and adhesion molecules like LFA-1. On other hand, the low levels of IL-6 concentration secreted by

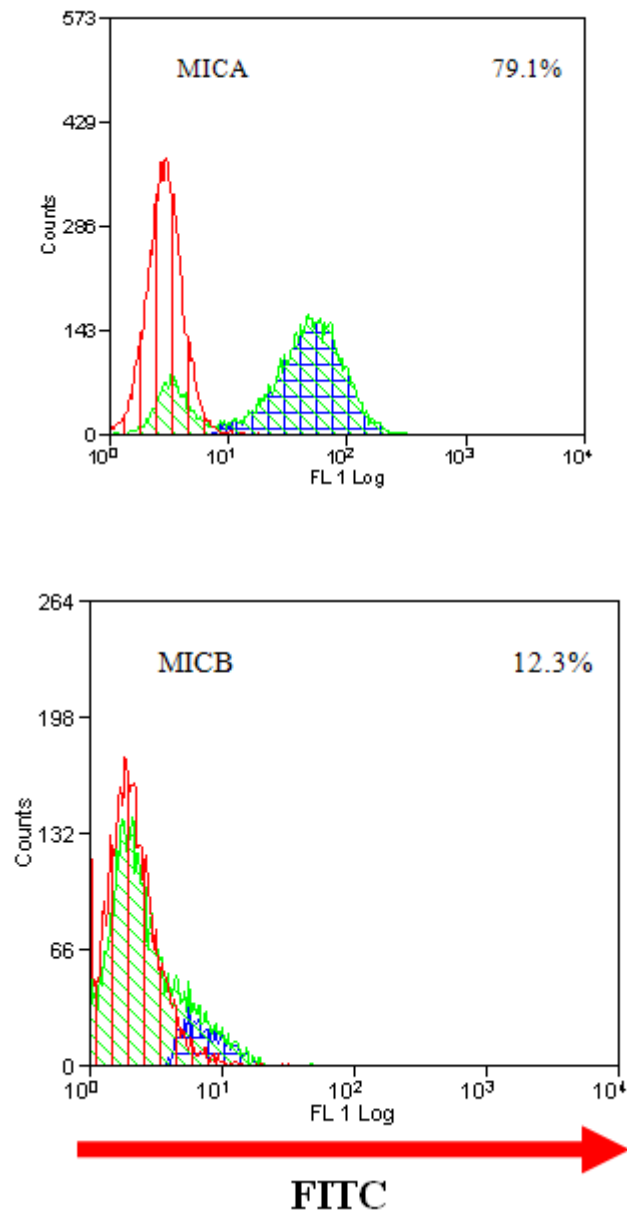
NK cells when co-cultured with NKG2D-transfected CHO cells might reflect the absence of human NK cell co-simulation and adhesion molecule co-expression. Nevertheless, the fact that IL-6 was detected in the supernatants collected from co-cultures with these NKG2D ligand transfected cells provided strong evidence for the direct involvement of the NKG2D pathway.

NK cells also express a family of receptors including DNAM-1, Tactile and CRTAM (class I-restricted T cell-associated molecule) (Stewart et al, 2006). Tactile and CRTAM are only expressed on the surface of activated NK cells. They mediate adhesion and stimulate cytotoxicity (Fuchs et al, 2004; Boles et al, 2005). CRTAM also promotes *in vitro* secretion of IFN- $\gamma$  by CD8<sup>+</sup> T-cells. DNAM-1 is associated with LFA-1 in NK cells and is phosphorylated by protein kinase C (Shibuya et al, 1999). The role of DNAM-1 in the secretion of cytokines by NK cells was investigated by Bryceson et al (2006). They found that DNAM -1 synergised strongly with 2B4 and, to a lesser extent, with NKG2D, NKp46 and CD2 in the secretion of IFN- $\gamma$  and TNF- $\alpha$  by NK cells. The ligands for DNAM-1 are Nectin-2 (CD112) and the poliovirus receptor (PVR) (Pende et al, 2005). These ligands are also expressed on HeLa cells (El-Sherbiny et al, 2005; Akhtar et al, 2008) giving another potential explanation for higher IL-6 secretion in HeLa co-cultures compared to NKG2D ligand-transfected CHO co-cultures.

#### **6.4.3 MICA involvement in the NKG2D-induced secretion of IL-6 could reflect either differences in surface expression or differences in the affinities of NKG2D ligands for their receptor**

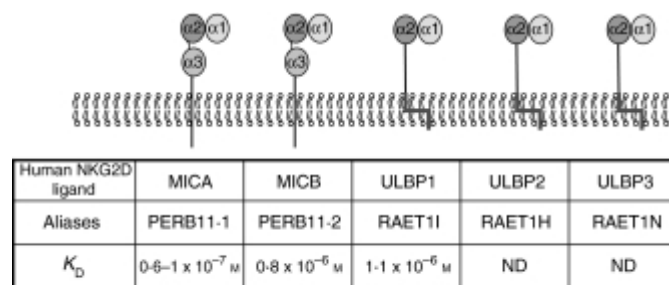
The findings also indicated that the MICA and MICB (to lesser extent) were involved in the NKG2D-mediated IL-6 secretion pathway in NK cells. ULBPs induced

no significant IL-6 secretion over resting NK cells while no IL-6 was detected in the supernatant collected from un-transfected CHO cells. These observations reflected either differences in the surface expression of these ligands by CHO cells or different affinities of the NKG2D ligands for NKG2D receptor. All the MICA-, MICB-, ULBP1-, ULBP2- and ULBP3-transfected CHO cells were checked and confirmed positive for the expression of these ligands by RT-PCR. Since MICA and MICB were the two ligands mostly involved in the NKG2D-induced secretion of IL-6, therefore the surface expression of these ligands was also tested by flow cytometry. Our observations indicated differences in the surface expression of these ligands as shown in Figure 6.3. Unfortunately, these experiments cannot establish whether the observed differences in IL-6 secretion were due to the different affinities of these ligands for the NKG2D or simply attributable to differences in surface expression levels on the transfected cells.



**Figure 6.3: Expression of MICA/B proteins by transfected CHO cells as determined by flow cytometry.** MICA- and MICB- transfected CHO cells were cultured for 3 days in Ham's F-12 medium at 37°C and 5% CO<sub>2</sub>. Thereafter, 1 x 10<sup>5</sup> cells were stained with 0.25 µg of mIgG2a k isotype control (Clone: MOPC-173, Biologend) (red histogram) or 0.25 µg of mouse anti-human MICA/B Purified (Clone: 6D4, ebioscience) (green histogram) followed by anti-mouse IgG FITC (ebioscience). Total viable cells were used for analysis. Gate was set by forward and side scatter to exclude dead cells, debris, and cell aggregates. The percentage of positive cells was 79.1% for MICA and 12.3 % for MICB as determined by DAKO Summit software (blue histogram).

Differences in the affinity of human and mouse NKG2D ligands for the NKG2D receptor have been demonstrated in many studies (O'Callaghan et al, 2001; Sutherland et al, 2002; Takada et al, 2008; Eagle et al, 2009). The affinities of the different human NKG2D ligands for NKG2D receptors range between 6 nM for MICA to 1.1  $\mu$ M for ULBP1 as determined by surface plasmon resonance studies (Mistry and O'Callaghan, 2007; Nausch and Cerwenka, 2008). The reported affinity of ligands to NKG2D receptor is MICA > MICB > ULBP1 (Figure 6.4).



**Figure 6.4: Affinities of different NKG2D ligands for NKG2D receptor as determined by surface plasmon resonance (SPR).** The equilibrium dissociation constant ( $K_D$ ) was used to describe the affinity between NKG2D ligands and receptors. The smaller the dissociation constant, the higher the affinity. ND = not determined. Reproduced from Mistry and O'Callaghan (2007).

It is still largely unknown whether the differences in the NKG2D ligand affinities for the NKG2D receptor have an impact on NK cell activation and function. Do these differences in the ligand affinity translate into functional differences in NK cell stimulation? In fact this is supported by the findings of others who observed that the differences in the binding affinity of different alleles for NKG2D within the same ligand itself (different MICA alleles) could have profound effect on NK cell activation and in the modulation of T-cell responses (Steinle et al, 2001). The results presented here may also suggest that this is the case. However, a definitive answer would require the standardisation of NKG2D ligand density. This, in the context of transfected cell lines is practically impossible.

# Chapter 7

## CD4<sup>+</sup> T Lymphocyte Migration in Response to IL-6 Secreted by NK Cells



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## **7.1 Introduction**

IL-6 is a pleiotropic cytokine with multiple functions that plays many roles in inflammation and immune responses (Kishimoto, 2006). Since IL-6 is secreted rather ubiquitously by many cells types (Naka et al, 2002), therefore it was necessary to show that IL-6 secreted from NK cells is biologically relevant in influencing the immune response. Although not defined as a chemokine, one of the reported functions of IL-6 is T cell recruitment both *in vitro* (Weissenbach et al, 2004) and *in vivo* (McLoughlin et al, 2005). This function of IL-6 seems at first to be secondary by regulation of chemokine secretion and/or chemokine receptor expression on target cells (Romano et al, 1997; McLoughlin et al, 2005). However, Weissenbach et al (2004) provided evidence that IL-6 could be a direct mediator of CD4<sup>+</sup> T cell migration. In this chapter the direct effect of IL-6 secreted by NK cells on *in vitro* migration of CD4<sup>+</sup> T cells was studied. It was hypothesized that IL-6 secreted by NK cells could contribute, at least partially, to the migration of other inflammatory and immune cells to the site of inflammation. This hypothesis was based on the observations that IL-6 could induce direct CD4<sup>+</sup> T cell migration. Given the findings that NK cells can secrete IL-6 one can speculate that NK cells could be responsible for the migration of lymphocytes that occurs after initial NK cell activation. This mechanism could facilitate the transition between innate and acquired immunity.

## **7.2 Approach used for *in vitro* trans-migration assay**

A modified Boyden chamber trans-migration assay was used to assess the migration of CD4<sup>+</sup> T cells in response to NK cell activation. Details of the protocol used were described in Chapter 2. NK cells were purified by negative selection. The purity of NK cells was more than 95% as shown by flow cytometry. At the time of NK

cell purification, the non-NK cell fraction from each donor was saved and CD4<sup>+</sup> T cells were purified by positive selection using a CD4<sup>+</sup> T cell isolation kit. After selection, the NK cells were activated overnight by co-culture with HeLa cells at a ratio of 5:1 and IL-2 at a dose of 100 U / ml. The next day lymphocyte migration in response to IL-6 secreted by NK cells was assessed in 24-well Transwell plates with two different pore size inserts (8 and 3 µm). Transwell inserts were pre-coated with fibronectin (5µg/cm<sup>2</sup>) since lymphocyte migration in response to IL-6 was shown to be fibronectin dependent (Weissenbach et al, 2004). Four wells were prepared for each sample. In the first well, 2 x 10<sup>5</sup> CD4<sup>+</sup> T cells were suspended in 100 µl RPMI culture medium and placed in the upper compartment. The same number of NK cells from the same donor was suspended in 600 µl of culture medium and placed in the lower compartment. In a second well CD4<sup>+</sup> T cells were seeded in the upper compartment and NK cells in the lower compartment but in the presence of a neutralizing anti-IL-6 antibody (0.15 µg / ml for each 2.5 ng / ml of human IL 6 as recommended by the manufacturer). Lymphocyte migration in response to activated NK cells was compared to migration in response to 20 ng/ml of recombinant human IL-6 (rhIL-6) contained in 600 µl of culture medium in a third well (positive control) and culture medium alone in the fourth well (negative control). After 4 hours incubation the cells in the lower compartment were mixed with a known number of counting beads before harvesting. Thereafter the cells and the beads were removed from the lower compartment, washed with 1X PBS and stained with a mouse anti-CD4<sup>+</sup>FITC antibody. The absolute number of migrating CD4<sup>+</sup> T cells was counted by flow cytometry and the level of migration was expressed as a migration percentage calculated in relation to the total number CD4<sup>+</sup> T cells used in the upper compartment.

### 7.3 Results

#### 7.3.1 Trans-migration assay using 8 µm pore size Transwell inserts

To start with, it was decided to use inserts with 8 µm pore size since these inserts are commonly used in the literature for trans-migration assay for lymphocytes. Before proceeding with the actual experiments it was necessary to check these inserts for the spontaneous migration of lymphocytes and compare this to that of test samples and positive control. Therefore, a preliminary experiment with only one donor was carried out as outlined in section 7.2. After 4 hours incubation the percentage of migration of CD4<sup>+</sup> T cells to the lower compartment was calculated as follows:

Absolute number of migrating CD4<sup>+</sup> cells/ 1 µl:

$$\frac{\text{Number of CD4}^+ \text{ cell events}}{\text{Number of beads events}} \times \frac{\text{Assigned bead count of the lot (25000 beads/25 } \mu\text{l)}}{\text{Volume of sample (} \mu\text{l) used in flow analysis}}$$

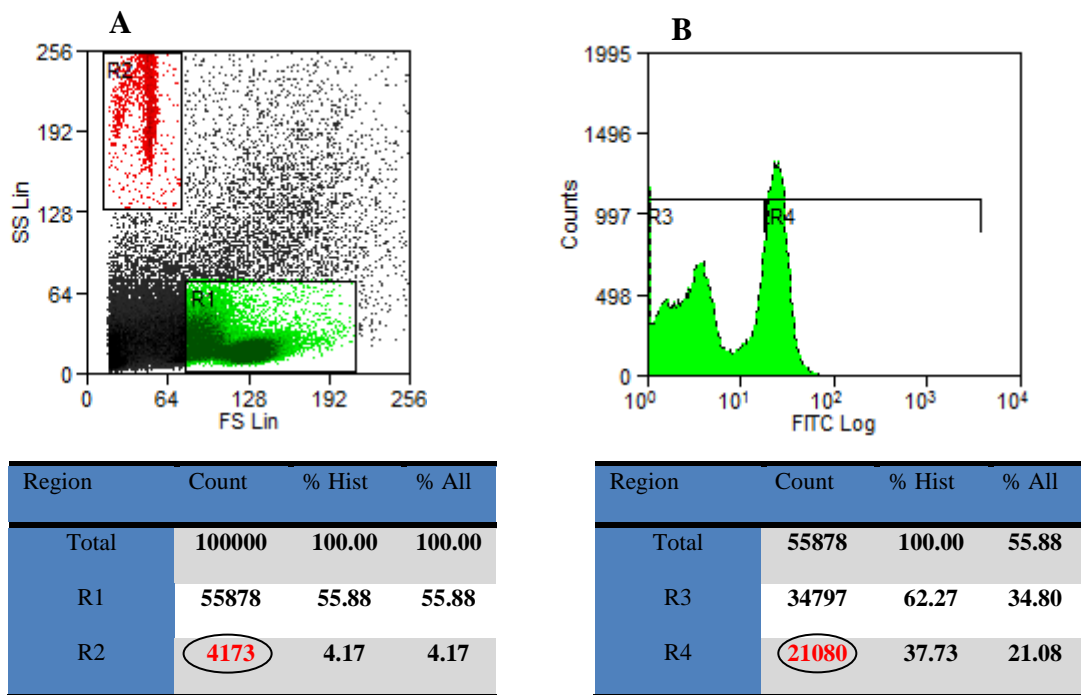
Total number of migrating CD4<sup>+</sup> T cells in the lower compartment:

$$\text{Absolute number of CD4}^+ \text{ T cells in lower compartment} / \mu\text{l} \times \text{volume of sample in lower compartment}$$

The percentage of migrating CD4<sup>+</sup> T cells:

$$\frac{\text{Total number of migrating CD4}^+ \text{ T cells}}{\text{Total number of CD4}^+ \text{ T cells}} \times 100$$

When no anti- IL-6 antibody was used, the percentage of migrating CD4<sup>+</sup> T cells elicited by activated NK cells was 37.8 % (Figure 7.1). However, in the presence of an anti- IL-6 antibody in the lower compartment the migration was inhibited to around 3.9 % (Figure 7.2). In the positive control well when only rhIL-6 was used, the migration percentage of CD4<sup>+</sup> T cells was 95.94% (Figure 7.3) compared to 11.4% in the negative control well, where only culture medium was used in the lower compartment (Figure 7.4). These preliminary results were very promising suggesting that IL-6 secreted by NK cells might, at least partially, participate in the migration of CD4<sup>+</sup>T lymphocytes. However, since the size of the insert pores was large (8  $\mu$ ) there is a possibility that some of these lymphocytes might diffuse through the insert pores by gravity and not by transmigration. For this reason it was decided not to continue with 8  $\mu$ m inserts and replace them with smaller pore size (3  $\mu$ m) inserts.

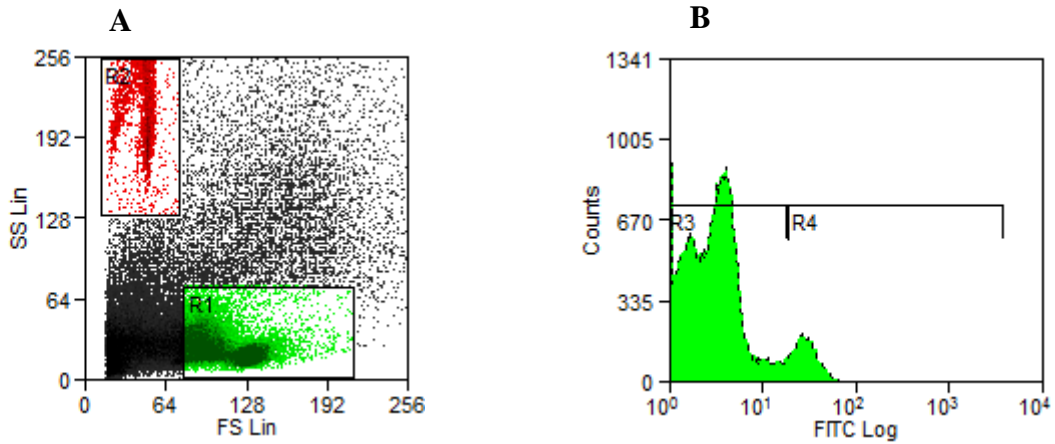


$$\frac{21080}{4173} \times \frac{25000}{1000} = 126 \text{ cells} / \mu\text{l}$$

**126 x 600 = 75600 cells.** This represents the total number of CD4<sup>+</sup> lymphocytes in the lower compartment

The migration percentage of CD4<sup>+</sup> T cells =  $75600/200000 \times 100 = 37.8 \%$ .

**Figure 7.1: CD4<sup>+</sup> T cell migration elicited by activated NK cells.** Transwell inserts (8 μm pore size) were pre-coated with fibronectin 45 -60 minutes before the experiment.  $2 \times 10^5$  CD4<sup>+</sup> T cells were placed in the upper compartment. The same number of activated NK cells was seeded in the lower compartment. After 4 hours of incubation, a known number of fluorescent counting beads were added to the lower compartment before collection. These beads were present during all following stages of processing, washing and staining with an anti- CD4<sup>+</sup>FITC antibody, allowing for correction for losses during these steps. **A)** Forward and sideward scatter. R1 refers to the gated cells (NK cells + migrating CD4<sup>+</sup> T cells) while R2 is the beads gate. **B)** Flow cytometry histogram showing the CD4<sup>-</sup> cells (R3) and the CD4<sup>+</sup> T cells (R4).



Region	Count	% Hist	% All
Total	100000	100.00	100.00
R1	40711	40.71	40.71
R2	7207	7.21	7.21

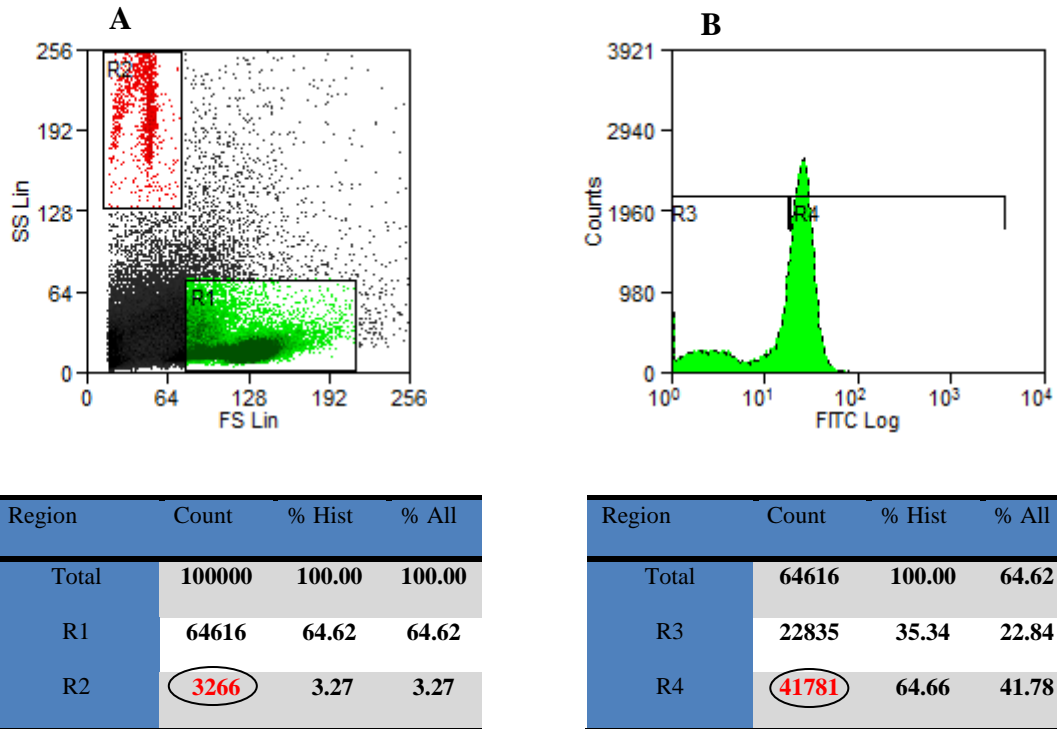
Region	Count	% Hist	% All
Total	40711	100.00	40.71
R3	36898	90.63	36.90
R4	3813	9.37	3.81

$$\frac{3813}{7207} \times \frac{25000}{1000} = 13 \text{ cells} / \mu\text{l}$$

The total number of CD4<sup>+</sup> lymphocytes in the lower compartment is: **13 x 600 = 7800 cells.**

The percentage of migration of CD4<sup>+</sup> T cells = **7800/200000 x 100 = 3.9 %.**

**Figure 7.2: CD4<sup>+</sup> T cell migration in response to NK cells in the presence of anti-IL-6 antibody.**  $2 \times 10^5$  CD4<sup>+</sup> T cells were placed in upper compartment. The same number of activated NK cells was seeded in lower compartment. IL-6 secreted by NK cells was neutralized by adding a monoclonal anti-IL-6 antibody to a final concentration of 0.15  $\mu\text{g} / \text{ml}$  for each 2.5  $\text{ng} / \text{mL}$  of human IL 6. The absolute number and the percentage of migrating CD4<sup>+</sup> T cells were calculated after 4 hours. **A)** Forward and side scatter. R1 represents the gated lymphocytes and NK cells, R2 is the beads gate. **B)** Flow cytometry histogram showing CD4<sup>-</sup> (R3) and CD4<sup>+</sup> cells (R4).

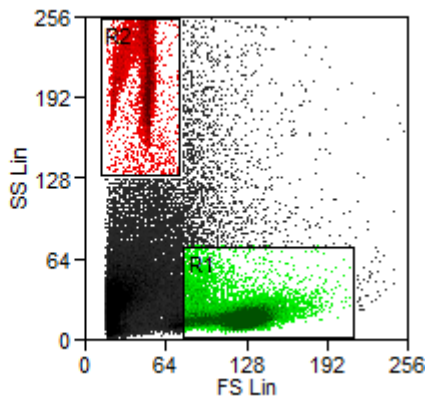


$$\frac{41781}{3266} \times \frac{25000}{1000} = 320 \text{ cells} / \mu\text{l}$$

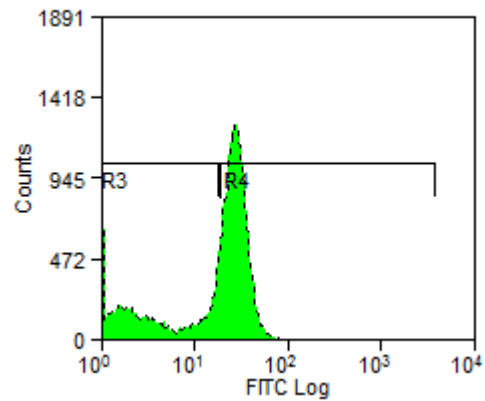
The total number of CD4<sup>+</sup> lymphocytes in the lower compartment is: **320 x 600 = 192000 cells.**

The percentage of migrating CD4<sup>+</sup> T cells = **192000/200000 x 100 = 96.0 %.**

**Figure 7.3: CD4<sup>+</sup> T cell migration in response to recombinant human IL-6 (rhIL-6).** 2 x 10<sup>5</sup> purified CD4<sup>+</sup> T cells were placed in the upper compartment of 8 μm pore size Transwell wells. Migration was induced by addition of 20 ng/ ml of rh IL-6 into the lower compartment. The number of migrating CD4<sup>+</sup> T cells and the migration percentage were calculated after 4 hours using flow cytometry. **A)** FACS analysis of the cells in the lower compartment showing the forward and side scatter. R1 represents lymphocytes and R2 is the beads gate. **B)** Flow cytometry histogram showing CD4<sup>-</sup> (R3) and CD4<sup>+</sup> T cells (R4).



Region	Count	% Hist	% All
Total	100000	100.00	100.00
R1	33501	33.50	33.50
R2	14330	14.33	14.33



Region	Count	% Hist	% All
Total	33501	100.00	33.50
R3	11695	34.91	11.70
R4	21806	65.09	21.81

$$\frac{21806}{14330} \times \frac{25000}{1000} = 38 \text{ cells / } \mu\text{l}$$

The total number of CD4<sup>+</sup> lymphocytes in the lower compartment is: **38 x 600 = 22800 cells.**

The percentage of migration of CD4<sup>+</sup> T cells = **192000/200000 x 100 = 11.4 %.**

**Figure 7.4: Spontaneous CD4<sup>+</sup> T cell migration in 8 μm pore size Transwell wells.**

2 x 10<sup>5</sup> of purified CD4<sup>+</sup> T cells were applied to the upper compartment. Only culture medium (RPMI) was placed in the bottom chamber. Migration of CD4<sup>+</sup> T cells was assessed at 4 hours as described above. **A)** FACS analysis of the cells in the lower compartment showing the forward and side scatter. R1 contains migrating CD4<sup>+</sup> T cells. R2 is the beads gate. **B)** Flow cytometry histogram showing the CD4<sup>-</sup> cells (R3) and the CD4<sup>+</sup> T cells (R4). Approximately 11 % of the CD4<sup>+</sup> T cells migrated spontaneously.



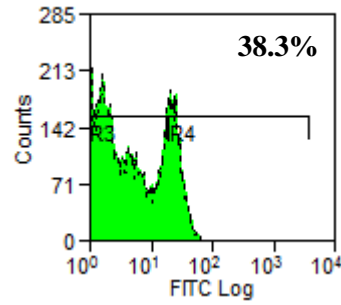
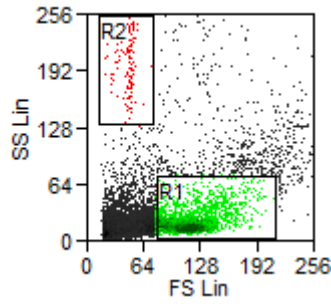
### **7.3.2 Trans-migration assay using 3 µm pore size inserts**

The trans-migration assay was repeated using 3 micron pore size Transwell inserts using cells from 4 additional healthy donors. As described previously, Transwell inserts were first coated with human fibronectin ( $5\mu\text{g}/\text{cm}^2$ ) suspended in 100 µl PBS and left for 45-60 minutes for sedimentation. The placement of the cells and controls was the same as for the preliminary experiment using the larger pore inserts. The results are expressed as percentage migration of  $\text{CD4}^+$  T cells. In sample 1 the migration of the  $\text{CD4}^+$  T cells in response to activated NK cells was 38.3%. However, this migration was inhibited by more than half in the presence of a neutralising anti-IL-6 antibody (15.8%). In sample 2, the migration of  $\text{CD4}^+$  T cells in response to NK cells was inhibited from 48.8% to only 12.1% in the presence of IL-6 antibody. A prominent inhibition was also seen in sample 3 where the migration of  $\text{CD4}^+$  T cells was inhibited from 61.5% to 18.8% in the presence of IL-6 antibody. Although the  $\text{CD4}^+$  T cell migration in sample 4 was as low as 9.6%, but the migration to the lower chamber was still inhibited by more than half in the presence of the anti IL-6 antibody (4.3%).

Regarding the positive control where the migration of  $\text{CD4}^+$  T cells was assessed in response to 20 ng/ml of recombinant IL-6 in the lower compartment, the migration percentage of T cells was 95.4%, 99.8%, 111.0% and 19.6% in samples 1, 2, 3 and 4 respectively. Spontaneous migration of  $\text{CD4}^+$  T cells in the absence of any external stimuli was also recorded. However, this migration did not exceed 8.8% in all samples. Overall, these results supported the preliminary findings and further suggested the involvement of IL-6 secreted by NK cells in the migration of  $\text{CD4}^+$  T cells.

**A**

*CD4<sup>+</sup>Tcells (upper compartment).  
NK cells (lower compartment).  
No anti- IL-6 antibody*

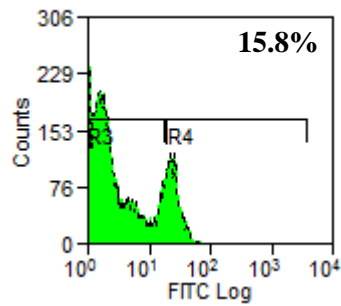
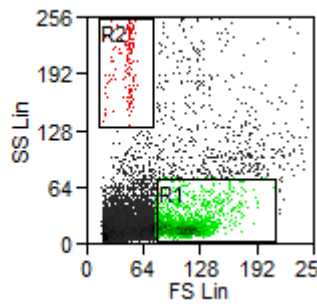


Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	13397	66.98	66.98
R2	584	2.92	2.92

Region	Count	% Hist	% All
Total	13397	100.00	66.98
R1	10414	77.73	52.07
R2	2983	22.27	14.92

**B**

*CD4<sup>+</sup>Tcells (upper compartment).  
NK cells (lower compartment).  
Anti- IL-6 antibody.*

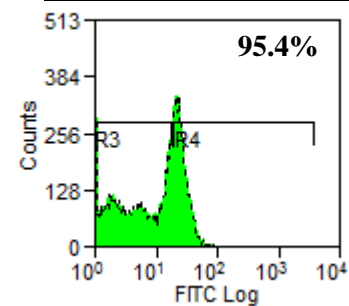
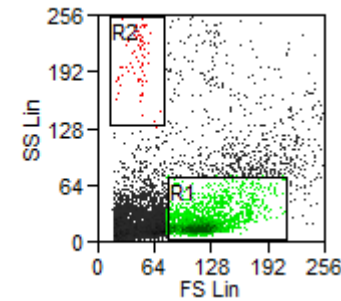


Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	10147	50.73	50.73
R2	861	4.30	4.30

Region	Count	% Hist	% All
Total	10147	100.00	50.73
R1	8336	82.15	41.68
R2	1811	17.85	9.05

**C**

*CD4<sup>+</sup>Tcells (upper compartment).  
Human IL-6 (lower compartment).*

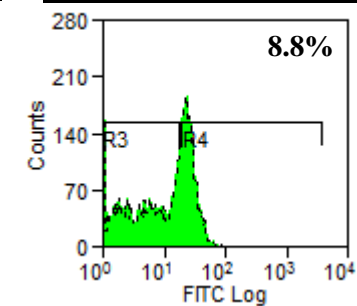
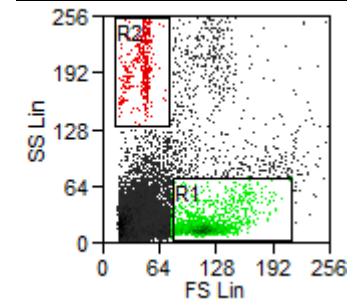


Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	13631	68.16	68.16
R2	414	2.07	2.07

Region	Count	% Hist	% All
Total	13631	100.00	68.16
R1	8361	61.34	41.81
R2	5268	38.65	26.34

**D**

*CD4<sup>+</sup>Tcells (upper compartment).  
Culture medium (lower compartment).*

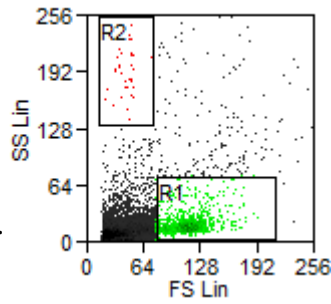


Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	7071	35.35	35.35
R2	2533	12.67	12.67

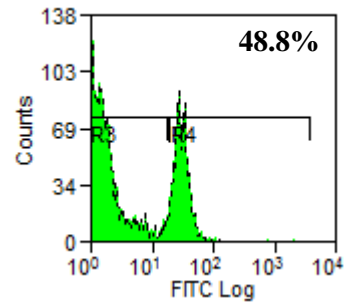
Region	Count	% Hist	% All
Total	7071	100.00	35.35
R1	4086	57.79	20.43
R2	2985	42.21	14.92

**Figure 7.5: Migration of CD4<sup>+</sup> T cells in sample 1 across the 3 μm pore size Transwell filters.** 2 x 10<sup>5</sup> purified CD4<sup>+</sup> T cells were placed in the upper chamber of Transwell wells and assessed for migration across the membranes in response to different stimuli in the lower chamber by flow cytometry. **A)** CD4<sup>+</sup> T cell migration across the membrane in response to 2 x 10<sup>5</sup> pre-stimulated NK cells from the same donor. The percentage of migration was 38.3%. **B)** CD4<sup>+</sup> T cell migration across the membrane in response to same concentration of pre-activated NK cells from same donor in the presence of mouse anti-human anti-IL-6 antibody in the lower chamber. **C)** CD4<sup>+</sup> T cell migration to the lower compartment in response to 20 ng/ml recombinant human IL-6 (positive control). **D)** Spontaneous CD4<sup>+</sup> T cell migration (negative control).

**A**  
*CD4<sup>+</sup>Tcells (upper compartment).*  
*NK cells (lower compartment).*  
*No anti- IL-6 antibody.*

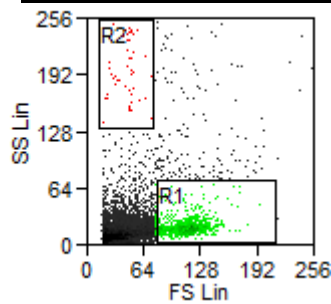


Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	4924	24.62	24.62
R2	219	1.10	1.10

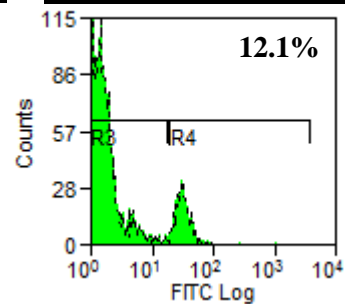


Region	Count	% Hist	% All
Total	4924	100.00	24.62
R1	3500	71.08	17.50
R2	1424	28.92	7.12

**B**  
*CD4<sup>+</sup>Tcells (upper compartment).*  
*NK cells (lower compartment).*  
*Anti- IL-6 antibody*

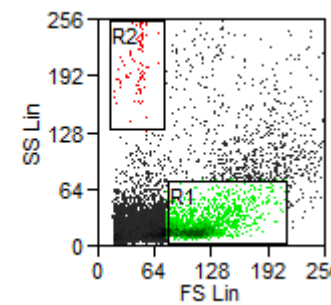


Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	3873	19.36	19.36
R2	295	1.47	1.47

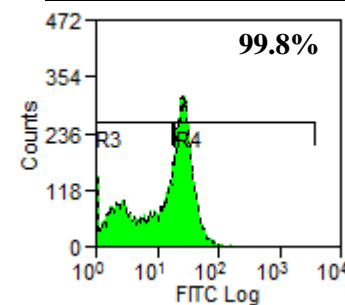


Region	Count	% Hist	% All
Total	3873	100.00	19.36
R1	3399	87.76	16.99
R2	474	12.24	2.37

**C**  
*CD4<sup>+</sup>Tcells (upper compartment).*  
*Human IL-6 (lower compartment).*

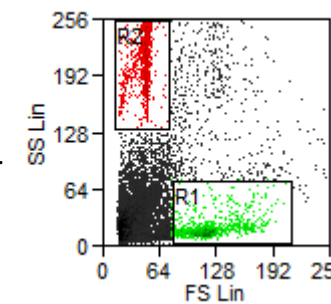


Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	12119	60.59	60.59
R2	466	2.33	2.33

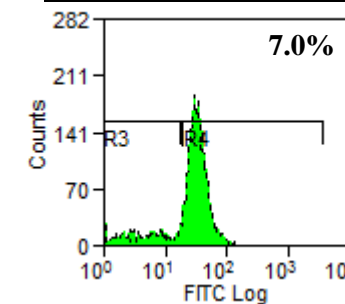


Region	Count	% Hist	% All
Total	12119	100.00	60.59
R1	5913	48.79	29.57
R2	6206	51.21	31.03

**D**  
*CD4<sup>+</sup>Tcells (upper compartment.)*  
*Culture medium (lower compartment).*



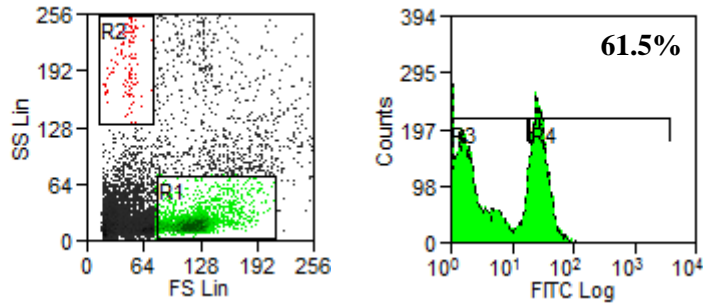
Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	7071	35.35	35.35
R2	2533	12.67	12.67



Region	Count	% Hist	% All
Total	7071	100.00	35.35
R1	4086	57.79	20.43
R2	2985	42.21	14.92

**Figure 7.6: Migration of CD4<sup>+</sup> T cells in sample 2 across the 3 μm pore size Transwell filters.** Isolated human CD4<sup>+</sup> T cells were seeded into the upper reservoir of the chamber. The number of migrated cells was determined 4 hrs after stimulation by counting the cells that found in the lower compartment of Transwell wells by flow cytometry. **A)** CD4<sup>+</sup> T cell migration across the membrane in response to pre-activated NK cells from the same donor. **B)** CD4<sup>+</sup> T cell migration across the membrane in response to pre- activated NK cells from same donor in the presence of mouse anti-human anti-IL-6 antibody in the lower chamber. **C)** CD4<sup>+</sup> T cell migration to the lower compartment in response to recombinant human IL-6 (positive control). **D)** Spontaneous CD4<sup>+</sup> T cell migration (negative control).The percentage of migration of CD4<sup>+</sup> T cells were given in relation to total number of CD4<sup>+</sup> T cells used.

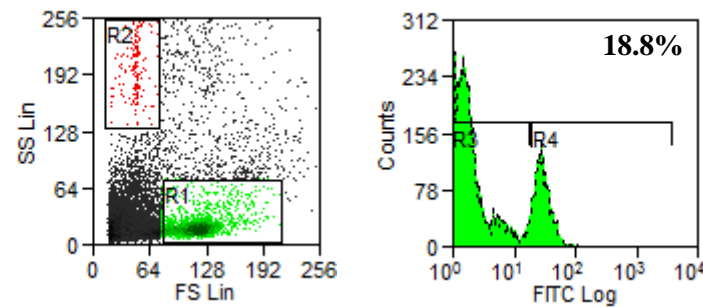
**A**  
*CD4<sup>+</sup>Tcells (upper compartment).*  
*NK cells (lower compartment).*  
*No anti- IL-6 antibody.*



Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	12819	64.09	64.09
R2	589	2.94	2.94

Region	Count	% Hist	% All
Total	12819	100.00	64.09
R1	7984	62.28	39.92
R2	4833	37.70	24.16

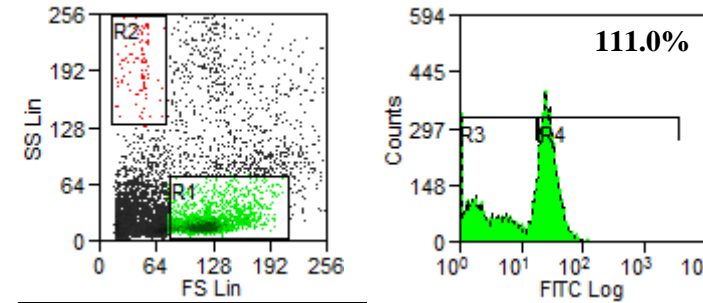
**B**  
*CD4<sup>+</sup>Tcells (upper compartment).*  
*NK cells (lower compartment).*  
*Anti- IL-6 antibody.*



Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	10878	54.39	54.39
R2	937	4.69	4.69

Region	Count	% Hist	% All
Total	10878	100.00	54.39
R1	8528	78.40	42.64
R2	2349	21.59	11.74

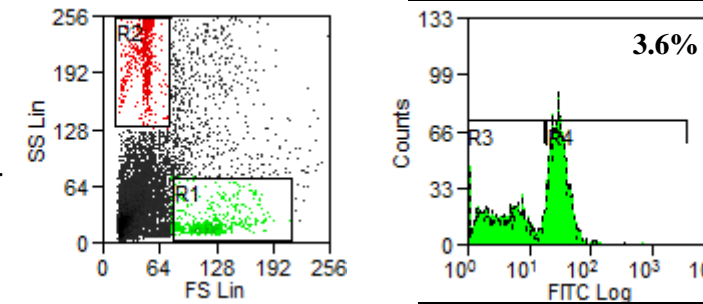
**C**  
*CD4<sup>+</sup>Tcells (upper compartment).*  
*Human IL-6 (lower compartment).*



Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	13747	68.73	68.73
R2	506	2.53	2.53

Region	Count	% Hist	% All
Total	13747	100.00	68.73
R1	6209	45.17	31.05
R2	7538	54.83	37.69

**D**  
*CD4<sup>+</sup>Tcells (upper compartment).*  
*Culture medium (lower compartment).*



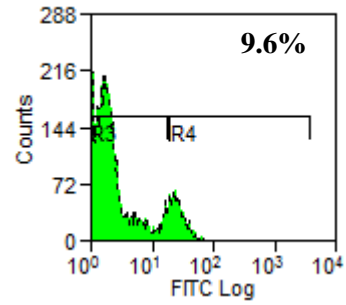
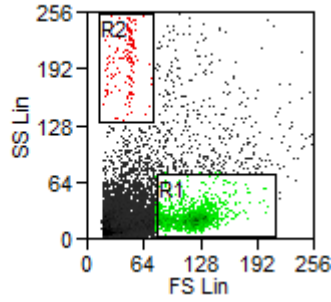
Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	2883	14.41	14.41
R2	3396	16.98	16.98

Region	Count	% Hist	% All
Total	2883	100.00	14.41
R1	1235	42.84	6.17
R2	1648	57.16	8.24

**Figure 7.7: Migration of CD4<sup>+</sup> T cells from sample 3 across the 3µm pore size Transwell inserts.** As described for sample 1 and 2, the migration of CD4<sup>+</sup> T cells across the membrane was assessed in response to different stimuli. **A)** 61.5% of the total CD4<sup>+</sup> T cells in the upper compartment migrated in response to pre-activated NK cells in the lower compartment from the same donor. **B)** The migration of T cells was inhibited to 18.8% when anti-IL-6 antibody was added to pre-activated NK cells in the lower compartment. **C)** All the cells in the upper compartment migrated across the membrane in response to recombinant human IL-6 in the lower compartment. **D)** Only 3.6% of the total T cell in the upper compartment migrated spontaneously to lower chamber in the absence of any external stimuli (negative control).

**A**

*CD4<sup>+</sup>Tcells (upper compartment.)*  
*NK cells (lower compartment.)*  
*No anti- IL-6 antibody.*

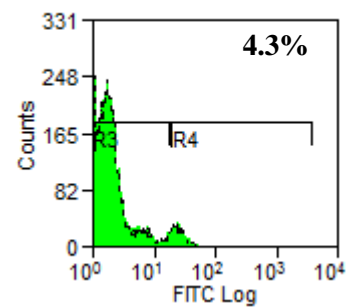
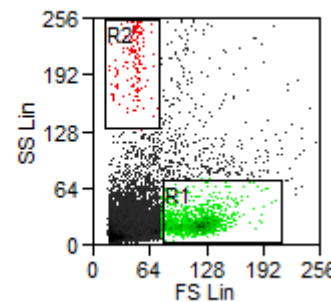


Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	7857	39.28	39.28
R2	794	3.97	3.97

Region	Count	% Hist	% All
Total	7857	100.00	39.28
R1	6838	87.03	34.19
R2	1019	12.97	5.10

**B**

*CD4<sup>+</sup>Tcells (upper compartment.)*  
*NK cells (lower compartment.)*  
*Anti- IL-6 antibody.*

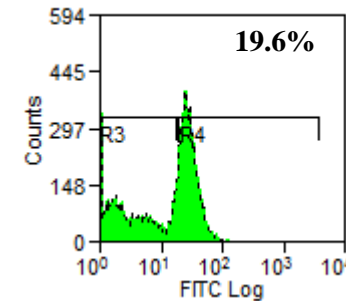
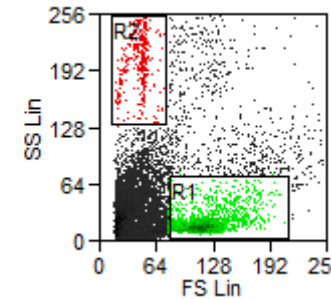


Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	7980	39.90	39.90
R2	834	4.17	4.17

Region	Count	% Hist	% All
Total	7980	100.00	39.90
R1	7497	93.95	37.48
R2	483	6.05	2.42

**C**

*CD4<sup>+</sup>Tcells (upper compartment.)*  
*Human IL-6 (lower compartment.)*

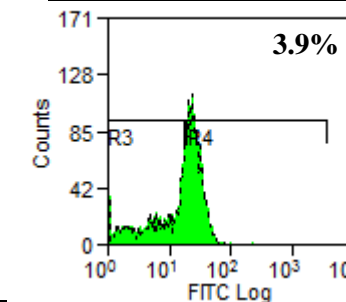
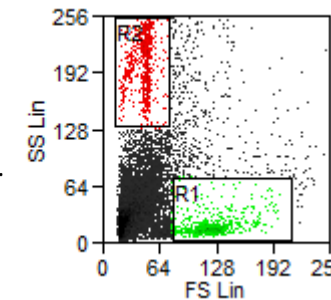


Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	8240	41.20	68.73
R2	1850	9.25	2.53

Region	Count	% Hist	% All
Total	8240	100.00	41.20
R1	33.98	41.24	16.99
R2	4842	58.76	24.21

**D**

*CD4<sup>+</sup>Tcells (upper compartment.)*  
*Culture medium (lower compartment.)*



Region	Count	% Hist	% All
Total	20000	100.00	100.00
R1	3236	16.18	16.18
R2	3549	17.75	17.75

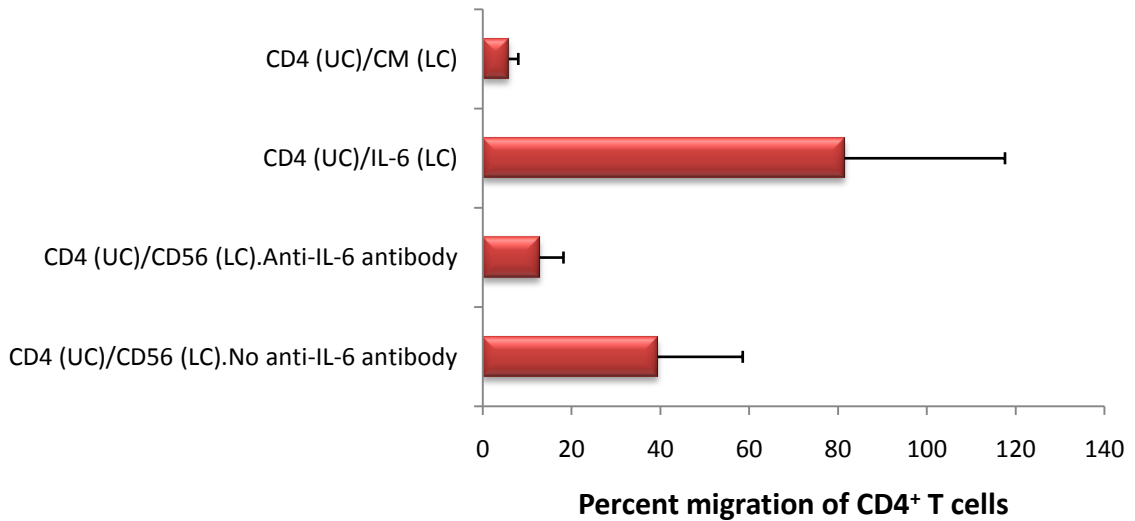
Region	Count	% Hist	% All
Total	3236	100.00	16.18
R1	1408	43.51	7.04
R2	1827	56.46	9.13



**Figure 7.8: CD4<sup>+</sup> T cell migration profiles in sample 4 in response to pre-activated NK cells, IL-6 and anti IL-6 antibody.** Although the CD4<sup>+</sup> T cell migration profiles were lower than those observed with the other 3 samples, the migration was still inhibited by more than half when the effect of IL-6 secreted by NK cells was neutralized with anti-IL-6 antibody.

### **7.3.3 CD4<sup>+</sup> T cell recruitment in response to activated NK cells was inhibited by more than 30% in the presence of neutralizing concentration of anti-IL-6 antibody**

Next the data obtained from the transmigration assay from all 4 samples were compared and the results were presented as means  $\pm$  SD. In response to pre-activated NK cells, the average CD4<sup>+</sup> T cell migration from the upper to lower chambers of 3 $\mu$ m Transwell wells was ~ 40 % ( $39.45 \pm 19.1$ ). This migration was inhibited by ~ 30% ( $12.75 \pm 5.4$ ) when the medium in the NK cell chamber contained a neutralizing anti-IL-6 antibody. In positive control wells where CD4<sup>+</sup> T cell migration was induced by rIL-6, the average migration was  $81\% \pm 36.2$  compared to only  $5.8 \pm 2.2$  in negative controls where the migration of CD4<sup>+</sup> T cells was assessed only in response to culture medium alone. The data obtained from the all 4 samples are summarized in Figure 7.9.



**Figure 7.9 Effect of IL-6 neutralization on CD4<sup>+</sup> T cell migration across 3  $\mu$ m Transwell membrane in response to activated NK cells.** Results comparing data obtained from all 4 samples. From the bottom, the 1<sup>st</sup> bar is the percentage of migration of CD4<sup>+</sup> T cells in response to activated NK cells; the 2<sup>nd</sup> bar represents the migration of CD4<sup>+</sup> T cells when the NK cells were incubated with neutralizing anti-IL-6 antibody; the 3<sup>rd</sup> bar is the positive control where the migration of T cells was induced by IL-6; the 4<sup>th</sup> bar is the percentage migration of CD4<sup>+</sup> T cells in response to culture medium (negative control). All data are presented as means  $\pm$  SD.

## **7.4 Discussion**

### **7.4.1 The role of NK cells in T cells recruitment**

NK cells represent a component of the innate immunity known for their cytotoxic activity against intracellular pathogens and transformed cells (Trinchieri et al, 1989). In addition to their cytotoxic activity NK cells can also regulate the development of subsequent adaptive immune response via direct cell-to cell interaction (Gerosa et al, 2002; Piccioli et al, 2002; Lünemann et al, 2009) or via the secretion of immunoregulatory cytokines and chemokines (Kos, 1998; Biron et al, 1999; French and Yokoyama, 2004). These immunoregulatory functions include DC maturation, CD4<sup>+</sup> T cell polarization, selective editing of antigen presenting cells (APC), B cell activation and immunoglobulin isotype switching (Lünemann et al, 2009).

The recruitment of lymphocytes to the site of inflammation is a critical step in the generation of an effective adaptive immune response. NK cells dominate among the early invading cells in allografts and virus infected tissues (Somersalo et al, 1994) and their appearance precedes the influx of T cells. T cell recruitment to the site of inflammation is a complex process during which chemotactic cytokines and chemokines play a pivotal role (Roda et al, 2006). Many studies have shown that NK cells could participate in the initiation of a specific immune response by facilitating the recruitment of T cells to the site of inflammation through the secretion of cytokines and chemokines. Somersalo et al (1994) showed that NK cells activated *in vitro* with histamine and IL-2, or with a combination of IL-2 and an anti-CD16 monoclonal antibody increased both CD4<sup>+</sup> and CD8<sup>+</sup> T cell migration through fibronectin coated filters by 30-70%. This migration was inhibited by ~ 60% when the NK cells supernatant were pre-incubated with neutralizing antibodies to IL-8. Moreover, MIP-

1 $\alpha$  (CCL3) and RANTES (CCL5), two additional chemokines known to be secreted by NK cells in response to FcR/cytokine receptor co-stimulation, were documented in mediating T-cell chemotaxis (Roda et al, 2006). Hedrick et al (1997) found that lymphotactin (XCL1), a member of the XC chemokine family, is produced by NK cells and is responsible for the *in vivo* recruitment of both NK and T cells to the site of inflammation. NK cell depletion also has been shown to result in inhibition of T cell recruitment to the brain of Plasmodium berghei -infected animals (Hansen et al, 2007). Overall, these observations confirm that NK cells have the potential to recruit T lymphocytes, a function that fits with their unique role in bridging innate and adaptive immunity.

#### **7.4.2 IL-6 involvement in the migration of inflammatory and immune cells: Evidence from *in vitro* and *in vivo* studies**

Although IL-6 is not a classical chemokine, there is strong evidence that it alone or in combination with other cytokines, has a role in both *in vitro* and *in vivo* migration of other inflammatory and immune cells. Initially the ability of IL-6 to stimulate cell migration was attributed to IL-6 induced expression of other chemokines such as MCP-1 (CCL2) and IL-8 (CXCL8) (Romano et al, 1997). However, it has been found that IL-6 itself has the potential to act as a chemotactic factor by direct stimulation of cell migration. In dissecting the role of IL-6 in the migration of lymphocytes, Weissenbach et al (2004) found that, in the presence of extracellular matrix, IL-6 induced migration of human CD4<sup>+</sup>/CD45RO<sup>+</sup> T cells and human Karpas-299 CD4<sup>+</sup> T cell line. Moreover, they showed that this activity was not due to release of secondary chemotactic factors but was a direct response to IL-6. These findings are of great significance since the recruitment of CD4<sup>+</sup> T cells to the site of inflammation

is a crucial step in inflammation as well as in many autoimmune diseases. The role of IL-6 in stimulating the migration of lymphocytes was also investigated by others. Bacon et al (1990) found that IL-6 together with IL-3 and IL-4 directly induced peripheral lymphocyte migration *in vitro*. Lawlor et al (1990) demonstrated that IL-6 was not only capable of inducing human CD4<sup>+</sup> lymphocyte migration but also CD8<sup>+</sup> migration *in vitro*.

The finding that IL-6 is involved in T-lymphocyte trafficking is also supported by observations from *in vivo* studies in both inflammation and autoimmunity. Through analysis of mononuclear cell infiltration in wild type (WT) and IL-6-deficient mice during peritoneal inflammation, McLoughlin et al (2005) reported that IL-6-deficient mice exhibited impaired T cell recruitment. They showed that IL-6 selectively governs T cell infiltration by regulating chemokine secretion (CXCL10, CCL4, CCL5, CCL11, and CCL17) and chemokine receptor (CCR3, CCR4, CCR5, and CXCR3) expression within the CD3<sup>+</sup> infiltrate. Moreover, CD4<sup>+</sup> T-cells were unable to enter the infected lung in the absence of IL-6 during the course of an infection (Longhi et al, 2008; Dienz and Rincon, 2009). Using a mouse model of multiple sclerosis (experimental autoimmune encephalomyelitis), Arima et al (2012) showed that auto-reactive CD4<sup>+</sup> T cells access the central nervous system via the lumbar spinal cord. This access into the central nervous system was IL-6 dependent through up-regulation of the chemokine CCL20 in associated dorsal blood vessels. Interestingly, IL-6 trans-signalling events in an animal model of arthritis not only increase the severity of the disease through maintaining lymphocytes within the inflamed joint, but also through increasing lymphocyte recruitment and infiltration into the joint (Nowell et al, 2003).

In addition to lymphocytes, IL-6 was also reported to induce migration of other cells. IL-6 has been shown to be involved in the migration of monocytic cells (Clahsen and Schaper, 2008), and in the switching of the immune response from innate to acquired response (Kaplanski et al, 2003). Vasse et al (1999) demonstrated that IL-6 was a direct chemo-attractant for human dermal microvasculature endothelial cells (HMEC-1s) *in vitro*. Dominguez et al (2008) found that IL-6 in association with CXCL10 is a potent chemoattractive factor for trophoblast cells in a human model.

#### **7.4.3 *In vitro* trans-migration assay: Important considerations**

To test the specificity of CD4<sup>+</sup> T cell migration in response to IL-6 secreted by NK cells a modified Boyden chamber assay was used. CD4<sup>+</sup> lymphocytes were placed in the upper compartment of a Transwell chamber and were separated from the NK cells, IL-6 or culture medium in the lower compartment by the membrane of the insert. Cells were allowed to migrate through the pores of the membrane into the lower compartment. After 4 hours of incubation time the cells in the lower compartment were mixed with a known amount of beads to facilitate accurate quantitation, stained with an anti- CD4<sup>+</sup> FITC antibody and analysed by flow cytometry.

Cell migration is an active process that requires the cells to change their shape in a process similar to diapedesis that occurs *in vivo* (Colantonio, 2004). For this reason the selection of the appropriate Transwell plate with suitable pore size inserts is important as part of the optimization process for trans-migration assay. If the pore size is too small, there will be no cells migrating through the membrane. On the other hand if the pore size is large, there would be cells diffusing across the membrane by gravity and not by true trans-migration (Sherman and Rothenberg, 2012). There is some controversy regarding the most suitable insert's pore size used for studying the

migration of lymphocytes. In some studies 3  $\mu\text{m}$  pore size was used (Marelli-Berg et al, 1999; Edelbauer et al, 2010; O'Boyle et, 2012). In other studies 5  $\mu\text{m}$  (Taub et al, 1995; Zang et al, 2000; Roda et al, 2006; Damsker et al, 2007; Knieke et al, 2009) and 8  $\mu\text{m}$  (Graeler and Goetzl , 2002; Graeler et al, 2002; Weissenbach et al, 2004; Qin et al,2009; Kim et al, 2010) pore sizes were utilised. Inserts with 8  $\mu\text{m}$  pore size are usually recommended for migration of large cells like endothelial and epithelial cells, while 3  $\mu\text{m}$  pore size inserts are recommended for small cells like leukocytes (Transwell manufacturers' recommendations). In the preliminary experiment using 8  $\mu\text{m}$  pore size inserts the non specific (spontaneous) migration of the  $\text{CD4}^+$  T cells was around 11 %. Because of the large pore size there was a possibility that some of the  $\text{CD4}^+$  cells collected from the lower compartment diffused through the Transwell membranes by gravity and not by migration. In an attempt to keep the spontaneous migration as low as possible and to obtain maximal signal compared to background the 8  $\mu\text{m}$  inserts were replaced with 3  $\mu\text{m}$  inserts.

Another important point to be considered in trans-migration assay is the method used to calculate the number of cells that migrated into the lower compartment. In general, there are two methods used when counting the migrating cells by flow cytometry: "time-dependent acquisition of events" and "bead-dependent acquisition of events" (Colantonio, 2004). The first method depends on running all the samples at same event acquisition rate and the same period of time. Although this method is simple and inexpensive, it is not always accurate. The second method depends on using known number of microsphere particles that are brightly fluorescent across a wide range of excitation and emission wave lengths. These particles should be added to a known volume of sample and the absolute number of the cells can then be calculated using an equation.

#### 7.4.4 NK cell - secreted IL-6 stimulates CD4<sup>+</sup> T cell migration

The current study examined the ability of activated PBNK cells to stimulate CD4<sup>+</sup> T cell migration *in vitro* through fibronectin coated Transwell inserts. The results demonstrated that in the presence of NK cells that were pre-activated by culturing them in the presence of target cells and IL-2, about 40% of all CD4<sup>+</sup> T cells in the upper compartment migrated into the lower compartment during the 4 hrs of the assay period. This migration was inhibited to ~ 13% (~ 30% inhibition) in the presence of an anti-IL-6 antibody. IL-8 (CXCL8), MIP-1 $\alpha$  (CCL3), and RANTES (CCL5) are among the chemokines secreted by NK cells that are known to stimulate T cell migration or recruitment. However, this is apparently the first study shows that IL-6 from NK cells could cause this migration. This adds IL-6 to the growing list of cytokines and chemokines that are secreted by NK cells and have been found to stimulate *in vitro* T cell migration.

The ability of human NK cells to recruit T lymphocytes cells was investigated by others (section 7.4.1) and found to be dependent on the method used for activating NK cells and the state of T lymphocytes (naïve or activated). Somersalo et al (1994) showed that NK cells which were purified in the presence of 10% foetal calf serum (FCS), or which were activated in the absence of FCS but with histamine, IL-2, or with a combination of IL-2 and anti-CD16 mAbs increased both CD4<sup>+</sup> and CD8<sup>+</sup> T cell migration through fibronectin coated inserts by 30-70%. This migration was inhibited by ~ 60% in the presence of neutralizing antibodies to IL-8. Roda et al (2006) found that naïve T cells exhibit reduced migration (less than 10%) in response to NK cells stimulated with antibody-coated tumour cells and IL-2. In contrast, they found that NK cells co-stimulated with antibody-coated tumour cells and IL-12 induced significant levels of chemotaxis (~36% of T cells migrated) as compared with



those derived from NK cells stimulated with single stimulation conditions. When activated T cells were used, ~ 20% of the total cells migrated when the NK cells were activated with IL-12 alone compared to more than 50% migration when the NK cells were activated with antibody-coated tumour cells and IL-12. In the current study NK cells were activated overnight with HeLa cells + IL-2 before using in the transmigration assay. This activation recruited ~ 40% of CD4<sup>+</sup> T lymphocytes in the upper compartment. Therefore, our results extend the findings obtained by the above two groups and showed that activation of NK cells with targets + IL-2 was also sufficient to induce T cell migration.

The main aim of this chapter was to examine the functional significance of IL-6 secreted by NK cells. Having shown that activated NK cells could stimulate CD4<sup>+</sup> T cell migration did not necessarily indicate that IL-6 was causing this migration. As stated before, IL-8 (CXCL8), MIP-1 $\alpha$  (CCL3) and RANTES (CCL5) are secreted by NK cells and these chemokines have been shown to stimulate T cell migration. To confirm that IL-6 secreted by NK cells could also cause migration it was very necessary to neutralize the effect of IL-6 by an anti-IL-6 antibody. The results showed that the migration of CD4<sup>+</sup> T cells in response to NK cells was inhibited by ~ 30% when the NK cells were maintained in the presence of a neutralizing concentration of anti-IL-6 antibody. These results confirmed that IL-6 secreted by NK cells could, at least partially, contribute to T cell migration. Comparing these results with other chemokines, it has been found that the migration of T lymphocytes was inhibited by about 50-60% when the NK were pre-incubated with neutralizing antibodies to IL-8 (Somersalo et al, 1994; Roda et al, 2006). Neutralizing of MIP-1 $\alpha$  or RANTES had only a slight (~10%) effect on the migration of both naïve and

activated T lymphocytes (Roda et al, 2006). Therefore, IL-6 comes only second to IL-8 as a single cytokine secreted by NK cells that contributes to T cell migration.

One can argue about the significance of IL-6 secreted by NK cells in stimulating T lymphocyte migration since many other cytokines and chemokines secreted by NK cells can recruit T cells. Indeed, there are many possible answers to this question. **1)** Neither these results nor the results of other investigators confirm that a single cytokine or chemokine secreted by NK cells could stimulate 100% migration by T cells. Even with the positive control when the percentage T cell migration was > 95%, very high doses of IL-6 were used (20 ng/ ml) in comparison to picogram concentrations secreted by NK cells (Chapter 3). Such artificially high doses of IL-6 are unlikely to persist in an inflammatory milieu. Therefore, it is highly likely that IL-6 acts in combination with other cytokines and chemokines (IL-8, MIP-1 $\alpha$  and RANTES) to stimulate maximal T cell migration by NK cells. In support of this view, Roda et al (2006) found that neutralization of IL-8 + MIP-1 $\alpha$  + RANTES together had a dramatic effect on the migration of activated T cells in response to NK cells, reducing chemotaxis to ~20% of the original level. It still remains a possibility that NK cell-derived IL-6 can induce autocrine or paracrine production of chemokines which then mediate the chemotactic effect. **2)** The significance of CD4<sup>+</sup> T cell migration in response to IL-6 secreted by NK cells derives from the significance of IL-6 itself in both inflammation and autoimmunity. IL-6 is not only known to induce T cell recruitment, but also T cell proliferation, activation and differentiation. Lotz et al (1988) found that IL-6 acts as a co-stimulatory molecule for T cell proliferation independently of IL-2 gene expression. IL-6 was also found to prolong the survival of CD4<sup>+</sup> T cells both *in vitro* (Teague et al, 1997; Dienz and Rincon, 2009) and *in vivo* (Takeda et al, 1998) due to its anti-apoptotic properties. Antigen specific CD4<sup>+</sup> T cells

also expand more vigorously *in vivo* when IL-6 is present during immunization, due to reduced apoptosis, suggesting that IL-6 may increase the effector/memory T cell population (Dienz and Rincon, 2009). IL-6 was also shown to act synergistically with IL-1 in promoting T cell activation (Mizutani et al, 1988). In the light of these findings it is likely that NK cells, through the secretion of IL-6, may enhance the defensive immune response by coupling the induction of trafficking with stimulation of proliferation, activation and differentiation of CD4<sup>+</sup> T cells. 3) NK cells suggested to have an important role in several CD4<sup>+</sup> T cell- driven autoimmune diseases such as rheumatoid arthritis (RA) in which IL-6 has a major impact. In this disease NK cells were found in abundance in the synovial fluid as well as in the serum of RA patients (Pridgeon, 2002; Langley, 2005). Since NK cells are found at inflammatory sites at very early stages where the concentration of CD4<sup>+</sup> T cells is still very low, the abundance of the NK cells in the synovium of these patients further raises the possibility that NK cells could play a role in the initiation and progression of the disease by enhancing the CD4<sup>+</sup> T cell recruitment through IL-6 secretion.

# Chapter 8

## General Discussion



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NK cells have been shown to be important in defence against viral infections such as lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus (MCMV), herpes simplex virus (HSV), vaccinia virus and influenza virus (Rager-Zisman et al, 1987; Biron et al, 1999). They are able to kill virally infected cells through direct lysis of the infected cells via the secretion of cytolytic granules or interaction via death receptors (Fas-FasL interaction). However, in most of these infections, secretion of anti-viral cytokines was observed and demonstrated to have an important protective role.

INF- $\gamma$  and TNF- $\alpha$  are the two major cytokines secreted by NK cells and are known to have antiviral activity. However, in some of these infections where the NK cells have an important role in the defence mechanism, IL-6 appears to play a significant anti-viral role. For example, studies using IL-6 deficient mice have shown that IL-6 is involved in protection against HSV (LeBlanc et al, 1999; Murphy et al, 2008). It also increases the rate of HSV clearance following infection (Murphy et al, 2008). Similarly, in vaccinia virus infections IL-6 has a significant antiviral role as demonstrated by widespread infection in IL-6 deficient mice (Kopf et al, 1994). In addition to viral infections, NK cells were also reported to play an important defence role against some bacterial, fungal and parasitic infections. These include, but are not limited to, Mycobacterium tuberculosis (Brill et al, 2001), Staphylococcus aureus (Small et al, 2008), Listeria monocytogenes (Naper et al, 2011) and Aspergillus fumigatus (Bouzani et al, 2011). In these infections NK cells directly mediate the defence functions by the release of cytokines (Small et al, 2008, Bouzani et al, 2011). Interestingly, in separate *in vivo* studies on IL-6 deficient mice for all of the infections listed above IL-6 was found to have a specific protective role (Dalrymple et al, 1995; Ladel et al, 1997; Cenci et al, 2001; Hume et al, 2006). Considering the role of both

IL-6 and NK cells in these infections raises the possibility that IL-6 secretion by NK cells could be a main defence mechanism against them.

During acute inflammation IL-6, together with IL-1 and TNF- $\alpha$ , is required for induction of the acute phase response. This response is composed of fever, corticosterone release, and hepatic production of acute phase proteins such as C reactive protein (CRP). The induction of the acute phase reaction by IL-6 has been regarded as part of an attempt to maintain homeostasis (Xing et al, 1998). In addition to the acute phase response, IL-6 was found to play a critical role in controlling the extent of local and systemic acute inflammatory responses by suppressing the level of pro-inflammatory cytokines without compromising the level of anti-inflammatory cytokines (Xing et al, 1998; Gabay, 2006). Its role is also crucial in the successful resolution of acute inflammation by facilitating the transition of initial neutrophil infiltrates into a more sustained mononuclear cell population such as monocytes and lymphocytes (Kaplanski et al, 2003; Fielding et al, 2008). Impaired IL-6 secretion may lead to impaired neutrophil clearance and increased tissue damage from the accumulation of neutrophil-secreted proteases at the site of inflammation (Fielding et al, 2008). Indeed, this situation has been implicated in the pathogenesis of many inflammatory diseases such as peritonitis, sepsis, inflammatory bowel diseases, obstructive pulmonary diseases and renal injury (Fielding et al, 2008). Overall, these studies demonstrate the protective role of IL-6 in acute inflammation. Therefore, the concept of IL-6 secretion by NK cells could fit with the latter protective role as a first line defence mechanism appearing very early at inflammation sites. It also fits with their role as cells of innate immunity that have the potential of influencing the later stages of the immune response.

During chronic inflammation IL-6 may exhibit a contrasting feature. IL-6 has been found to be responsible for the persistent mononuclear cell infiltrates at the site of inflammation in a number of chronic inflammatory and autoimmune diseases such as rheumatoid arthritis (RA). In these diseases IL-6 acts as a pro-inflammatory rather than anti-inflammatory cytokine. Recent findings clearly indicate that IL-6 is a key factor involved in the regulation of T cell differentiation, activation and survival (Dienz and Rincon, 2009). Memory CD4<sup>+</sup> T lymphocytes have been shown to expand more vigorously *in vivo* when IL-6 is present due to the anti-apoptotic properties of this cytokine (Rochman et al, 2005). In addition, IL-6 stimulation has been linked to increased migration of activated CD4<sup>+</sup> T cells both *in vitro* and *in vivo*, which could explain their inability to enter the infected tissues in the absence of IL-6 during an infection (Weissenbach et al, 2004; Dienz and Rincon, 2009). NK cells have been shown to constitute a significant fraction of the infiltrating cells in RA and these cells are predominantly of the CD56<sup>bright</sup> phenotype (Pridgeon et al, 2003; Poli et al, 2009; Conigliaro et al, 2011). Interestingly, in concordance with the pro-inflammatory role of IL-6, NK cells were also found to play a significant pathogenic role in these diseases. Taking into consideration that cells with the CD56<sup>bright</sup> phenotype are mainly cytokine secretors (Cooper et al, 2001) together with that fact that IL-6 is a powerful regulator of CD4<sup>+</sup> T cell response (Dienz and Rincon, 2009) one can speculate that NK cells could be a major source of IL-6 secretion in these patients. Furthermore, the preliminary findings in this laboratory that NK cells isolated from the blood of patients with RA produce significantly more IL-6 after *in vitro* stimulation than NK cells isolated from the blood of healthy donors supports the hypothesis that NK cells could augment the T-cell response in these patients via IL-6 secretion.

In addition to RA, surprising evidence has arisen regarding the co-involvement of both NK cells and IL-6 in the pathogenesis of several other CD4<sup>+</sup> T cell driven autoimmune diseases and therefore it is plausible that these cells might be the source of IL-6 secretion in these diseases. For example, disorders characterised by increasing number of NK cells such as NK cell lymphocytosis and leukaemia are often associated with autoimmune diseases such as vasculitis (Tefferi et al, 1994; French and Yokoyama, 2004). Interestingly, IL-6 is often found to play an important pathogenic role in vasculitis as demonstrated by increasing serum concentration of IL-6 in these diseases (Roche et al, 1993; Emilie et al, 1994) as well as by the induction of disease remission using anti-IL-6 therapy (Seitz et al, 2011; Salvarani et al, 2012). Similarly, in animal models of autoimmunity, such as experimental autoimmune uveoretinitis (EAU), depletion of NK cells before immunization resulted in significantly less severe EAU demonstrating that NK cells participate in the development of EAU either by directly mediating cellular damage or by supporting rather than suppressing autoreactive T cells (Kitaichi et al, 2002; French and Yokoyama, 2004). A murine model of autoimmune-mediated diabetes after viral infection with Coxsackie B4 virus (CVB4) provides another example of NK cell-mediated damage (Flodström et al, 2002; French and Yokoyama, 2004). In this model, depletion of NK cells before infection with CVB4 prevented the development of diabetes, implying that NK cells contributed to the destruction of the infected pancreatic beta cells. Surprisingly, separate studies on the role of IL-6 in these diseases have shown IL-6 to play an important role in pathogenesis of these diseases and IL-6 blockade is often observed to be beneficial (Hohki et al, 2010). Based on these observations from both human and animal models of autoimmunity, it may be



useful to look at NK cell-induced IL-6 secretion in these disorders to see how this secretion could contribute to the abnormalities seen in these diseases.

The work presented in this thesis demonstrated that IL-6 concentration was significantly increased following exposure of NK cells to target cells against which these cells are known to exhibit direct cytotoxicity (either K562 or HeLa cells). The described secretory response was rapid with IL-6 being significantly higher as early as 1 hr in HeLa co-cultures compared to 6 hours in the case of K562 co-cultures. Since both the K562 and HeLa cells are able to secrete IL-6 endogenously, a question arose whether the increase in IL-6 secretion following co-culture of NK cells with the target cells was coming from these cells rather than from NK cells. This issue was addressed when NK cells were activated alone with high doses of IL-2 or with non specific chemical activators (PMA+ ionomycin). The result of these experiments clearly showed that NK cells alone have the potential to secrete IL-6 in the absence of the target cells. The finding that NK cells secrete this multifaceted cytokine within a short period of time has its impact on the subsequent immune response. Taking into consideration the effect of IL-6 on the cells of specific (adaptive) immunity together with the fact that NK cells appear at inflammatory sites during the very early stages when the number of other cells such as T cells is negligible, NK cell-mediated IL-6 secretion has the potential to influence the later stages of the immune response. Thus this mechanism may provide a critical link between the innate and adaptive immune response of the host. Furthermore, as described above, NK cell-induced IL-6 secretion in various autoimmune diseases, for example RA, may be relevant to the pathogenesis of these disorders.

Investigating the activation pathway which triggers the secretion of IL-6 by NK cells was the next step. This is an important point to clarify from both a biological

and a clinical perspective in order to fully understand the role of NK cells in regulating adaptive immune response. It was known from the Transwell experiments presented in Chapter 3 that when NK cells come in contact with target cells, direct cell–cell contact is required for IL-6 production. For many years it was thought that the NK cells recognize the target cells as “friends or enemies” depending on the surface expression of MHC class I molecules on these cells; the “missing self hypothesis”. However, this concept represents an oversimplification of the way that NK cells recognize their targets. In the last decade it has been demonstrated that NK cells could attack some targets despite the expression of MHC class I molecules. On the other hand, some other cells are not sensitive to NK cell-lysis despite low or absent class I MHC expression. These observations necessitated the presence of other mechanisms, in addition to the “missing self hypothesis” by which NK cell target recognition is mediated. It is now clear that NK cells are controlled by complex combinations of activating and inhibitory receptors and the integration of signals derived from these receptors can determine whether the NK cell would become activated or not. The fact that HeLa, an MHC class I positive target cell line, provides a stimulus for IL-6 production by NK cells indicates that KIR receptors recognising MHC class I proteins as an inhibitory molecule, play no role in the initiation of IL-6. This observation also indicates that the pathway involved provides an activating signal not “switched off” by inhibitory KIR signalling. On other hand, the finding that NK cells exposed to HeLa cells secrete more IL-6 than those exposed to K562 suggesting the involvement of different NK cell activating receptors in this secretion. Some studies using K562 cells as targets have demonstrated that NKG2C is involved in the NK cell induced lysis of these cells (Düchler et al, 1995). Despite this study, because K562 cells lack the surface expression of MHC-class I molecules, therefore the involvement of NKG2C

(recognise HLA-E alleles) in K562 lysis is less likely. The involvement of this receptor in NK cell induced lysis and cytokine secretion in case of HeLa cells seems to be unlikely also. Moretta et al (2001) suggested that in the case of MHC class I<sup>+</sup> cells, NKG2C (and activated KIRs) could only initiate NK cell triggering if the target cells do not express ligands for NKG2D and the NK cells are of NCR<sup>dull</sup> phenotype i.e. when the involvement of NKG2D and NCR pathways is lacking. Since HeLa cells are NKG2D ligand and NCR ligand positive (Moretta et al, 2001), therefore NKG2D and NCRs receptors are the most likely cell surface receptors satisfying this criterion, although some of the other natural killer receptors such as CD16, as well as other, less widely studied pathways, may also be theoretically responsible. One of the easiest ways to identify the molecular pathways involved in IL-6 secretion by NK cells would be the use of blocking monoclonal antibodies (antagonist mAbs) against different activating NK cell receptors to see if blocking of these receptors resulted in significantly reduced levels of IL-6 production by NK cells. Blocking some of these receptors such as NKG2D was indeed attempted (data not shown). The experiment was carried out on four healthy donors and the NK cells were co-cultured with HeLa cells in the presence and absence of an anti-NKG2D antibody (clone 1D11) at a concentration of 10 µg/ml. This antibody clone was used by others to block NKG2D receptors in NK and CD8<sup>+</sup> T cells (Carbone et al, 2005; Mincheva - Nilsson et al, 2006; Crane et al, 2010). In these experiments the results were inconsistent. Only one sample showed a prominent inhibition of IL-6 secretion by NK cells in the presence of blocking antibody while two samples showed only mild inhibition and one sample showed no inhibition at all. These findings, together with the fact that monoclonal antibodies with blocking properties are not commercially available against all NK receptors, made it necessary to consider other ways to study the differential

involvement of NK cell receptors in IL-6 secretion. An alternative approach to receptor blocking experiments was to stimulate NK cells in the presence of stimulating (agonist) antibodies. Immobilized agonist mAbs against a wide range of NK receptors, namely anti-CD16 (clone 3G8), anti-NKG2D (clone 149810), anti-NKp46 (clone 195314), anti-NKp44 (clone 253415) and anti-NKp30 (clone 210845) are widely available and were used to activate NK cells (Chapter 4). In concordance with the HeLa co-culture experiments, the results demonstrated that NKG2D and NKp46 (in addition to CD16 to lesser extent) were the main receptors involved in inducing IL-6 expression / or secretion. This response was detectable as early as 1 hour following stimulation, increased further at 3 and 6 hours and reached a peak at 24 hours. The finding that different receptors can induce IL-6 secretion suggests the involvement of different activating pathways in this secretion. Although structurally different, both NKp46 and CD16 NK cell activating receptors signalling through the same ITAM containing subunits (CD3 $\zeta$  or Fc $\epsilon$ RI $\gamma$ ). Activation of these receptors on the surface of NK cells results in Ca<sup>2+</sup> flux followed by the phosphorylation of CD3 $\zeta$  or Fc $\epsilon$ RI $\gamma$  by Lymphocyte-specific protein tyrosine kinase (Lck) and the recruitment of SYK family kinases-tyrosine kinase (Syk) and Zeta-chain associated protein kinase 70kDa (ZAP70). NKG2D receptors are not associated with CD3 $\zeta$  or Fc $\epsilon$ RI $\gamma$  and instead they are associated with another ITAM bearing subunit (DAP10). Activation of NKG2D receptors on the surface of NK or CD8<sup>+</sup> T cells stimulates another signalling cascade characterised by recruitment of phosphatidylinositol 3-kinase (PI3K). Therefore different NK cell receptors activate different signalling pathways. However, in contrast to other cytokines secreted by NK cells such as IFN- $\gamma$  and TNF- $\alpha$ , the observation that the engagement of a single receptor was sufficient for the secretion of IL-6 by NK cells may reflect a lower threshold for IL-6 secretion by these cells. In fact

this lower threshold for IL-6 secretion by NK cells fits with the pivotal role of IL-6 in inflammation.

In the relative real time experiments fold change differences in IL-6 mRNA expression was detected as early as 1 hour, increased further at 3 and 6 hours but was then inhibited at 24 hours regardless of whether the NK cells were stimulated with immobilized antibodies to NKG2D, NKp46 or CD16. The fact that IL-6 mRNA expression was detected as early as 1 hour post activation is an important finding into the activation mechanism. It may indicate that IL-6 message is constitutively expressed by NK cells. However, the subsequent findings that IL-6 mRNA increased further at later stages also indicate that IL-6 expression is regulated at transcription level. Therefore, the assumption is that IL-6 expression by NK cells following appropriate activation is regulated both at transcriptional and translational levels. However, transcriptional regulation seemed to be transient since there was a decline at 24 hours to a level comparable to base line background.

It is known that activating mouse NK cells with anti-NKG2D mAbs can lead to a potent NKG2D - dependent stimulation of cytokine release (Diefenbach and Raulet, 2001; Jamieson et al, 2002). However, the role of NKG2D in cytokine production by human NK cells upon mAbs triggering remains unclear. In contrast to mouse studies, cross linking human NKG2D receptors by mAbs failed to stimulate IFN- $\gamma$  secretion by NK cells (Billadeau et al, 2003; André et al, 2004). These contradictory findings were attributed to structural differences in the NKG2D receptors between these two species. In the mouse two alternative splice products of the NKG2D gene co-exist on the surface of the activated NK cells (mNKG2D-L and mNKG2D-S) whereas there is only one human NKG2D product (Rosen et al, 2004). Since NKG2D receptors in both mouse and human lack intrinsic signalling capability,

therefore they need to be associated with adaptor molecules within their transmembrane (TM) region. The mNKG2D-L and hNKG2D are selectively associated with DAP10; while the mNKG2D-S can be associated with either DAP10 or DAP12 molecules. In other words, mouse NKG2D receptors can be associated with both DAP10 and DAP12 while human NKG2D can only be associated with DAP10. It has been reported that mAbs activation of DAP10 associated NKG2D receptors stimulates NK cell cytotoxicity but not cytokine secretion, while activation of receptors in association with DAP12 can stimulate both cytotoxicity and cytokine secretion (André et al, 2004). These findings led to the assumption that while NKG2D can act as a primary activating receptor on mouse NK cells, it functions only as a co-receptor in humans (Diefenbach et al, 2002). In contrast, we found that triggering of hNKG2D receptors with immobilized mAbs can stimulate both mRNA expression and secretion of IL-6. This is apparently the first work demonstrating that human NK cells can be induced to secrete a cytokine upon NKG2D triggering via a monoclonal antibody. This discrepancy can, however, be easily explained. All other studies focused on the secretion of IFN- $\gamma$ , a cytokine that is regulated by different signalling elements than IL-6. As discussed in Chapter 4, Syk and ZAP70 kinases are the main signalling element involved in the secretion of IFN- $\gamma$  by human NK cells. Since triggering of hNKG2D receptors by a mAbs activates totally different kinases, therefore it is not surprising that cross-linking of these receptors cannot activate IFN- $\gamma$  secretion. As explained above, the activation of DAP10 associated NKG2D stimulates another signalling pathway, PI3K. Moreover, NKG2D activation in association with DAP10 has also shown to activate other activating pathways namely phospholipase-C  $\gamma$  and VAV (Billadeau et al, 2003; Bryceson et al, 2006). Stimulation of PI3K, phospholipase-C  $\gamma$  and VAV signalling elements has been linked to increased IL-6

secretion in a variety of normal as well as malignant human cells. Taken together these results may reveal distinct activation mechanisms for different cytokines by NK cells.

As stated before, these results demonstrated that NKG2D and NKp46 were the main receptors involved in inducing IL-6 expression. To assess which NKG2D ligands were involved in this process, the expression of the NKG2D ligands as fusion proteins was attempted. Unfortunately, the expression of these fusion proteins was unsuccessful. As an alternative strategy target cells transfected with the individual ligands were used. Results showed that MICA and, and to a lesser extent, MICB were involved in NKG2D induced secretion of IL-6 by NK cells. Although this might indicate variation in the affinity of different ligands to NKG2D (which is supported in the literature), differences in the surface expression of these ligands on the target cells cannot be excluded as a potential explanation.

It is known that the recruitment of leukocytes to the site of inflammation is a crucial step in the immune response. Chemokines play a central role in this process. Initially neutrophils are recruited first to the site of inflammation followed by mononuclear cells such as monocytes and lymphocytes. Although not a classical chemokine, IL-6 has been found to play an important role in recruitment of lymphocytes to the site of inflammation and therefore facilitating the switching of the immune response from the innate to the more specific adaptive response. The potential of IL-6 to induce cell migration was attributed to **1)** induction of expression of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and IL-8. **2)** a direct effect of IL-6 on T cell migration. Given the role of NK cells as cells of innate immunity with immunoregulatory functions, it is possible that NK cells recruit these cells to the site of inflammation via IL-6 secretion. To test this hypothesis, an *in vitro* transmigration assay using a modified Boyden chamber was used. The results

demonstrated that CD4<sup>+</sup> T cell migration in response to NK cells was inhibited by about 30% in the presence of neutralizing antibody to IL-6. These results signify the relative biological importance of IL-6 induced secretion by NK cells.

After this research, more questions are raised and needed to be explored in the future. It is known that NK cells are not a homologous group of cells and are divided into two phenotypic subsets according to their surface expression of CD56. These are CD56<sup>dim</sup> cells which constitute ~ 90% of PBNK cells and CD56<sup>bright</sup> cells which constitute ~10% of PBNK cells but are enriched in secondary lymphoid tissues (SLTs). For many years it was generally accepted that CD56<sup>dim</sup> cells are mainly responsible for cytotoxicity and target cell killing, while CD56<sup>bright</sup> cells are mainly cytokine secretors and acquire cytotoxicity only after prolonged activation. However, this concept is now challenged by the recent findings that CD56<sup>dim</sup> cells are also a potent source of cytokine and chemokines that are secreted within relatively short times (hours) after target cell recognition (Fauriat et al,2010 ; Juelke et al, 2010 ; Moretta, 2010). Since CD56<sup>bright</sup> cells express more monokine receptors (IL-2, IL-12, IL-15 and IL-18) than CD56<sup>dim</sup> cells (Cooper et al, 2001), therefore it may be possible that this NK subtype are the main subset responsible for cytokine secretion upon monokine activation. However, CD56<sup>dim</sup> cells seem to be the main cytokine secretors upon target cell recognition. Based on these observations, it would be interesting in the future to see which of these differential subsets contributes to IL-6 secretion by NK cells. Identification of the phenotype of the NK cell subset responsible for IL-6 secretion would facilitate a better understanding of the biology of NK cell subsets. Moreover, it may also have an important clinical implication in monitoring the role of NK cells in pathologic conditions in which these effectors may contribute to defence or, possibly, to tissue damage.



There is some preliminary evidence in this laboratory that NK cells isolated from patients with autoimmune diseases such as RA persistently produce more IL-6 than control groups (unpublished data). Whether this reflects differences in the activation state of NK cells between the patients and controls or represents an inherent genetic feature of the disease needs to be further clarified in the future.

Another important direction for future study will be an *in vivo* evaluation of the biological significance of IL-6 secretion by NK cells. IL-6 is secreted rather ubiquitously by many cell types. To see if IL-6 production by NK cells is biologically relevant in influencing antiviral and antibacterial defence mechanisms, the use of animal models with selective NK cell deficiency are planned. Here two animal models are suggested. In the first model IL-6 knockout mice will be subjected to viral infection such as LCMV, HSV or vaccinia virus. The susceptibility of mice to these infections would be assessed later by measuring viral load in the blood and local tissues by PCR. IL-6 knockout mice will then be divided into three groups. The first group will be left untreated (negative control), the second group will be injected with NK cells derived from wild type (WT)-IL-6 secreting congenic animals and the third group will be injected with PBMCs from WT donors, which have been depleted of NK cells. The mice will then be monitored over an appropriate period of time to see if the function of any of the three groups will be re-constituted. If giving WT NK cells improves the outcome then the argument can be made that IL-6 secretion by NK cells is a critical step in protecting the animals against the infection. On the other hand, if the giving WT NK cells afford little or no benefit, while injecting other mononuclear cells from animals that can produce IL-6 proves beneficial, this would indicate that IL-6 secretion by cells other than NK cells is the critical step in generating a protective immune response. The second model is a more direct evaluation of the importance of

NK cell-secreted IL-6. During the course of the *in vivo* experiments three groups of mice will be subjected to NK cell depletion by injecting them with rabbit antiserum to asialo GM1, a neutral glycosphingolipid present in high concentration on the surface of mouse NK cells. The first group of animals will then be left untreated – negative control – while the second group of mice will be reconstituted with NK cells that can produce IL-6 while the third group will be injected with NK cells derived from IL-6 knockout animals. A few hours later these three groups will be challenged with a virus. Over a period of days post infection the animals will be evaluated for viral load in the blood. If the IL-6 produced by NK cells is biologically relevant and contributes to the defence mechanisms of the body against infection, one would expect that the first and the third groups of mice will develop a more serious infection while the second group will be at least partially protected.

One of the future aims would be to expand this work to see if IL-6 production by NK cells can affect the activity of B cells and DCs in a variety of experimental models as well as in autoimmune diseases. For example, it is known that IL-6 plays a central role in B cell activation, survival and differentiation. Thus it is a critical component of the regulation of both protective and autoreactive B cells (Hirano et al, 1985; Kishimoto, 1989). At the same time, activated PBNK cells have been shown to directly stimulate resting B cells to produce IgM and IgG (Yuan et al, 1992; Gray and Horwitz, 1995). Combining these findings together, it is possible that NK cells stimulate resting B cells through IL-6 secretion. Moreover, it has been demonstrated that both NK cells and IL-6 play a crucial role in the pathogenesis of some of B cell autoimmune diseases such as experimental autoimmune myasthenia gravis (French and Yokoyama, 2004; Arecha et al, 2011). Therefore, it would be interesting to assess role of NK cell-induced IL-6 in this disease.

# Chapter 9

## Appendix



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## 9.1 Human MICA sequence

Human MICA sequence, transcript variant 1 (allele MICA\*00801) (NCBI Reference Sequence: NM\_001177519) is presented below. Basically, it consists of a leader peptide (L), alpha-1 ( $\alpha$ 1), alpha-2 ( $\alpha$ 2), alpha-3 ( $\alpha$ 3), transmembrane portion (TM) and cytoplasmic tail (Bahram et al, 1996a). The sequence encoding for extracellular region includes L,  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 and it is 891 bp in length (Bahram et al, 1996a). It starts at position 40 and ends at position 930.

```
CACTGCTTGA GCCGCTGAGA GGGTGGCGAC GTCGGGGCCA TGGGGCTGGG CCCGGTCTTT
CTGCTTCTGG CTGGCATCTT CCCTTTTGCA CCTCCGGGAG CTGCTGCTGA GCCCCACAGT
CTTCGTTATA ACCTCACGGT GCTGTCCTGG GATGGATCTG TGCAGTCAGG GTTCTTTGCT
GAGGTACATC TGGATGGTCA GCCCTTCCTG CGCTATGACA GGCAGAAATG CAGGGCAAAG
CCCCAGGGAC AGTGGGCAGA AGATGTCCTG GGAAATAAGA CATGGGACAG AGAGACCAGG
GACTTGACAG GGAACGGAAA GGACCTCAGG ATGACCCTGG CTCATATCAA GGACCAGAAA
GAAGGCTTGC ATTCCCTCCA GGAGATTAGG GTCTGTGAGA TCCATGAAGA CAACAGCACC
AGGAGCTCCC AGCATTTCTA CTACGATGGG GAGCTCTTCC TCTCCAAAAA CCTGGAGACT
GAGGAATGGA CAGTGCCCCA GTCTCCAGA GCTCAGACCT TGGCCATGAA CGTCAGGAAT
TTCTTGAAGG AAGATGCCAT GAAGACCAAG ACACACTATC ACGCTATGCA TGCAGACTGC
CTGCAGGAAC TACGGCGATA TCTAGAATCC GCGTAGTCC TGAGGAGAAC AGTGCCCCC
ATGGTGAATG TCACCCGCGAG CGAGGCCTCA GAGGGCAACA TCACCGTGAC ATGCAGGGCT
TCCAGTTCT ATCCCCGAA TATCATACTG ACCTGGCGTC AGGATGGGGT ATCTTTGAGC
CACGACACCC AGCAGTGGGG GGATGTCTG CCTGATGGGA ATGGAACCTA CCAGACCTGG
GTGGCCACCA GGATTTGCCG AGGAGAGGAG CAGAGGTTCA CCTGCTACAT GGAACACAGC
GGGAATCACA GCACTCACCC TGTGCCCTCT GGGAAAGTGC TGGTGCTTCA GAGTCATTGG
CAGACATTCC ATGTTTCTGC TGTGTCTGCT GGCTGCTGCT ATTTTGTGTA TTATTATTTT
CTATGTCCGT TGTGTAAGA AGAAAACATC AGCTGCAGAG GTTCCAGAGC TCGTGAGCCT
GCAGGTCTCT GATCAACACC CAGTTGGGAC GAGTGACCAC AGGGATGCCA CACAGCTCGG
ATTTAGCCT CTGATGTCAG CTCTTGGGTC CACTGGCTCC ACTGAGGGCA CCTAGACTCT
ACAGCCAGGC GGCTGGAATT GAATTCCTG CCTGGATCTC ACAAGCACTT TCCCTCTTGG
TGCCTCAGTT TCCTGACCTA TGAACAGAG AAAATAAAAG CACTTATTTA TTGTGTTGG
AGGCTGCAAA ATGTTAGTAG ATATGAGGCA TTTGCAGCTG TGCCATATT (1369 bp)
```

Red: Leader sequence = 69 nucleotides

Blue: Alpha 1 domain = 256 nucleotides

Green: Alpha 2 domain = 286 nucleotides

Purple: Alpha 3 domain = 280 nucleotides

### 9.1.1 Recombinant (Chimeric) hMICA-mFc1 sequence

Recombinant hMICA-mFc1 sequence cloned (1590 bp) is presented below (5' to 3' orientation). The cloned sequence started with the start codon **ATG** and end with the stop codon **TGA**. No other stop codons (TAG, TAA, and TGA) were found through the entire sequence.

```
ATGGGGCTGGGCCCCGGTCTTCCCTGCTTCTGGCTGGCATCTTCCCTTTTGCACCTCCGGGAGCTGCTGCTG
AGCCCCACAGTCTTCGTTATAACCTCACGGTGTCTGCTGGGATGGATCTGTGCAGTCAGGGTTTCTCAC
TGAGGTACATCTGGATGGTCAGCCCTTCCCTGCGCTGTGACAGGCAGAAATGCAGGGCAAAGCCCCAGGGA
CAGTGGGCAGAAGATGTCCTGGGAAATAAGACATGGGACAGAGAGACCAGAGACTTGACAGGGAACGGAA
AGGACCTCAGGATGACCCTGGCTCATATCAAGGACCAGAAAGAAGGCTTGCATTCCCTCCAGGAGATTAG
GGTCTGTGAGATCCATGAAGACAACAGCACCAGGAGCTCCCAGCATTTCTACTACGATGGGGAGCTCTTC
CTCTCCAAAAACCTGGAGACTAAGGAATGGACAATGCCCCAGTCCCTCCAGAGCTCAGACCTTGGCCATGA
ACGTCAGGAATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTATCACGCTATGCATGCAGACTG
CCTGCAGGAACCTACGGCGATATCTAAAATCCGGCGTAGTCTGAGGAGAACAGTGCCCCCATGGTGAAT
GTCACCCGCAGCGAGGCCTCAGAGGGCAACATTACCGTGACATGCAGGGCTTCTGGCTTCTATCCCTGGA
ATATCACACTGAGCTGGCGTCAGGATGGGGTATCTTTGAGCCACGACACCCAGCAGTGGGGGGATGTCTT
```

GCCTGATGGGAATGGAACCTACCAGACCTGGGTGGCCACCAGGATTTGCCAAGGAGAGGAGCAGAGGGTTC  
 ACCTGCTACATGGAACACAGCGGGAATCACAGCACTCACCTGTGCCCTCTCCAGAGGGCCCACAATCA  
 AGCCCTGTCCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCC  
 AAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAG  
 GATGACCCAGATGTCCAGATCAGCTGGTTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCC  
 ATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCACTGCCCTCCCCATCCAGCACCAGGACTGGATGAG  
 TGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCCATCGAGAGAACCATCTCAAAA  
 CCCAAAGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAAC  
 AGGTCACCTCTGACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGGACCAACAACGG  
 GAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCTGGACTCTGATGGTTCTTACTTCATGTACAGC  
 AAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAAGAAATAGCTACTCCTGTTTCAGTGGTCCACGAGGGTC  
 TGCACAATCACCACACGACTAAGAGCTTCTCCCGGACTCCGGGTAAA (1590 bp)

Black: Leader sequence = 69 nucleotides

Red: Sequence encodes for extracellular portion of MICA protein = 822 bp

Blue: Sequence encodes for Fc region of mIgG2A = 699 bp

TCATGA: BspHI restriction site

Translation (5' to 3' frame of the above nucleotides sequence)

MGLGPVFLLLAGIFPFAPPAAAAEPHSLRYNLTVLSWDGVSQSGFLTEVHLDGQPFRLRCRQKCRAPQ  
 GQWAEDVLGNKTWDRETRDLTGNGKDLRMTLAHIKDQKEGLHSLQEIIRVCEIHEDNSTRSSQHFYYDGE  
 LFLSQNLETKEWTPQSSRAQTLAMNVRNFLKEDAMKTKTHYHAMHADCLQELRRYLKSGVLRRTVPP  
 MVNVRSEASEGNITVTCRASGFYPWNITLSWRQDGVLSHDTQQWGDVLPDNGTYQTWVATRICQGE  
 EQRFTCYMEHSGNHSTHPVPSPRGPTIKPCPPCKCPAPNLLGGPSVFI FPPKIKDVLMI SLSPIVTCVV  
 VDVSEDDPDVQISWFVNNVEVHTAQQTTHREDYNSTLRVVSALPIQHQDWMMSGKEFKCKVNNKDLPAPI  
 ERTISKPKGSVRAPQVYVLPPEEEMTKKQVTLTCMVTFMPEDIYVEWTNNGKTELNYKNTEPVLDS  
 GSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

Black: Leader protein sequence

Red: Extracellular portion of MICA protein

Blue: Fc portion of mIgG2A

Molecular weight = 60011.35 Daltons

### 9.1.1.1 Alignment of the cloned nucleotide sequence to hMICA-mFc1 sequence

The result of hMICA - mFc1 sequencing was aligned to the sequence described in section 9.1.1 using BLAST tool from NCBI website. Note that the *Query sequence* is the sequencing result, while the *Sbjct sequence* is the sequence described in section 9.1.1

#### Sequencing result

CGCCTACCTTGAAGATCCACCCGATGAATTCATGGGGCTGGGCCCGGTCTTCCCTGCTTCTGGCTGGCATCTTCCC  
 TTTTGACCTCCGGGAGCTGCTGCTGAGCCCCACAGTCTTCGTTATAACCTCACGGTCTGTCTGGGATGGATCT  
 GTGCAGTCAGGGTTTCTCACTGAGGTACATCTGGATGGTCAAGCCCTTCCCTGCGCTGTGACAGGCAGAAATGCAGGG  
 CAAAGCCCCAGGGACAGTGGGCAGAAGATGTCTGGGAAATAAGACATGGGACAGAGAGACCAGAGACTTGCAGG  
 GAACGGAAAGGACCTCAGGATGACCTGGCTCATATCAAGGACCAGAAAGAAGGCTTGCATTCCCTCCAGGAGATT  
 AGGGTCTGTGAGATCCATGAAGACAACAGCACCAGGAGCTCCCAGCATTTCTACTACGATGGGGAGCTCTTCCCTCT  
 CCAAAAACCTGGAGACTAAGGAATGGACAATGCCCGAGTCTCCAGAGCTCAGACCTTGGCCATGAACGTCAGGAA  
 TTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTATCACGCTATGCATGCAGACTGCCTGCAGGAACACGG  
 CGATATCTAGAATCCGGCGTAGTCTGAGGAGAACAGTGCCCCCATGGTGAATGTCAACCCGACGCGAGGCGCTCAG  
 AGGGCAACATCACCGTGACATGCAGGGCTTCTGGCTTCTATCCCTGGAATATCACACTGAGCTGGCGTCAGGATGG

GGTATCTTTGAGCCACGACACCCAGCAGTGGGGGGATGTCTCGCTGATGGGAATGGAACCTACCAGACCTGGGTG  
GCCACCAGGATTTGCCAAGGAGAGGAGCAGAGGTTACCTGCTACATGGAACACAGCGGGAATCACAGCACTCACC  
CTGTGCCCTCTCCAGAGGGGCCCAATCAAGCCCTGTCTCCATGCAANNNNNNCCNCTN  
AATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTGGGTGG  
ACCATCCGTCTTCATCTTCGCTCCAAAGATCAAGGATGTAC TCTCCCTGAGCCCCATAGTCACATGTGTG  
GTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACAGCTC  
AGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTG  
GATGAGTGGCAAGGAGNNNNNTNNNNNGN

**GAATTC**: EcoRI restriction site

**TCATGA**: BspHI restriction site

### Alignment

Query	33	ATGGGGCTGGGCCCGGTCTTCTCGCTTCTGGCTGGCATCTTCCCTTTTGCACCTCCGGGA	92
Sbjct	1	ATGGGGCTGGGCCCGGTCTTCTCGCTTCTGGCTGGCATCTTCCCTTTTGCACCTCCGGGA	60
Query	93	GCTGCTGCTGAGCCCCACAGTCTTCGTTATAACCTCACGGTGTCTCTGGGATGGATCT	152
Sbjct	61	GCTGCTGCTGAGCCCCACAGTCTTCGTTATAACCTCACGGTGTCTCTGGGATGGATCT	120
Query	153	GTGCAGTCAGGGTTTCTCACTGAGGTACATCTGGATGGTCAGCCCTTCTGCGCTGTGAC	212
Sbjct	121	GTGCAGTCAGGGTTTCTCACTGAGGTACATCTGGATGGTCAGCCCTTCTGCGCTGTGAC	180
Query	213	AGGCAGAAATGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAGATGTCCTGGGAAATAAG	272
Sbjct	181	AGGCAGAAATGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAGATGTCCTGGGAAATAAG	240
Query	273	ACATGGGACAGAGAGACCAGAGACTTGACAGGGAACGGAAAGGACCTCAGGATGACCCCTG	332
Sbjct	241	ACATGGGACAGAGAGACCAGAGACTTGACAGGGAACGGAAAGGACCTCAGGATGACCCCTG	300
Query	333	GCTCATATCAAGGACCAGAAAGAAGGCTTGCAATCCCTCCAGGAGATTAGGGTCTGTGAG	392
Sbjct	301	GCTCATATCAAGGACCAGAAAGAAGGCTTGCAATCCCTCCAGGAGATTAGGGTCTGTGAG	360
Query	393	ATCCATGAAGACAACAGCACCAGGAGCTCCCAGCATTTCTACTACGATGGGGAGCTCTTC	452
Sbjct	361	ATCCATGAAGACAACAGCACCAGGAGCTCCCAGCATTTCTACTACGATGGGGAGCTCTTC	420
Query	453	CTCTCCCAAACCTGGAGACTAAGGAATGGACAATGCCCCAGTCCCTCCAGAGCTCAGACC	512
Sbjct	421	CTCTCCCAAACCTGGAGACTAAGGAATGGACAATGCCCCAGTCCCTCCAGAGCTCAGACC	480
Query	513	TTGGCCATGAACGTCAGGAATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTAT	572
Sbjct	481	TTGGCCATGAACGTCAGGAATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTAT	540
Query	573	CACGCTATGCATGCAGACTGCCTGCAGGAACTACGGCGATATCTA GAATCCGGCGTAGTC	632
Sbjct	541	CACGCTATGCATGCAGACTGCCTGCAGGAACTACGGCGATATCTA GAATCCGGCGTAGTC	600
Query	633	CTGAGGAGAACAGTGCCCCCATGGTGAATGTACCCCGCAGCGAGGCCCTCAGAGGGCAAC	692
Sbjct	601	CTGAGGAGAACAGTGCCCCCATGGTGAATGTACCCCGCAGCGAGGCCCTCAGAGGGCAAC	660
Query	693	ATCACCGTGACATGCAGGGCTTCTGGCTTCTATCCCTGGAATATCACACTGAGCTGGCGT	752
Sbjct	661	ATCACCGTGACATGCAGGGCTTCTGGCTTCTATCCCTGGAATATCACACTGAGCTGGCGT	720
Query	753	CAGGATGGGGTATCTTTGAGCCACGACACCCAGCAGTGGGGGGATGTCTGCTGATGGG	812
Sbjct	721	CAGGATGGGGTATCTTTGAGCCACGACACCCAGCAGTGGGGGGATGTCTGCTGATGGG	780
Query	813	AATGGAACCTACCAGACCTGGGTGGCCACCAGGATTTGCCAAGGAGAGGAGCAGAGGTTT	872
Sbjct	781	AATGGAACCTACCAGACCTGGGTGGCCACCAGGATTTGCCAAGGAGAGGAGCAGAGGTTT	840

```

Query 873 ACCTGCTACATGGAACACAGCGGGAATCACAGCACTCACCCCTGTGCCCTCTCCAGAGGG 932
          |||
Sbjct 841 ACCTGCTACATGGAACACAGCGGGAATCACAGCACTCACCCCTGTGCCCTCTCCAGAGGG 900

Query 933 CCCACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCA 992
          |||
Sbjct 901 CCCACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCA 960

Query 993 TCCGTCTTCATCTTCCTCCAAAGATCAAGGATGTACTCATGA|TCTCCCTGAGCCCCATA 1052
          |||
Sbjct 961 TCCGTCTTCATCTTCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATA 1020

Query 1053 GTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTT 1112
          |||
Sbjct 1021 GTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTT 1080

Query 1113 GTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGT 1172
          |||
Sbjct 1081 GTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGT 1140

Query 1173 ACTCTCCGGGTGGTCAGTGCCCTCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAG 1232
          |||
Sbjct 1141 ACTCTCCGGGTGGTCAGTGCCCTCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAG 1200

```

### 9.1.2 Recombinant hMICA-mFc2 sequence

The cloned sequence of recombinant hMICA-mFc2 (1581 bp) is presented below (5' to 3' orientation). Natural leading sequence for MICA protein were removed and replaced by the leader sequence of IL-2 protein. Start codon **ATG** and stop codon **TGA** were highlighted in colours.

```

ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACCTGTGCAGAAATTCGGAGCCCCAC
AGTCTTCGTTATAACCTCACGGTGTCTGCTGGGATGGATCTGTGCAGTCAGGGTTTCTTGCTGAGGTA
CATCTGGATGGTCAGCCCTTCTGCGCTATGACAGGCAGAAATGCAGGGCAAAGCCCCAGGGACAGTGG
GCAGAAGATGTCTGGGAAATAAGACATGGGACAGAGAGACCAGGGACTTGACAGGGAACGGAAAGGAC
CTCAGGATGACCCTGGCTCATATCAAGGACCAGAAAGAAGGCTTGCATTCCCTCCAGGAGATTAGGGTC
TGTGAGATCCATGAAGACAACAGCACCAGGAGCTCCCAGCATTTCTACTACGATGGGGAGCTCTTCCTC
TCCCAAACCTGGAGACTGAGGAATGGACAGTGCCCCAGTCCCTCCAGAGCTCAGACCTTGCCCATGAAC
GTCAGGAATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTATCACGCTATGCATGCAGACTGC
CTGCAGGAACTACGGCGATATCTAGAATCCGGCGTAGTCCTGAGGAGAACAGTGCCCCCATGGTGAAT
GTCACCCGCAGCGAGGCCTCAGAGGGCAACATCACCGTGACATGCAGGGCTTCCAGCTTCTATCCCCGG
AATATCATACTGACCTGGCGTCAGGATGGGGTATCTTTGAGCCACGACACCCAGCAGTGGGGGGATGTC
CTGCCTGATGGGAATGGAACCTACCAGACCTGGGTGGCCACCAGGATTTGCCGAGGAGAGGAGCAGAGG
TTCACCTGCTACATGGAACACAGCGGGAATCACAGCACTCACCCCTGTGCCCTCTCCAGAGGGCCACA
ATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTC
CTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTG
AGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACA
CAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCATCCAGCACCAGGAC
TGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCAGCGCCCATCGAGAGAACC
ATCTCAAAACCCAAAGGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATG
ACTAAGAAACAGGTCACTCTGACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGG
ACCAACAACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCCCTGGACTCTGATGGTTCTTAC
TTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAGAAATAGCTACTCCTGTTCAGTG
GTCCACGAGGGTCTGCACAATCACCACACGACTAAGAGCTTCTCCCGGACTCCGGGTAATGA (1581
bp)

```

Green: Leader sequence for human IL-2 secreting protein (hIL-2 signal sequence) = 60 nucleotides

Red: Sequence encoding for the extracellular region of hMICA = 822 nucleotides

Blue: Sequence encoding for Fc region of mIgG2A = 699 nucleotides



### Translation (5' to 3' orientation)

MYR**MQLLSCIALSLALVTNSEPHSLRYNLT**TVLSWDG**SVQSGFLAEVHLDGQPF**FLRYDRQK**CRAK**PQ**QW**  
AEDV**LG**NKTWDRETRDL**TGNGKDLRMTLAHIKDQKEGLHSLQEI**RVCEIHEDNSTRSSQH**FYYDGE**LFL  
SQ**NLE**TEEW**TV**PQSSRAQ**T**LAMNVR**NFLKEDAMKTK**THYHAMHAD**CLQELRRYLESGV**VLRRT**V**PPMVN  
VTRSEASEGNIT**VT**CRASS**FY**PRNI**IL**TRQD**GV**LS**SHDTQ**Q**W**GD**V**LPD**GN**TY**Q**T**W**VAT**R**IC**R**GEE**Q**R  
F**TCY**MEH**SGNHSTH**VP**SP**RGPT**IK**PCPP**CK**CPAP**NLLGG**PS**V**F**I**FP**PK**IKD**V**LMI**SL**SP**I**V**T**CV**V**VD**V**  
SEDD**P**D**V**Q**I**SW**F**VNN**VE**VHTA**Q**T**Q**THRED**YN**ST**L**RV**V**SAL**P**I**Q**H**Q**D**W**MS**G**KE**F**CK**V**NN**K**D**L**P**A**P**I**ERT  
I**SK**PK**G**SV**R**AP**Q**V**V**LP**P**PEE**E**MT**K**Q**V**TL**T**CM**V**TD**F**MP**E**D**I**Y**V**E**W**T**N**NG**K**TEL**N**Y**K**NT**E**VL**D**SD**G**SY**F**  
M**Y**SK**L**R**V**E**K**KN**W**VER**N**SY**S**CS**V**VE**H**GL**H**N**H**HT**T**KS**F**S**R**TP**G**K

Green: Leader sequence for IL-2 protein

Red: Extracellular region of hMICA protein

Blue: Fc region of mIgG2A

Molecular weight = 60308.58

#### 9.1.2.1 Alignment of the cloned nucleotide sequence to hMICA-mFc2 sequence

The result of hMICA-mFc2 sequencing was aligned to the sequence described in section 9.1.2 using BLAST tool from NCBI website. *Query sequence* is the sequencing result, while *Subject sequence* is the sequence described in section 9.1.2

#### Sequencing result

ATGTACAGGATGCAACTCCTGTCTTGCAATTGCACTAAGTCTTGCACTTGTCA**GAATTC**GGAGCCCCACAGTCTTCG  
TTATAACCTCACGGTGCTGTCCTGGGATN  
NNNNNNNNN**C**NNNGT**N**NGT**C**CTGGGATGN**A**CTGTG**C**AGTCAGGGTT**T**CTTGCTGAGGTACATCTGGATGGTCAGC  
C**T**TCCTGCGCTATGACAGGCAGAAATGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAAGATGTCTGGGAAATAAG  
ACATGGGACAGAGACCAGGGACTTGACAGGGAACGAAAGACCTCAGGATGACCTGGCTCATATCAAGGACCA  
GAAAGAAGGCTTGCA**T**CCCTCCAGGAGATTAGGGTCTGTGAGATCCATGAAGACAACAGCACCAGGAGCTCC**C**AGC  
ATTTCTACTACGATGGGGAGCTCTCCTCTCCCAA**A**ACTGGAGACTGAGGAATGGACAGT**G**CCCCAGTCCCTCCAGA  
GCTCAGACCTTGCCATGAACGTCAGGAATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTATCAGCCTAT  
GCATGCAGACTGCCTGCAGGA**A**CTACGGCGATATCTAGAATCCGGCGTAGTCCTGAGGAGAACAGT**G**CCCCCATGG  
TGAATGTCACCCGCAGCGAGG**C**CTCAGAGGGCAACATCACCGTGACATGCAGGGCTTCCAGCTTCTATCCCGGAAT  
ATCATACTGACCTGGCGTCAGGATGGGGTATCTTTGAGCCACGACACCCAGCAGTGGGGGGATGTCTGCTGATGG  
GAATGGAACCTACCAGACCTGGGTGCCACCAGGATTTGCCAGGAGAGGAGCAGAGGTT**C**ACCTGCTACATGGAAC  
ACAGCGGGAATCACAGCACTAC**C**CTGTGCCCTCTCCAGAGGGCCCAATCAAGCCCTGTCTCCATGCAAA**T**GC  
CCAGCACCTAACCTCTTGGGTGGACCATCCGTCTCATCTTCCCTCCAAAGATCAAGGATGTAC**TCATGA**TCTCCCT  
GAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCANATGTCCAGATCAGCTGGTTTGTGAACA  
ACGTNNNTACACACAGCTCANANN**C**ANANCCNATANANN**G**

**GAATTC**: EcoRI restriction site

**TCATGA**: BspHI restriction site

## Alignment

		EcoRI	
Query	1	ATGTACAGGATGCAACTCCTGTCTTGCAATGCACTAAGTCTTGCACTTGTCACTGAATTCG	60
Sbjct	1	ATGTACAGGATGCAACTCCTGTCTTGCAATGCACTAAGTCTTGCACTTGTCACTGAATTCG	60
Query	61	GAGCCCCACAGTCTTCGTTATAACCTCACGGTGCTGCTCCTGGGATGATCTGTGCAGTCA	120
Sbjct	61	GAGCCCCACAGTCTTCGTTATAACCTCACGGTGCTGCTCCTGGGATGATCTGTGCAGTCA	120
Query	121	GGGTTTCTTGCTGAGGTACATCTGGATGGTCAGCCCTTCCTGCGCTATGACAGGCAGAAA	180
Sbjct	121	GGGTTTCTTGCTGAGGTACATCTGGATGGTCAGCCCTTCCTGCGCTATGACAGGCAGAAA	180
Query	181	TGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAGATGTCCTGGGAAATAAGACATGGGAC	240
Sbjct	181	TGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAGATGTCCTGGGAAATAAGACATGGGAC	240
Query	241	AGAGAGACCAGGGACTTGACAGGGAACGGAAGGACCTCAGGATGACCCTGGCTCATATC	300
Sbjct	241	AGAGAGACCAGGGACTTGACAGGGAACGGAAGGACCTCAGGATGACCCTGGCTCATATC	300
Query	301	AAGGACCAGAAAGAAGGCTTGCATTCCTCCAGGAGATTAGGGTCTGTGAGATCCATGAA	360
Sbjct	301	AAGGACCAGAAAGAAGGCTTGCATTCCTCCAGGAGATTAGGGTCTGTGAGATCCATGAA	360
Query	361	GACAACAGCACCAGGAGCTCCCAGCATTTCTACTACGATGGGGAGCTTTCCTCTCCCAA	420
Sbjct	361	GACAACAGCACCAGGAGCTCCCAGCATTTCTACTACGATGGGGAGCTTTCCTCTCCCAA	420
Query	421	AACCTGGGAGACTGAGGAATGGACAGTGCCCCAGTCCCTCCAGAGCTCAGACCTTGGCCATG	480
Sbjct	421	AACCTGGGAGACTGAGGAATGGACAGTGCCCCAGTCCCTCCAGAGCTCAGACCTTGGCCATG	480
Query	481	AACGTCAGGAATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTATCACGCTATG	540
Sbjct	481	AACGTCAGGAATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTATCACGCTATG	540
Query	541	CATGCAGACTGCCTGCAGGAACTACGGCGATATCTAGAATCCGGCGTAGTCTGAGGAGA	600
Sbjct	541	CATGCAGACTGCCTGCAGGAACTACGGCGATATCTAGAATCCGGCGTAGTCTGAGGAGA	600
Query	601	ACAGTGCCCCCATGGTGAATGTCACCCGACGCGAGGCCTCAGAGGGCAACATCACCGTG	660
Sbjct	601	ACAGTGCCCCCATGGTGAATGTCACCCGACGCGAGGCCTCAGAGGGCAACATCACCGTG	660
Query	661	ACATGCAGGGCTTCCAGCTTCTATCCCCGGAATATCATACTGACCTGGCGTCAGGATGGG	720
Sbjct	661	ACATGCAGGGCTTCCAGCTTCTATCCCCGGAATATCATACTGACCTGGCGTCAGGATGGG	720
Query	721	GTATCTTTGAGCCACGACACCCAGCAGTGGGGGGATGTCCTGCCTGATGGGAATGGAACC	780
Sbjct	721	GTATCTTTGAGCCACGACACCCAGCAGTGGGGGGATGTCCTGCCTGATGGGAATGGAACC	780
Query	781	TACCAGACCTGGGTGGCCACCAGGATTTGCCGAGGAGAGGAGCAGAGGTTACCTGCTAC	840
Sbjct	781	TACCAGACCTGGGTGGCCACCAGGATTTGCCGAGGAGAGGAGCAGAGGTTACCTGCTAC	840
Query	841	ATGGAACACAGCGGAATCACAGCACTCACCTGTGCCCTCTCCCAGAGGGCCACAATC	900
Sbjct	841	ATGGAACACAGCGGAATCACAGCACTCACCTGTGCCCTCTCCCAGAGGGCCACAATC	900
Query	901	AAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC	960
Sbjct	901	AAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC	960
Query	961	ATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGT	1020
Sbjct	961	ATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGT	1020
Query	1021	GTGGTGGTGGATGTGAGCGAGGATGACCCANATGTCCAGATCAGCTGGTTTGTGAACAAC	1080
Sbjct	1021	GTGGTGGTGGATGTGAGCGAGGATGACCCANATGTCCAGATCAGCTGGTTTGTGAACAAC	1080

### 9.1.3 hMICA-mFc2 sequence with EcoRI and BglII restriction sites

When MICA sequence was planned to be inserted between the EcoRI (5'GAATTC3') and BglII (5' AGATCT 3') restriction sites of pFUSE-mIgG2A-Fc2 vector by simple ligation, the BglII restriction site separated the hMICA sequence from the mFc sequence.

```
ATGTACAGGATGCAACTCCTGTCTTGCACTTAAGTCTTGCACTTGTACGAATTCGGAGCCCCAC
AGTCTTCGTTATAACCTCACGGTGTCTGCTGGGATGGATCTGTGCAGTCAGGGTTTCTTGCTGAGGTA
CATCTGGATGGTCAGCCCTTCTGCGCTATGACAGGCAGAAATGCAGGGCAAAGCCCCAGGGACAGTGG
GCAGAAGATGTCTGGGAAATAAGACATGGGACAGAGAGACCAGGGACTTGACAGGGAACGGAAAGGAC
CTCAGGATGACCCTGGCTCATATCAAGGACCAGAAAGAAGGCTTGCAATCCCTCCAGGAGATTAGGGTC
TGTGAGATCCATGAAGACAACAGCACCAGGAGCTCCCAGCATTTCTACTACGATGGGGAGCTCTTCTCT
TCCCAAAACCTGGAGACTGAGGAATGGACAGTGCCTCCAGTCTCCAGAGCTCAGACCTTGCCCATGAAC
GTCAGGAATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTATCACGCTATGCATGCAGACTGC
CTGCAGGAACTACGGCGATATCTAGAATCCGGCGTAGTCCTGAGGAGAACAGTGCCCCCATGGTGAAT
GTCACCCGCAGCGAGGCCTCAGAGGGCAACATCACCGTGACATGCAGGGCTTCCAGCTTCTATCCCCGG
AATATCATACTGACCTGGCGTCAGGATGGGGTATCTTTGAGCCACGACACCCAGCAGTGGGGGGATGTC
CTGCCTGATGGGAATGGAACCTACCAGACCTGGGTGGCCACCAGGATTTGCCGAGGAGAGGAGCAGAGG
TTCACCTGCTACATGGAACACAGCGGGAATCACAGCACTCACCTGTGCCCTCTAGATCTCCAGAGGG
CCCACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC
ATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTG
GATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCT
CAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCAC
CAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAGACCTCCCAGCGCCCATCGAG
AGAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAGAAGAA
GAGATGACTAAGAAACAGGTCACTCTGACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTG
GAGTGGACCAACAACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCTGGACTCTGATGGT
TCTTACTTTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAGAAATAGCTACTCTGT
TCAGTGGTCCACGAGGGTCTGCACAATCACACACGACTAAGAGCTTCTCCCGACTCCGGGTAA
```

(1587 bp)

#### 9.1.3.1 Alignment of the cloned nucleotide sequence to hMICA-mFc2 sequence (with EcoRI and BglII restriction sites)

##### Results of sequencing

```
ATGCAACTCCTGTCTTGCACTTAAGTCTTGCACTTGTACGAATTCGGAGCCCCACAGTCTTCGTTATAACCT
CACGGTGTCTTGGGATGGATCTGTGCAGTCAGGGTTTCTTGCTGAGGTACATCTGGATGGTCAGCCCTTCTGC
GCTATGACAGGCAGAAATGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAGATGTCTGGGAAATAAGACATGGGAC
AGAGAGACCAGGGACTTGACAGGGAACGGAAAAGGACCTCAGGATGACCCTGGCTCATATCAAGGACCAGAAAAGG
CTTGCAATCCCTCCAGGAGATTAGGGTCTGTGAGATCCATGAAGACAACAGCACCAGGAGCTCCCAGCATTTCTACT
ACGATGGGGAGCTCTTCTCTCCAAAACCTGGGACTGAGGAATGGACAGTGCCTCCAGAGCTCAGACC
TTGGCCATGAACGTGAGGAATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTATCACGCTATGCATGCAGA
CTGCCTGCAGGAACTACGGCGATATCTAGAATCCGGCGTAGTCCTGAGGAGAACAGTGCCCCCATGGTGAATGTCA
CCCCGAGCGAGGCCTCAGAGGGCAACATCACCGTGACATGCAGGGCTTCCAGCTTCTATCCCCGGAATATCATACTG
ACCTGGCGTCAGGATGGGGTATCTTTGAGCCACGACACCCAGCAGTGGGGGGATGTCTGCCTGATGGGAATGGAAC
CTACCAGACCTGGGTGGCCACCAGGATTTGCCGAGGAGAGGAGCAGANNANNNNGNNNNNNN
TGCCGAGGAGAGGAGCAGAGGTTACCTGCTACATGGAACACAGCGGGA
ATCACAGCACTCACCTGTGCCCTCTAGATCTCCAGANGGCCACAATCAAGCCCTGTCTCCATGCAAATGCCCA
GCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAG
CCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCANATGTCCAGATCAGCTGGTTTGTGAACAACG
TNNNNTACACACAGCTCANANNANANCCNATANANNNGATTACANNANNTNNNNGGNGNCANTGCCCTCCCATC
NGCACAGGACTGGATNANTGCAAGGATTCATGCAAGNNACANANNACNNCCNNCCATCNANAGAANCNTCTNA
ANCNAAAGNCANTANN
```

**GAATTC**: EcoRI restriction site

**AGATCT**: BglII restriction site

## Alignment

```

                                                    EcoRI
Query 1      ATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGGCACTTGTACCAATTCGGAGCCCCAC 60
            |
Sbjct 10     ATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGGCACTTGTACGAATTCGGAGCCCCAC 69

Query 61     AGTCTTCGTTATAAacctCACGGTGTCTTGGGATGGATCTGTGCAGTCAGGGTTTCTT 120
            |
Sbjct 70     AGTCTTCGTTATAAacctCACGGTGTCTTGGGATGGATCTGTGCAGTCAGGGTTTCTT 129

Query 121    GCTGAGGTACATCTGGATGGTCAGCCCTTCTGCGTATGACAGGCAGAAATGCAGGGCA 180
            |
Sbjct 130    GCTGAGGTACATCTGGATGGTCAGCCCTTCTGCGTATGACAGGCAGAAATGCAGGGCA 189

Query 181    AAGCCCAGGGACAGTGGGCAGAAGATGTCTGGGAAATAAGACATGGGACAGAGAGACC 240
            |
Sbjct 190    AAGCCCAGGGACAGTGGGCAGAAGATGTCTGGGAAATAAGACATGGGACAGAGAGACC 249

Query 241    AGGGACTTGACAGGGAACGAAAGGACCTCAGGATGACCCTGGCTCATATCAAGGACCAG 300
            |
Sbjct 250    AGGGACTTGACAGGGAACGAAAGGACCTCAGGATGACCCTGGCTCATATCAAGGACCAG 309

Query 301    AAAGAAGGCTTGCATTCCCTCCAGGAGATTAGGGTCTGTGAGATCCATGAAGACAACAGC 360
            |
Sbjct 310    AAAGAAGGCTTGCATTCCCTCCAGGAGATTAGGGTCTGTGAGATCCATGAAGACAACAGC 369

Query 361    ACCAGGAGCTCCCAGCATTTCTACTACGATGGGGAGCTCTTCTCTCCAAAACCTGGAG 420
            |
Sbjct 370    ACCAGGAGCTCCCAGCATTTCTACTACGATGGGGAGCTCTTCTCTCCAAAACCTGGAG 429

Query 421    ACTGAGGAATGGACAGTGCACCAGTCTCCAGAGCTCAGACCTTGGCCATGAACGTGAGG 480
            |
Sbjct 430    ACTGAGGAATGGACAGTGCACCAGTCTCCAGAGCTCAGACCTTGGCCATGAACGTGAGG 489

Query 481    AATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTATCACGCTATGCATGCAGAC 540
            |
Sbjct 490    AATTTCTTGAAGGAAGATGCCATGAAGACCAAGACACACTATCACGCTATGCATGCAGAC 549

Query 541    TGCCTGCAGGAACtACGGCGATATCTAGAATCCGGCGTAGTCTTGAGGAGAACAGTGCCT 600
            |
Sbjct 550    TGCCTGCAGGAACtACGGCGATATCTAGAATCCGGCGTAGTCTTGAGGAGAACAGTGCCT 609

Query 601    CCCATGGTGAATGTcACCCGCAGCGAGGCCCTCAGAGGGCAACATCACCGTGACATGCAGG 660
            |
Sbjct 610    CCCATGGTGAATGTcACCCGCAGCGAGGCCCTCAGAGGGCAACATCACCGTGACATGCAGG 669

Query 661    GCTTCCAGCTTCTATCCCCGGAATATCATACTGACCTGGCGTCAGGATGGGGTATCTTTG 720
            |
Sbjct 670    GCTTCCAGCTTCTATCCCCGGAATATCATACTGACCTGGCGTCAGGATGGGGTATCTTTG 729

Query 721    AGCCACGACACCcAGCAGTGGGGGATGTCTGCCTGATGGGAATGGAACCTACCAGACC 780
            |
Sbjct 730    AGCCACGACACCcAGCAGTGGGGGATGTCTGCCTGATGGGAATGGAACCTACCAGACC 789

Query 781    TGGGTGGCCACCAGGATTTGCCGAGGAGAGGAGCAGAGGTTcACCTGCTACATGGAACAC 840
            |
Sbjct 790    TGGGTGGCCACCAGGATTTGCCGAGGAGAGGAGCAGAGGTTcACCTGCTACATGGAACAC 849

Query 841    AGCGGGAATcACAGCACTCACCCGTGTGCCCTCTAGATCTCCAGAGGCCcACAATCAAG 900
            |
Sbjct 850    AGCGGGAATcACAGCACTCACCCGTGTGCCCTCTAGATCTCCAGAGGCCcACAATCAAG 909

Query 901    CCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATC 960
            |
Sbjct 910    CCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATC 969

Query 961    TTCCCTCCAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTG 1020
            |
Sbjct 970    TTCCCTCCAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTG 1029

                                                    BglII
```

## 9.2 Human MICB sequence

The MICB mRNA nucleotide sequence was submitted to NCBI (Reference Sequence: NM\_005931.3) and presented below. Like MICA, MICB consists of a leader peptide (L),  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , transmembrane portion (TM) and cytoplasmic tail (Bahram et al, 1996*b*). The sequence encoding for extracellular portion includes L,  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and it is 882 bp in length (Bahram et al, 1996*b*). It starts at position 117 and ends at position 998.

```

ACTGGATAAG CGGTCGCTGA GCGGGGCGCA GGTGACTAAA TTTCGACGGG GTCTTCTCAC
GGGTTTCATT CAGTTGGCCA CTGCTGAGCA GCTGAGAAGG TGCGGACGTA GGGGCCATGG
GGCTGGGCCG GGTCTGTCTG TTTCTGGCCG TCGCCTTCCC TTTTGCACCC CCGGCAGCCG
CCGTGAGCC CCACAGTCTT CGTTACAACC TCATGGTGCT GTCCAGGAT GGATCTGTGC
AGTCAGGGTT TCTCGCTGAG GGACATCTGG ATGGTCAGCC CTCCTGCGC TATGACAGGC
AGAAACGCAG GGCAAAGCCC CAGGGACAGT GGGCAGAAAA TGTCTGGGA GCTAAGACCT
GGGACACAGA GACCAGGAC TTGACAGAGA ATGGCAAGA CCTCAGGAG ACCCTGACTC
ATATCAAGGA CCAGAAAGGA GGCTGCATT CCCTCCAGGA GATTAGGGTC TGTGAGATCC
ATGAAGACAG CAGCACCAGG GGCTCCCGGC ATTTCTACTA CGATGGGGAG CTCTTCCTCT
CCCAAAACCT GGAGACTCAA GAATCGACAG TGCCCCAGTC CTCCAGAGCT CAGACCTTGG
CTATGAACGT CACAAATTTT TGAAGGAAG ATGCCATGAA GACCAAGACA CACTATCGCG
CTATGCAGGC AGACTGCCTG CAGAAACTAC AGCGATATCT GAAATCCGGG GTGGCCATCA
GGAGAACAGT GCCCCCATG GTGAATGTCA CCTGCAGCGA GGTCTCAGAG GGCAACATCA
CCGTGACATG CAGGGCTTCC AGCTTCTATC CCCGGAATAT CACTGACC TGGCGTCAGG
ATGGGGTATC TTTGAGCCAC AACACCCAGC AGTGGGGGGA TGTCTGCCT GATGGGAATG
GAACCTACCA GACCTGGGTG GCCACCAGGA TTCGCCAAGG AGAGGAGCAG AGGTTACCT
GCTACATGGA ACACAGCGG AATCACGGCA CTCACCCTGT GCCCTCTGGG AAGGCGCTGG
TGCTTCAGAG TCAACGGACA GACTTCCAT ATGTTTCTGC TGCTATGCCA TGTTTTGTTA
TTATATTAT TCTCTGTGTC CCTGTGTTGCA AGAAGAAAAC ATCAGCGGCA GAGGGTCCAG
AGCTTGTGAG CCTGCAGGTC CTGGATCAAC ACCCAGTTGG GACAGGAGAC CACAGGGATG
.....
TAAATGTTTA CTCTTTAAAT AGAAAAAAA AAAAAA (2497 bp)

```

- Red: Leader sequence = 69 nucleotides
- Blue: Alpha 1 domain = 256 nucleotides
- Green: Alpha 2 domain = 288 nucleotides
- Purple: Alpha 3 domain = 270 nucleotides

### 9.2.1 Recombinant (Chimeric) hMICB-mFc1 sequence (1581 bp)

```

ATGGGGCTGGGCCGGGTCTGCTGTTTCTGGCCGTCGCCTTCCCTTTTGCACCCCCGGCAGCCGCCGCT
GAGCCCCACAGTCTTCGTTACAACCTCATGGTGCTGTCCAGGATGGATCTGTGCAGTCAGGGTTTCTC
GCTGAGGGACATCTGGATGGTCAGCCCTTCTGCGCTATGACAGGCAGAAACGCAGGGCAAAGCCCCAG
GGACAGTGGGCAGAAAATGTCTGGGAGCTAAGACCTGGGACACAGAGACCGAGGACTTGACAGAGAAT
GGCAAGACCTCAGGAGGACCCTGACTCATATCAAGGACCAGAAAGGAGGCTTGCATTCCTCCAGGAG
ATTAGGGTCTGTGAGATCCATGAAGACAGCAGCACCAGGGGCTCCCGCATTCTACTACGATGGGGAG
CTCTTCTCTCCCAAAACCTGGAGACTCAAGAATCGACAGTGCCCCAGTCTCCAGAGCTCAGACCTTG
GCTATGAACGTCAAAATTTCTGGAAGGAAGATGCCATGAAGACCAAGACACACTATCGCGCTATGCAG
GCAGACTGCCTGCAGAAACTACAGCGATATCTGAAATCCGGGTGGCCATCAGGAGAACAGTGCCCCC
ATGGTGAATGTACCTGCAGCGAGGTCTCAGAGGGCAACATCACCGTGACATGCAGGGCTTCCAGCTTC
TATCCCCGGAATATCACACTGACCTGGCGTCAGGATGGGGTATCTTTGAGCCACAACCCAGCAGTGG
GGGGATGTCCTGCCTGATGGGAATGGAACCTACCAGACCTGGGTGGCCACCAGGATTCGCCAAGGAGAG
GAGCAGAGGTTACCTGCTACATGGAACACAGCGGGAATCACGGCACTCACCTCCAGAGGGCCACA
ATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTC
CCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTG
AGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTTGTGAACAACGTGGAAGTACACACAGCTCAGACA
CAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTTCAGTGCCCTCCCCATCCAGCACCAGGAC
TGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCAGCGCCCATCGAGAGAACC
ATCTCAAAACCCAAAGGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATG

```

ACTAAGAAACAGGTCACCTCTGACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGG  
ACCAACAACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCTGGACTCTGATGGTTCTTAC  
TTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAAGAAATAGCTACTCTGTTCAGTG  
GTCCACGAGGGTCTGCACAATCACCACACGACTAAGAGCTTCTCCCGGACTCCGGGTAAT  
(1581 bp)

Black: Leader sequence = 69 bp

Red: Sequence encodes for extracellular portion of MICB protein = 813 bp

Blue: Sequence encoding for Fc region of mIgG2A = 699 bp.

TCATGA: BspHI restriction site

### Translation (5' to 3' orientation)

MGLGRVLLFLAVAFPFPAAAAEPHSLRYNLMVLSQDGSVQSGFLAEGHLDGQPFLRYDRQKRRAKPQG  
QWAENVLGAKTWDTEDELTENGQDLRRTLTHIKDQKGLHSLQEIIRVCEIHEDSSTRGSRHFYYDGELF  
LSQNLETQESTVPQSSRAQTLAMNVTNFWKEDAMKTKTHYRAMQADCLQKLQRYLKSGVAIRRTVPPMVN  
VTCSEVSEGNITVTCRASSFYPRNITLWTRQDGVLSHNTQOWGDVLPDNGNTYQTWVATRIRQGEQRF  
TCYMEHSGNHGTHPRGPTIKPCPPCKCPAPNLLGGPSVFI FPPKIKDVLMI SLSPIVTCVVVDVSEDDP  
DVQISWVFNNEVHTAQQTQTHREDYNSTLRVVSALPIQHQDWMMSGKEFKCKVNNKDLPAPIERTISKPKG  
SVRAPQVYVLPPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSGYSYFMYSKLR  
VEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

Black: Leader protein sequence

Red: Extracellular portion of MICB protein

Blue: Fc portion of mIgG2A

Molecular weight = 59566.72 Daltons

### 9.2.1.1 Alignment of the cloned nucleotide sequence to hMICB-mFc1 sequence

#### Result of sequencing

NNNAANTGGNNNTNNCNGNNNNANGGTAANGTTTAAANNNNNAGNTNGAAGACCGAGGNCNTTNTCCGNNCGNTCC  
CNTGNAGGCCCTACNTAGNTCANNNGNNNNNACGCTTTCGCTGACCGCTTGNTCAANNNTANGTNTTGTTCGTTTT  
CTGTNTTGCGCCGTACAGANNNAAGCTGTGACCGGGCGCNTACCTGAGATCACCGGTGAATTCATGGGGCTGGGCC  
GGGTCTGCTGTTTCTGGCCGTCGCCCTCCCTTTTGACCCCCGGCAGCCGCCGCTGAGCCCCACAGTCTTCGTTAC  
AACCTCATGGTGTCTCCCGGGATGGATCTGTGCAGTCAGGGTTTCTCGCTGAGGGACATCTGGATGGTCAGCCCTT  
CCTGCGCTATGACAGGCAGAAACGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAAATGTCTGGGAGCTAAGACCT  
GGGACACAGAGACCGAGGACTTGACAGAGAATGGGCAAGACCTCAGGAGGACCCTGACTCATATCAAGGACCAGAAA  
GGAGGCTTGCATTCCTCCAGGAGATTAGGGTCTGTGAGATCCATGAAGACAGCAGCACCAGGGGCTCCCGGCATTT  
CTACTACGATGGGGAGCTCTTCTCTCCCAAACCTGGAGACTCAGGAATCGACAGTCCCCAGTCCCTCAGAGCTC  
AGACCTTGGCTATGAACGTCAAAATTTCTGGAAGGAAGATGCCATGAAGACCAAGACACACTATCACGCTATGCAG  
GCAGACTGCCTGCAGAACTACAGCGATATCTGAAATCCGGGTGGCCATGAGGAGAACAGTCCCCCATGGTGAA  
TGTACCTGCAGCGAGGTCTCAGANNNNNNNNNNNNNNNNNN  
TGTACCTGCAGCGAGGTCTCAGAGGGCAACATCACCGTGACATGCAGGGCTTCCAGCTTCTATCCCCGGAATATCA  
CACTGACCTGGCGTCAGGATGGGGTATCTTTGAGCCACAACCCAGCAGTGGGGGATGTCTGCCTGATGGGAAT  
GGAACCTACCAGACTGGGTGGCCACCAGGATTCGCCAAGGAGAGGAGCAGAGGTTACCTGCTACATGGAACACAG  
CGGGAATCACGGCACTCACCTCCAGAGGGCCCCAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACC  
TCTTGGGTGGACCATCCGTCTTACTCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTC  
ACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACA  
CACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCTCCCCATCCAGCACC  
AGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAGACCTCCAGCGCCCATCGAGAGAACCATC  
TCAAACCCAAAGGGTCAGTAAGAGCTCCACAGNNNNNNNNNNNN

GAATTC: EcoRI restriction site

TCATGA: BspHI restriction site

## Alignment

```
Query 219 ATGGGGCTGGGCCGGGTCCTGCTGTTTCTGGCCGTCGCCTTCCCTTTTGCACCCCCGGCA 278
Sbjct 1 ATGGGGCTGGGCCGGGTCCTGCTGTTTCTGGCCGTCGCCTTCCCTTTTGCACCCCCGGCA 60

Query 279 GCCGCCGCTGAGCCCCACAGTCTTCGTTACAACCTCATGGTGTGTCCCAGGATGGATCT 338
Sbjct 61 GCCGCCGCTGAGCCCCACAGTCTTCGTTACAACCTCATGGTGTGTCCCAGGATGGATCT 120

Query 339 GTGCAGTCAGGGTTTCTCGCTGAGGGACATCTGGATGGTCAGCCCTTCCTGCGCTATGAC 398
Sbjct 121 GTGCAGTCAGGGTTTCTCGCTGAGGGACATCTGGATGGTCAGCCCTTCCTGCGCTATGAC 180

Query 399 AGGCAGAAACGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAAATGTCCTGGGAGCTAAG 458
Sbjct 181 AGGCAGAAACGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAAATGTCCTGGGAGCTAAG 240

Query 459 ACCTGGGACACAGAGACCGAGGACTTGACAGAGAATGGGCAAGACCTCAGGAGGACCTG 518
Sbjct 241 ACCTGGGACACAGAGACCGAGGACTTGACAGAGAATGGGCAAGACCTCAGGAGGACCTG 300

Query 519 ACTCATATCAAGGACCAGAAAGGAGGCTTGCAATCCCTCCAGGAGATTAGGGTCTGTGAG 578
Sbjct 301 ACTCATATCAAGGACCAGAAAGGAGGCTTGCAATCCCTCCAGGAGATTAGGGTCTGTGAG 360

Query 579 ATCCATGAAGACAGCAGCACCAGGGGCTCCCGGCATTTCTACTACGATGGGGAGCTCTTC 638
Sbjct 361 ATCCATGAAGACAGCAGCACCAGGGGCTCCCGGCATTTCTACTACGATGGGGAGCTCTTC 420

Query 639 CTCTCCAAAACCTGGAGACTCAAGAAATCGACAGTGCCCCAGTCCCTCCAGAGCTCAGACC 698
Sbjct 421 CTCTCCAAAACCTGGAGACTCAAGAAATCGACAGTGCCCCAGTCCCTCCAGAGCTCAGACC 480

Query 699 TTGGCTATGAACGTCAAAATTTCTGGAAGGAAGATGCCATGAAGACCAAGACACACTAT 758
Sbjct 481 TTGGCTATGAACGTCAAAATTTCTGGAAGGAAGATGCCATGAAGACCAAGACACACTAT 540

Query 759 CACGCTATGCAGGCAGACTGCCTGCAGAAACTACAGCGATATCTGAAATCCGGGGTGGCC 818
Sbjct 541 CACGCTATGCAGGCAGACTGCCTGCAGAAACTACAGCGATATCTGAAATCCGGGGTGGCC 600

Query 819 ATCAGGAGAACAGTGCCCCCATGGTGAATGTCACCTGCAGCGAGGTCTCAGAGGGCAAC 878
Sbjct 601 ATCAGGAGAACAGTGCCCCCATGGTGAATGTCACCTGCAGCGAGGTCTCAGAGGGCAAC 660

Query 879 ATCACCGTGACATGCAGGGCTTCCAGCTTCTATCCCGGAATATCACACTGACCTGGCGT 938
Sbjct 661 ATCACCGTGACATGCAGGGCTTCCAGCTTCTATCCCGGAATATCACACTGACCTGGCGT 720

Query 939 CAGGATGGGGTATCTTTGAGCCACAACACCAGCAGTGGGGGGATGTCCTGCCTGATGGG 998
Sbjct 721 CAGGATGGGGTATCTTTGAGCCACAACACCAGCAGTGGGGGGATGTCCTGCCTGATGGG 780

Query 999 AATGGAACCTACCAGACCTGGGTGGCCACCAGGATTCGCCAAGGAGAGGAGCAGAGGTTT 1058
Sbjct 781 AATGGAACCTACCAGACCTGGGTGGCCACCAGGATTCGCCAAGGAGAGGAGCAGAGGTTT 840

Query 1059 ACCTGCTACATGGAACACAGCGGGAATCACGGCACTCACCTCCCAGAGGGCCCAATC 1118
Sbjct 841 ACCTGCTACATGGAACACAGCGGGAATCACGGCACTCACCTCCCAGAGGGCCCAATC 900

Query 1119 AAGCCCTGTCTCCATGCAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC 1178
Sbjct 901 AAGCCCTGTCTCCATGCAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC 960

Query 1179 ATCTTCCCTCCAAAGATCAAGGATGTAATCATGATCTCCCTGAGCCCCATAGTCACATGT 1238
Sbjct 961 ATCTTCCCTCCAAAGATCAAGGATGTAATCATGATCTCCCTGAGCCCCATAGTCACATGT 1020

Query 1239 GTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAAC 1298
Sbjct 1021 GTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAAC 1080
```

BspHI

```

Query 1299 GTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGG 1358
          |||
Sbjct 1081 GTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGG 1140

Query 1359 GTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCAATGC 1418
          |||
Sbjct 1141 GTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCAATGC 1200

Query 1419 AAGGTCAACAACAAGACCTCCCAGCGCCCATCGAGAGAACCATCTCAAAACCCAAAGGG 1478
          |||
Sbjct 1201 AAGGTCAACAACAAGACCTCCCAGCGCCCATCGAGAGAACCATCTCAAAACCCAAAGGG 1260

Query 1479 TCAGTAAGAGCTCCACAG 1496
          |||
Sbjct 1261 TCAGTAAGAGCTCCACAG 1278

```

### 9.2.2 Recombinant hMICB-mFc2 sequence

```

ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACCTTGTACAGGAGCCCCAC
AGTCTTCGTTACAACCTCATGGTGTGTCCCAGGATGGATCTGTGCAGTCAGGGTTTCTCGCTGAGGGA
CATCTGGATGGTCAGCCCTTCCTGCGCTATGACAGGCAGAAACGCAGGGCAAAGCCCCAGGGACAGTGG
GCAGAAAATGTCCTGGGAGCTAAGACCTGGGACACAGAGACCCGAGGACTTGACAGAGAATGGGACATG
CTCAGGAGGACCCTGACTCATATCAAGGACCAGAAAGGAGGCTTGCATTCCCTCCAGGAGATTAGGTC
TGTGAGATCCATGAAGACAGCAGCACCAGGGGCTCCCGGCATTTCTACTACGATGGGGAGCTCTTCCTC
TCCAAAACCTGGAGACTCAAGAATCGACAGTGCCCCAGTCCCTCCAGAGCTCAGACCTTGGCTATGAAC
GTCACAAATTTCTGGAAGGAAGATGCCATGAAGACCAAGACACACTATCGCGCTATGCAGGCAGACTGC
CTGCAGAACTACAGCGATATCTGAAATCCGGGGTGGCCATCAGGAGAACAGTGCCCCCATGGTGAAT
GTCACCTGCAGCGAGGTCTCAGAGGGCAACATCACCGTGACATGCAGGGCTTCCAGCTTCTATCCCCGG
AATATCACACTGACCTGGCGTCAGGATGGGGTATCTTTGAGCCACAACACCAGCAGTGGGGGGATGTC
CTGCCTGATGGGAATGGAACCTACCAGACCTGGGTGGCCACCAGGATTCCCAAGGAGAGGAGCAGAGG
TTCACCTGCTACATGGAACACAGCGGGAATCACGGCACTCACCTCCAGAGGGCCCACAATCAAGCCC
TGTCCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAG
ATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGAT
GACCCAGATGTCCAGATCAGCTGGTTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCAT
AGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGAGT
GGCAAGGAGTTCAAATGCAAGGTCAACAACAAGACCTCCCAGCGCCCATCGAGAGAACCATCTCAAAA
CCCAAAGGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAA
CAGGTCACCTTGACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGGACCAACAAC
GGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCCCTGGACTCTGATGGTTCTTACTTCATGTAC
AGCAAGCTGAGAGTGGAAAAGAAGAAGTGGGTGGAAAGAAATAGCTACTCCTGTTTCAGTGGTCCACGAG
GGTCTGCACAATCACACACGACTAAGAGCTTCTCCCGGACTCCGGGTAAA (1572 bp)

```

Green: Leader sequence for IL-2 secreting protein (hIL-2 signal sequence) = 60 nucleotides.

Red: Sequence encoding for the extracellular region of hMICB = 822 nucleotides

Blue: Sequence encoding for Fc region of mIgG2A = 699 nucleotides.

### Translation

```

MYRMQLLSCIALSLALVTNSEPHSLRYNLMVLSQDGSVQSGFLAEGHLDGQPFLRYDRQKRRRAKPQGQWA
ENVLGAKTWDTEDELTENGQDLRRTLTHIKDKKGLHSLQEIRVCEIHEDSSSTRGRHFYDGELEFLSQ
NLETQESTVPPQSSRAQTLAMNVTNFWKEDAMKTKTHYRAMQADCLQKLQRYLKSQVAIIRRTVPPMVNVC
SEVSEGNITVTCRASSFYPRNITLTWRQDGVLSHNTQQWGDVLPDNGTYQTWVATRIRQGEERFTCY
MEHSGNHGTHPPRGPTIKPCPPCKCPAPNLLGGPSVFI FPPKIKDVLMI SLSPIVTCVVVDVSEDDPDVQ
ISWVFNNEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMMSGKEFKCKVNNKDLPAPIERTISKPKGSVR
APQVYVLPPEEEMTKKQVTLTCMVTDFMPEDIYVEWVTNNGKTELNYKNTEPVLDSGDSY
FMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

```

Green: Leader sequence for IL-2 protein

Blue: Fc region of mIgG2A

Red: Extracellular region of hMICA protein

Molecular weight = 59493.63



### 9.2.2.1 Alignment of the cloned nucleotide sequence to hMICB-mFc2 sequence

#### Results of sequencing

GTGACCGGCGCCTACCTGAGATCACCGGCGAAGGAGGGCCACCATGTACAGGATGCAACTCCTGTCTTG  
 CATTGCACTAAGTCTTGCACCTTGTACGAATTCGGAGCCCCACAGTCTTCGTTACAACCTCATGGTGCT  
 GTCCCAGGATGGATCTGTGCAGTCAGGGTTTCTCGCTGAGGGACATCTGGATGGTCAGCCCTTCCTGCG  
 CTATGACAGGCAGAAACGCAGGGCAAAGCCCCAGGGACAGTGG  
 NNNNNNCNNNNNNNNNNNNNNNGCNAAGCCCCAGGGACAGTGGGCAGAAAATGTCCTGGGAGCTAAGAC  
 CTGGGACACAGAGACCGAGGACTTGACAGAGAATGGGCAAGACCTCAGGAGGACCCTGACTCATATCAA  
 GGACCAGAAAGGAGGCTTGCATTCCCTCCAGGAGATTAGGGTCTGTGAGATCCATGAAGACAGCAGCAC  
 CAGGGGCTCCCGGCATTTCTACTACGATGGGGAGCTCTTCTCTCCCAAACCTGGAGACTCAAGAATC  
 GACAGTGCCCCAGTCTCCAGAGCTCAGACCTTGGCTATGAACGTCACAAATTTCTGGAAGGAAGATGC  
 CATGAAGACCAAGACACACTATCGCGCTATGCAGGCAGACTGCCTGCAGAAACTACAGCGATATCTGAA  
 ATCCGGGGTGGCCATCAGGAGAACAGTGCCCCCATGGTGAATGTCACCTGCAGCGAGGTCTCAGAGGG  
 CAACATCACCGTGACATGCAGGGCTTCCAGCTTCTATCCCCGGAATATCACACTGACCTGGCGTCAGGA  
 TGGGGTATCTTTGAGCCACAACACCCAGCAGTGGGGGGATGTCCTGCCTGATGGGAATGGAACCTACCA  
 GACCTGGGTGGCCACCAGGATTCGCCAAGGAGAGGAGCAGAGGTTACCTGCTACATGGAACACAGCGG  
 GAATCACGGCACTCACCTCCAGAGGGCCACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACC  
 TAACCTTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCT  
 GAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGNN  
 NNNN

**GAATTC**: EcoRI restriction site

**TCATGA**: BspHI restriction site

#### Alignment

			EcoRI	
Query	44	ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACCTTGTAC <b>GAATTC</b>		103
Sbjct	1	ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACCTTGTACCGAATTCG		60
Query	104	GAGCCCCACAGTCTTCGTTACAACCTCATGGTGCTGTCCCAGGATGGATCTGTGCAGTCA		163
Sbjct	61	GAGCCCCACAGTCTTCGTTACAACCTCATGGTGCTGTCCCAGGATGGATCTGTGCAGTCA		120
Query	164	GGGTTTCTCGCTGAGGGACATCTGGATGGTCAGCCCTTCCTGCGCTATGACAGGCAGAAA		223
Sbjct	121	GGGTTTCTCGCTGAGGGACATCTGGATGGTCAGCCCTTCCTGCGCTATGACAGGCAGAAA		180
Query	224	CGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAAATGTCCTGGGAGCTAAGACCTGGGAC		283
Sbjct	181	CGCAGGGCAAAGCCCCAGGGACAGTGGGCAGAAAATGTCCTGGGAGCTAAGACCTGGGAC		240
Query	284	ACAGAGACCAGGACTTGACAGAGAATGGGCAAGACCTCAGGAGGACCCTGACTCATATC		343
Sbjct	241	ACAGAGACCAGGACTTGACAGAGAATGGGCAAGACCTCAGGAGGACCCTGACTCATATC		300
Query	344	AAGGACCAGAAAGGAGGCTTGCATTCCCTCCAGGAGATTAGGGTCTGTGAGATCCATGAA		403
Sbjct	301	AAGGACCAGAAAGGAGGCTTGCATTCCCTCCAGGAGATTAGGGTCTGTGAGATCCATGAA		360
Query	404	GACAGCAGCACCAGGGGCTCCCGGCATTTCTACTACGATGGGGAGCTCTTCTCTCCCAA		463
Sbjct	361	GACAGCAGCACCAGGGGCTCCCGGCATTTCTACTACGATGGGGAGCTCTTCTCTCCCAA		420
Query	464	AACCTGGAGACTCAAGAATCGACAGTGGCCAGTCCCTCCAGAGCTCAGACCTTGGCTATG		523
Sbjct	421	AACCTGGAGACTCAAGAATCGACAGTGGCCAGTCCCTCCAGAGCTCAGACCTTGGCTATG		480
Query	524	AACGTCACAAATTTCTGGAAGGAAGATGCCATGAAGACCAAGACACACTATCGCGCTATG		583
Sbjct	481	AACGTCACAAATTTCTGGAAGGAAGATGCCATGAAGACCAAGACACACTATCGCGCTATG		540

```

Query 584 CAGGCAGACTGCCTGCAGAACTACAGCGATATCTGAAATCCGGGGTGGCCATCAGGAGA 643
          |||
Sbjct 541 CAGGCAGACTGCCTGCAGAACTACAGCGATATCTGAAATCCGGGGTGGCCATCAGGAGA 600

Query 644 ACAGTGCCCCCATGGTGAATGTCACCTGCAGCGAGGTCTCAGAGGGCAACATCACCGTG 703
          |||
Sbjct 601 ACAGTGCCCCCATGGTGAATGTCACCTGCAGCGAGGTCTCAGAGGGCAACATCACCGTG 660

Query 704 ACATGCAGGGCTTCCAGCTTCTATCCCCGGAATATCACACTGACCTGGCGTCAGGATGGG 763
          |||
Sbjct 661 ACATGCAGGGCTTCCAGCTTCTATCCCCGGAATATCACACTGACCTGGCGTCAGGATGGG 720

Query 764 GTATCTTTGAGCCACAACACCCAGCAGTGGGGGGATGTCCTGCCTGATGGGAATGGAACC 823
          |||
Sbjct 721 GTATCTTTGAGCCACAACACCCAGCAGTGGGGGGATGTCCTGCCTGATGGGAATGGAACC 780

Query 824 TACCAGACCTGGGTGGCCACCAGGATTCGCCAAGGAGAGGAGCAGAGGTTACCTGCTAC 883
          |||
Sbjct 781 TACCAGACCTGGGTGGCCACCAGGATTCGCCAAGGAGAGGAGCAGAGGTTACCTGCTAC 840

Query 884 ATGGAACACAGCGGGAATCACGGCACTCACCTCCCAGAGGGCCACAATCAAGCCCTGT 943
          |||
Sbjct 841 ATGGAACACAGCGGGAATCACGGCACTCACCTCCCAGAGGGCCACAATCAAGCCCTGT 900

Query 944 CCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCT 1003
          |||
Sbjct 901 CCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCT 960

Query 1004 CCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTG 1063
          |||
          BspHI
          |||
Sbjct 961 CCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTG 1020

Query 1064 GATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGG 1102
          |||
Sbjct 1021 GATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGG 1059

```

### 9.3 Human ULBP1 sequence

The human ULBP1 mRNA sequence (NCBI Reference Sequence: NM\_025218.2) is presented below. It consists of a leader peptide (L),  $\alpha 1$ ,  $\alpha 2$  (but not  $\alpha 3$ ) domains and GPI anchor signal. The sequence encoding for extracellular region includes L,  $\alpha 1$  and  $\alpha 2$  and it is 609 bp in length (Cosman et al, 2001). It starts from position 44 bp and ends at position 652.

```

GGGAACCATC AGCGCCTCCT GTCCACGGAG CTCCAGGTCT ACAATGGCAG CGGCCGCCAG
CCCCGGTTC CTCTGTGCC TCCGCTTCT GCACCTGCTG TCTGGCTGGT CCGGGCAGG
ATGGGTCGAC ACACACTGTC TTTGCTATGA CTTCATCATC ACTCCTAAGT CCAGACCTGA
ACCACAGTGG TGTGAAGTTC AAGGCCTGGT GGATGAAAGG CCTTTTCTTC ACTATGACTG
TGTTAACCAC AAGGCCAAG CCTTGCTTC TCTGGGGAAG AAAGTCAATG TCACAAAAC
CTGGGAAGAA CAACTGAAA CACTAAGAGA CGTGGTGGAT TTCCTTAAAG GGCAACTGCT
TGACATTCAA GTGGAGAATT TAATACCCAT TGAGCCCTC ACCCTGCAGG CCAGGATGTC
TTGTGAGCAT GAAGCCCATG GACACGGCAG AGGATCTTGG CAGTTCCTCT TCAATGGCA
GAAGTTCCTC CTCTTTGACT CAAACAACAG AAAGTGGACA GCACTTCATC CTGGAGCCAA
GAAGATGACA GAGAAGTGGG AGAAGAACAG GGATGTGACC ATGTTCTTCC AGAAGATTTT
ACTGGGGGAT TGTAAGATGT GGCTTGAAGA ATTTTGTATG TACTGGGAAC AAATGCTGGA
TCCAACAAA CCACCTCTC TGGCCCCAGG CACAACCCAA CCAAGGCCA TGGCCACCAC
CCTCAGTCCC TGGAGCCTTC TCATCATCTT CCTCTGCTTC ATTCTAGCTG GCAGATGAGG
AGAGTTGTTT AGAGTGACAG GTGGAAGTG ATATCAAGAA GCCTCTGTTA GCCTGGTCTG
GGTCTGCTC TCCCTTCAGG GAGGCCGCT GTCTACTCAC CACTGTGCC TTTCTGGAAAG
CAGGAGTTCA AGCCTTAGCA AGCCAGAGG CCCCAGCAG ACGATGAGGA CATTTGTCGGC
TCAACATCTC AGGCCACTCA TTACCTTCGC TCATGATCCC AGCAGCCATT TTTCTTAACA
CCTTCTGCCA CTTTCTGTCG GTGCTAATGG ATGGAACTCC TGACAAGTT TTAAGTGAAC
.....
AATTAACAC TGTATAATGG AAAAAAAAAA AAAAAAAAAA (3160 bp)

```

Red: Leader sequence = 147 nucleotides  
Blue: Alpha 1 domain = 306 nucleotides  
Green: Alpha 2 domain = 156 nucleotides

### 9.3.1 Recombinant hULBP1-mFc1 sequence (1308 bp)

```
ATG GCAGCGGCCGCCAGCCCCGCGTTCCTTCTGTGCCTCCCGCTTCTGCACCTGCTGTCTGGCTGGTCC
CGGGCAGGATGGGTGCACACACACTGTCTTTGCTATGACTTCATCATCACTCCTAAGTCCAGACCTGAA
CCACAGTGGTGTGAAGTTCAAGGCCTGGTGGATGAAAGGCCTTTTCTTCACTATGACTGTGTTAACCAC
AAGGCCAAAGCCTTTGCTTCTCTGGGGAAGAAAGTCAATGTCACAAAAACCTGGGAAGAACAACTGAA
ACACTAAGAGACGTGGTGGATTTCTTAAAGGGCAACTGCTTGACATTCAAGTGGAGAATTTAATACCC
ATTGAGCCCCCTCACCTGCAGGCCAGGATGTCTTGTGAGCATGAAGCCCATGGACACGGCAGAGGATCT
TGGCAGTTCCTCTTCAATGGACAGAAGTTCCTCCTCTTTGACTCAAACAACAGAAAGTGGACAGCACTT
CATCCTGGAGCCAAGAAGATGACAGAGAAGTGGGAGAAGAACAGGGATGTGACCATGTTCTTCCAGAAG
ATTTCACTGGGGGATTGTAAGATGTGGCTTGAAGAATTTTTGATGTACTGGGAACAACCCAGAGGGCCC
ACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATC
TTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGAT
GTGAGCGAGGATGACCCAGATGTCCAGATCAGTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAG
ACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACCAG
GACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCCATCGAGAGA
ACCATCTCAAACCCAAAGGGTCAAGTAAAGAGCTCCACAGGTATATGTCTTGCTCCACCAGAAGAAGAG
ATGACTAAGAAACAGGTCACTCTGACCTGCATGGTACAGACTTCATGCCTGAAGACATTTACGTGGAG
TGGACCAACAACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCTGGACTCTGATGGTTCT
TACTTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAGAAATAGCTACTCCTGTTCA
GTGGTCCACGAGGGTCTGCACAATCACCACACGACTAAGAGCTTCTCCGGACTCCGGGTAAATG
```

Black: Leader sequence = 147 bp  
Red: Sequence encodes for extracellular portion of hULBP1 protein = 462 bp.  
Blue: Sequence encoding for Fc region of mIgG2A = 699 bp  
TCATGA: BspHI restriction site

### Translation

```
MAAAASPAFLLLCLPLLHLLSGWSRAGWVDTHCLCYDFIITPKSRPEPQWCEVQGLVDERPFLHYDCVNHK
AKAFASLGKKVNVTKTWEEQTETLRDVVDFLKQQLLDIQVENLIPIEPLTLQARMSCEHEAHGHRGSWQ
FLFNGQKFLLFDSNNRKWTALHPGAKKMTEKWEKNRDVTMFFQKISLGDCKMWLEEFILMYWEQPRGPTIK
PCPPCKCPAPNLLGGPSVFIFFPKIKDVLMIISLPIVTCVVVDVSEDDPDVQISWVFNNEVHTAQTQTH
REDYNSTLRVVSALPIQHQDWMGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPEEEMTKKQ
VTLTLCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDSGSYFMYSKLRVEKKNWVERNSYSCSVVHEGL
HNHHTTKSFSRTPGK
```

Black: Leader protein sequence  
Red: Extracellular portion of ULBP1 protein  
Blue: Fc portion of mIgG2A  
Molecular weight = 49859.29

### 9.3.1.1 Alignment of the cloned nucleotide sequence to hULBP1-mFc1 sequence

#### Results of sequencing

NNNGNNNNNACGCTNNNCCTNNCCNNCTTGNTCAANNNTANGTNNNNNGTTTTCTGTNTGCGCCGTACAGANN  
 AAGCTGTGACCGGGCGCNTACCTGAGATCACCGGTGAATTCATGGCAGCGGCCGCCAGCCCCGCTTCCTTCTGTGC  
 CTCCCGTTCTGCACCTGTGTCTGGCTGGTCCCAGGATGGGTGACACACACTGTCTTTGCTATGACTTCAT  
 CATCACTCCTAAGTCCAGACCTGAACCACAGTGGTGTGAAGTTCAAGGCCTGGTGGATGAAAGGCCTTTCTTCACT  
 ATGACTGTGTTAACCACAAGGCCAAAGCCTTTGCTTCTCTGGGGAAGAAAGTCAATGTCACAAAACCTGGGAAGAA  
 CAACTGAAACACTAAGAGACGTGGTGGATTTCTTAAAGGGCACTGCTTGACATTCAAGTGGAGAAATTAATACC  
 CATTGAGCCCCCTCACCTGCAGGCCAGGATGTCTTGTGAGCATGAAGCCCATGGACACGGCAGAGGATCTGGCAGT  
 TCCTCTTCAATGGACAGAAGTTCCTCTTTGACTCAAACAACAGAAAGTGGACAGCACTTCATCTGGAGCCAAG  
 AAGATGACAGAGAAGTGGGAGAAGAACAGGGATGTGACCATGTTCTTCCAGAAGATTTCACTGGGGGATTGTAAGAT  
 GTGGCTTGAAGAATTTTGTGACTGGGAACAACCCAGAGGGCCACAATCAAGCCCTGTCTCCATGCAAATGCC  
 CAGCACCTAACCTCTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTG  
 AGCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAA  
 CGTGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACACAGTACTCTCCGGGTGGTCACTGCCCTCC  
 CCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTNCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCCATC  
 GAGAGAACCATCTCAAACCCAAAGGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCTCCACCAGAAGAAGAGAT  
 GACTAAGAAAACAGGTCACCTGTACCTGCATGGTCCAGACTTCATGCCTGAAGACATTTACGTGGAGTGGACCAACA  
 ACGGAAAAACAGAGCTAAACTACAAGAACTGAACCAGTCCNNNACT

#### Alignment

Query	119	ATGGCAGCGGCCGCCAGCCCCGCTTCCTTCTGTGCCTCCCGCTTCTGCACCTGCTGTCT	178
Sbjct	1	ATGGCAGCGGCCGCCAGCCCCGCTTCCTTCTGTGCCTCCCGCTTCTGCACCTGCTGTCT	60
Query	179	GGCTGGTCCCAGGATGGGTGACACACACTGTCTTTGCTATGACTTCATCATCACT	238
Sbjct	61	GGCTGGTCCCAGGATGGGTGACACACACTGTCTTTGCTATGACTTCATCATCACT	120
Query	239	CCTAAGTCCAGACCTGAACCACAGTGGTGTGAAGTTCAAGGCCTGGTGGATGAAAGGCCT	298
Sbjct	121	CCTAAGTCCAGACCTGAACCACAGTGGTGTGAAGTTCAAGGCCTGGTGGATGAAAGGCCT	180
Query	299	TTTCTTCACTATGACTGTGTTAACCACAAGGCCAAAGCCTTTGCTTCTCTGGGGAAGAAA	358
Sbjct	181	TTTCTTCACTATGACTGTGTTAACCACAAGGCCAAAGCCTTTGCTTCTCTGGGGAAGAAA	240
Query	359	GTCAATGTCACAAAACCTGGGAAGAACAACCTGAAACACTAAGAGACGTGGTGGATTTTC	418
Sbjct	241	GTCAATGTCACAAAACCTGGGAAGAACAACCTGAAACACTAAGAGACGTGGTGGATTTTC	300
Query	419	CTTAAAGGGCAACTGCTTGACATTCAAGTGGAGAATTTAATACCCATTGAGCCCCCTCACC	478
Sbjct	301	CTTAAAGGGCAACTGCTTGACATTCAAGTGGAGAATTTAATACCCATTGAGCCCCCTCACC	360
Query	479	CTGCAGGCCAGGATGTCTTGTGAGCATGAAGCCCATGGACACGGCAGAGGATCTTGGCAG	538
Sbjct	361	CTGCAGGCCAGGATGTCTTGTGAGCATGAAGCCCATGGACACGGCAGAGGATCTTGGCAG	420
Query	539	TTCTCTTCAATGGACAGAAGTTCCTCTCTTTGACTCAAACAACAGAAAGTGGACAGCA	598
Sbjct	421	TTCTCTTCAATGGACAGAAGTTCCTCTCTTTGACTCAAACAACAGAAAGTGGACAGCA	480
Query	599	CTTCATCTGGAGCCAAGAAGATGACAGAGAAGTGGGAGAAGAACAGGGATGTGACCATG	658
Sbjct	481	CTTCATCTGGAGCCAAGAAGATGACAGAGAAGTGGGAGAAGAACAGGGATGTGACCATG	540
Query	659	TTCTTCCAGAAGATTTCACTGGGGGATTGTAAGATGTGGCTTGAAGAATTTTGTGATGAC	718
Sbjct	541	TTCTTCCAGAAGATTTCACTGGGGGATTGTAAGATGTGGCTTGAAGAATTTTGTGATGAC	600
Query	719	TGGGAACAACCCAGAGGGCCACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCT	778
Sbjct	601	TGGGAACAACCCAGAGGGCCACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCT	660
Query	779	AACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATG	838
Sbjct	661	AACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATG	720

BspHI

Query 839 ATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGAT 898  
 Sbjct 721 ATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGAT 780

Query 899 GTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCAT 958  
 Sbjct 781 GTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCAT 840

Query 959 AGAGAGGATTACAACAGTACTCTCCGGGTGGTTCAGTGCCTCCCATCCAGCACCAGGAC 1018  
 Sbjct 841 AGAGAGGATTACAACAGTACTCTCCGGGTGGTTCAGTGCCTCCCATCCAGCACCAGGAC 900

Query 1019 TGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAGACCTCCCAGCGCCCATC 1078  
 Sbjct 901 TGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAGACCTCCCAGCGCCCATC 960

Query 1079 GAGAGAACCATCTCAAAACCCAAAGGGTTCAGTAAGAGCTCCACAGGTATATGTCTTGCCT 1138  
 Sbjct 961 GAGAGAACCATCTCAAAACCCAAAGGGTTCAGTAAGAGCTCCACAGGTATATGTCTTGCCT 1020

Query 1139 CCACCAGAAGAAGAGATGACTAAGAAACAGGTCACTCTGACCTGCATGGTTCACAGACTTC 1198  
 Sbjct 1021 CCACCAGAAGAAGAGATGACTAAGAAACAGGTCACTCTGACCTGCATGGTTCACAGACTTC 1080

Query 1199 ATGCCTGAAGACATTTACGTGGAGTGGACCAACAACGGGAAAACAGAGCTAAACTACAAG 1258  
 Sbjct 1081 ATGCCTGAAGACATTTACGTGGAGTGGACCAACAACGGGAAAACAGAGCTAAACTACAAG 1140

Query 1259 AACACTGAACCAGTCC 1274  
 Sbjct 1141 AACACTGAACCAGTCC 1156

### 9.3.2 Recombinant hULBP1-m-Fc2 sequence (1221 bp)

ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCATTGTCACTGAATTCGTGTGAAGTT  
 CAAGGCCTGGTGGATGAAAGGCCTTTTCTTCACTATGACTGTGTTAACCACAAGGCCAAAGCCTTTGCT  
 TCTCTGGGGAAGAAAGTCAATGTCACAAAAACCTGGGAAGAACAACCTGAAACACTAAGAGACGTGGTG  
 GATTTCTTAAAGGGCAACTGCTTGACATTCAAGTGGAGAATTTAATACCCATTGAGCCCTCACCTG  
 CAGGCCAGGATGTCTTGTGAGCATGAAGCCCATGGACACGGCAGAGGATCTTGGCAGTTCCTCTTCAAT  
 GGACAGAAGTTCCTCCTCTTTGACTCAAACAACAGAAAGTGGACAGCACTTCATCCTGGAGCCAAGAAG  
 ATGACAGAGAAGTGGGAGAAGAACAGGGATGTGACCATGTTCTTCCAGAAGATTTCACTGGGGGATTGT  
 AAGATGTGGCTTGAAGAATTTTGTATGACTGGGAACAACCCAGAGGGCCACAATCAAGCCCTGTCTT  
 CCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTTCATCTTCCCTCCAAAGATCAAG  
 GATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCA  
 GATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAG  
 GATTACAACAGTACTCTCCGGGTGGTTCAGTGCCTCCCATCCAGCACCAGGACTGGATGAGTGGCAAG  
 GAGTTCAAATGCAAGGTCAACAACAAGACCTCCAGCGCCCATCGAGAGAACCATCTCAAAACCCAAA  
 GGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAACAGGTC  
 ACTCTGACCTGCATGGTTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGGACCAACAACGGGAAA  
 ACAGAGCTAAACTACAAGAACACTGAACCAGTCCCTGGACTCTGATGGTTCTTACTTCATGTACAGCAAG  
 CTGAGAGTGGAAAAGAAGAACTGGGTGGAAAGAAATAGCTACTCCTGTTTCAGTGGTCCACGAGGGTCTG  
 CACAATCACACGACTAAGAGCTTCTCCCGGACTCCGGGTAAA

Green: IL-2 leader sequence = 60 bp.

Red: Sequence encodes for extracellular portion of ULBP1 protein = 462

Blue: Sequences encoding for Fc region of mIgG2A = 699 bp.

GAATTC: EcoRI restriction site

TCATGA: BspHI restriction site

## Translation

MYRMQLLSICIALSLALVTNSCEVQGLVDERPFLHYDCVNHKAKAFASLGKKVNVTKTWEEQTETLRDVVD  
 FLKGQLLDIQVENLIPIEPLTLQARMSCEHEAHGHGRGSWQFLFNGQKFLLEDSDNNRKTALHPGAKKMT  
 EKWEKNRDVTFMFQKISLGDCKMWLEEFILMYWEQPRGPTIKPCPPCKCPAPNLLGGPSVFIFFPKIKDVL  
 MISLSPIVTCVVVDVSEDDPDVQISWVFNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMMSGKEFKC  
 KVNNKDLPAPIERTISKPKGSVRAPQVYVLPPEEEMTKKQVTLTCMVTFDMPEDIYVEWTNNGKTELNY  
 KNTEPVLDSGYSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

Green: IL-2 Leader protein sequence

Blue: Fc portion of mIgG2A

Red: Extracellular portion of ULBP1 protein

Molecular weight = 46602.6 Daltons

### 9.3.2.1 Alignment of the cloned nucleotide sequence to hULBP1-mFc2 sequence

#### Results of sequencing

NNNNGCCGGCNCNTCCANGCTTTGCGCTGACCCTGCTTGCTCAACTCTACGCTTTTGTTCGTTTCTGT  
 CTGCGCCGTTACAGATCCAAGCTGTGACCGGCCTACCTGAGATCACCGCGAAGGAGGGCCACCATG  
 TACAGGATGCAACTCCTGTCTTGCACTGACTAAGTCTTGCACTTGTGACGAATTCGTGTGAAGTTCAA  
 GGCCTGGTGGATGAAAGGCCTTTTCTTCACTATGACTGTGTTAACCAAGGCCAAAGCCTTTGCTTCT  
 CTGGGGAAGAAAGTCAATGTCACAAAAACCTGGGAAGAACAACCTGAAACACTAAGAGACGTGGTGGAT  
 TTCCTTAAAGGGCAACTGCTTGACATTCAAGTGGAGAATTTAATACCCATTGAGCCCCCACCCTGCAG  
 GCCAGGATGTCTTGTGAGCATGAAGCCATGGACACGGCAGAGGATCTTGGCAGTTCCCTCTTCAATGGA  
 CAGAAGTTCCCTCTTTGACTCAAACAACAGAAAGTGGACAGCACTTCATCCTGGAGCCAAGAAGATG  
 ACAGAGAAGTGGGAGAAGAACAGGGATGTGACCATGTTCTTCCAGAAGATTTCACTGGGGGATTGTAAG  
 ATGTGGCTTGAAGAATTTTTGATGTACTGGGAACAACCCAGAGGGCCACAATCAAGCCCTGTCTCCA  
 TGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCAAAGATCAAGGAT  
 GTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGAT  
 GTCCAGANNNTN

#### Alignment

			EcoRI	
Query	136	ATGTACAGGATGCAACTCCTGTCTTGCACTGACTAAGTCTTGCACTTGTGACGAATTCG	GAATTCG	195
Sbjct	1	ATGTACAGGATGCAACTCCTGTCTTGCACTGACTAAGTCTTGCACTTGTGACGAAATTCG		60
Query	196	TGTGAAGTTCAAGGCCTGGTGGATGAAAGGCCTTTTCTTCACTATGACTGTGTTAACCA		255
Sbjct	61	TGTGAAGTTCAAGGCCTGGTGGATGAAAGGCCTTTTCTTCACTATGACTGTGTTAACCA		120
Query	256	AAGGCCAAAGCCTTTGCTTCTCTGGGGAAGAAAGTCAATGTCACAAAAACCTGGGAAGAA		315
Sbjct	121	AAGGCCAAAGCCTTTGCTTCTCTGGGGAAGAAAGTCAATGTCACAAAAACCTGGGAAGAA		180
Query	316	CAAACGAAACACTAAGAGACGTGGTGGATTTCCCTTAAAGGGCAACTGCTTGACATTCAA		375
Sbjct	181	CAAACGAAACACTAAGAGACGTGGTGGATTTCCCTTAAAGGGCAACTGCTTGACATTCAA		240
Query	376	GTGGAGAATTTAATACCCATTGAGCCCCCACCCTGCAGGCCAGGATGTCTTGTGAGCAT		435
Sbjct	241	GTGGAGAATTTAATACCCATTGAGCCCCCACCCTGCAGGCCAGGATGTCTTGTGAGCAT		300
Query	436	GAAGCCCATGGACACGGCAGAGGATCTTGGCAGTTCCCTCTTCAATGGACAGAAGTTCCCT		495
Sbjct	301	GAAGCCCATGGACACGGCAGAGGATCTTGGCAGTTCCCTCTTCAATGGACAGAAGTTCCCT		360
Query	496	CTCTTTGACTCAAACAACAGAAAGTGGACAGCACTTCATCCTGGAGCCAAGAAGATGACA		555
Sbjct	361	CTCTTTGACTCAAACAACAGAAAGTGGACAGCACTTCATCCTGGAGCCAAGAAGATGACA		420
Query	556	GAGAAGTGGGAGAAGAACAGGGATGTGACCATGTTCTTCCAGAAGATTTCACTGGGGGAT		615
Sbjct	421	GAGAAGTGGGAGAAGAACAGGGATGTGACCATGTTCTTCCAGAAGATTTCACTGGGGGAT		480

```

Query 616 TGTAAAGATGTGGCTTGAAGAATTTTTGATGTACTGGGAACAACCCAGAGGGCCCAACATC 675
          |||
Sbjct 481 TGTAAAGATGTGGCTTGAAGAATTTTTGATGTACTGGGAACAACCCAGAGGGCCCAACATC 540

Query 676 AAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC 735
          |||
Sbjct 541 AAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC 600

Query 736 ATCTTCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGT 795
          |||
Sbjct 601 ATCTTCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGT 660

Query 796 GTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGA 835
          |||
Sbjct 661 GTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGA 700

```

BspHI

#### 9.4 Human ULBP2 sequence

The human ULBP2 sequence (NCBI Reference Sequence: NM\_025217.2) is submitted below. Like ULBP1, ULPB2 consists of a leader peptide (L),  $\alpha 1$ ,  $\alpha 2$  domains, and GPI anchor signal (Cosman et al, 2001). The sequence encoding for extracellular region includes L,  $\alpha 1$  and  $\alpha 2$  and it is 609 bp in length (Cosman et al, 2001). It starts from position 73 bp and ends at position 682.

```

AAAACCTTGA GGTGATTCAT CTTCCAGGCT CTCCTTCCAT CAAGTCTCTC CTCCTTAGCG
CTCTGGGTCC TTAATGGCAG CAGCCGCCGC TACCAAGATC CTTCTGTGCC TCCCGTTTCT
GCTCCTGTGTG TCCGGCTGGT CCCGGGCTGG GCGAGCCGAC CCTCACTCTC TTTGCTATGA
CATCACCGTC ATCCCTAAGT TCAGACCTGG ACCACGGTGG TGTGCGGTTT AAGGCCAGGT
GGATGAAAAG ACTTTTCTTC ACTATGACTG TGGCAACAAG ACAGTCACAC CTGTCAAGTCC
CCTGGGGAAG AACTAAATG TCACAACGGC CTGGAAAGCA CAGAACCCAG TACTGAGAGA
GGTGGTGGAC ATACTTACAG AGCAACTGCG TGACATTCAG CTGGAGAATT ACACACCCAA
GGAACCCCTC ACCCTGCAGG CCAGGATGTC TTGTGAGCAG AAAGCTGAAG GACACAGCAG
TGGATCTTGG CAGTTCAGTT TCATGAGGCA GATCTTCTC CTCTTTGACT CAGAGAAGAG
AATGTGGACA ACGTTCATC CTGGAGCCAG AAAGATGAAA GAAAAGTGGG AGAATGACAA
GGTTGTGGCC ATGTCCTTCC ATTACTTCTC AATGGGAGAC TGTATAGGAT GCCTGAGGA
CTTCTTGATG GGCATGGACA GCACCTGGA GCCAAGTGCA GGAGCACCAC TCGCCATGTC
CTCAGGCACA..... —————> TCCCAAAAAA AAAAAAAAAA AA (1362 bp)

```

Red: Leader sequence = 147 nucleotides

Blue: Alpha 1 domain = 310 nucleotides

Green: Alpha 2 domain = 152 nucleotides

#### 9.4.1 Recombinant hULBP2-mFc1 sequence

```

ATG GCAGCAGCCGCCGCTACCAAGATCCTTCTGTGCCTCCCGCTTCTGCTCCTGCTGTCCGGCTGGTCC
CGGGCTGGGCGAGCCGACCCTCACTCTCTTTGCTATGACATCACCGTCATCCCTAAGTTCAGACCTGGA
CCACGGTGGTGTGCGGTTCAAGGCCAGGTGGATGAAAAGACTTTTCTTCACTATGACTGTGGCAACAAG
ACAGTCACACCTGTCAGTCCCCTGGGGAAGAACTAAATGTCAACAACGGCCTGGAAAGCACAGAACCCA
GTACTGAGAGAGGTGGTGGACATACTTACAGAGCAACTGCGTGACATTCAGCTGGAGAATTACACACCC
AAGGAACCCCTCACCTGCAGGCCAGGATGTCTTGTGAGCAGAAAGCTGAAGGACACAGCAGTGGATCT
TGGCAGTTCAGTTTCGATGGGCAGATCTTCTCCTCTTTGACTCAGAGAAGAGAATGTGGACAACGGTT
CATCCTGGAGCCAGAAAGATGAAAGAAAAGTGGGAGAATGACAAGGTTGTGGCCATGTCCTTCCATTAC
TTCTCAATGGGAGACTGTATAGGATGGCTTGAGGACTTCTTGTATGGGCATGGACAGCCAGAGGGCCC
ACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATC
TTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGAT
GTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTTGTGAACAACGTGGAAGTACACACAGCTCAG

```

ACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACCAG  
 GACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCCATCGAGAGA  
 ACCATCTCAAACCCAAAGGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCCTCCACCAGAAGAAGAG  
 ATGACTAAGAAACAGGTCACTCTGACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAG  
 TGGACCAACAACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCCTGGACTCTGATGGTTCT  
 TACTTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAAGAAATAGCTACTCCTGTTCA  
 GTGGTCCACGAGGGTCTGCACAATCACCACACGACTAAGAGCTTCTCCCGGACTCCGGGTAAA **TCATGA**  
 (1308 bp)

Black: Leader sequence = 147 bp

Red: Sequence encodes for extracellular portion of hULBP2 protein = 462 bp

Blue: Sequence encoding for Fc region of mIgG2A = 699 bp.

**TCATGA**: BspHI restriction site

### Translation

**M**AAAAATKILLCLPLLLLLLSGWSRAGRADPHSLCYDITVI**P**KFRPGPRWC**AVQ**GQVDEK**TFLHYDCGNKT**  
**VTPV**SPLGK**KLNVTTAWKAQN**PVLR**EVVDILTEQLRDIQ**LENY**TPKEPLTLQ**ARMS**CEQKAEGHSSGSWQ**  
**FSFDGQIFLLF**DE**SEKRMWTTVHPG**ARK**MKEKWENDKVVAMS**FHY**F**SMG**DCIGWLED**FLMG**MDS**PRG**PTIK**  
 PC**PPCKCPAPNLLGGPSVFI**FPPK**IKDVL**MISL**SPIVTCVVVDVSEDDPDVQISWFV**NNVEVHTA**QTQTH**  
 RED**YNSTLRVVSALPIQH**QDWMSG**KEFKCKVNNKDL**PAP**IER**ISK**PKGSVRAPQVYVLP**PP**EEEMTKKQ**  
 VT**LTCMVTDFMPEDIYVEWT**NNG**KTELNYKNTE**PVLDSDGS**YFMYSKLRVEKKNW**VERNS**YS**CS**SVVHEGL**  
 H**NHHTTKSFSRTPGK**

Black: Leader protein sequence

Blue: Fc portion of mIgG2A

Red: Extracellular portion of ULBP2 protein

Molecular weight = 49244.13 Daltons

#### 9.4.1.1 Alignment of the cloned nucleotide sequence to hULBP2-mFc1 sequence

##### Results of sequencing

**GAATTC**ATGGCAGCAGCCGCCGCTACCAAGATCCTTCTGTGCCTCCCGCTTCTGCTCCTGCTGTCCGGC  
 TGGTCCCAGGCTGGGCGAGCCGACCCTCACTCTCTTTGCTATGACATCACCGTCATCCCTAAGTTCAGA  
 CCTGGACCACGGTGGTGTGCGGTTCAAGGCCAGGTGGATGAAAAGACTTTTCTTCACTATGACTGTGGC  
 AGCAAGACAGTACACCTGTGAGTCCCCTGGGGAAGAACTAAATGTCACAACGGCCTGGAAAAGCACAG  
 AACCCAGTACTGAGAGAGGTGGTGGACATACTTACAGAGCAACTGCGTGACATTCAGCTGGAGAATTAC  
 ACACCCAAGGAACCCCTCACCTGCAGGCCAGGATGTCTTGTGAGCAGAAAAGCTGAAGGACACAGCAGT  
 GGATCTTGGCAGTTCAGTTTCGATGGGCAGATCTTCCCCCTCTTTGACTCAGAGAAGAGAATGTGGACA  
 ACGGTTTCATCTGGAGCCAGAAAGATGAAAGAAAAGTGGGAGAATGACAAGGTTGTGGCCATGTCTCTC  
 CATTACTTCTCAATGGGAGACTGTATAGGATGGCTTGAGGACTTCTTGATGGGCATGGACAGC**CC**CAGA  
 GGGCCACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTC  
 TTCATCTTCCCTCAAAGATCAANNATGNNCNNNTNAN  
 TTCNNNNCCNNNNAANNNNCAAGGATGTAC**TCATGA**TCTCCCTGAGCCCCATAGTCACATGTGTGGTG  
 GTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTTGTGAACAACGTGGAAGTACACACA  
 GCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAG  
 CACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCCATC  
 GAGAGAACCATCTCAAACCCAAAGGGTCAGTAAGAGCTCC

**GAATTC**: EcoRI restriction site

**TCATGA**: BspHI restriction site



## Alignment

```

Query 7      ATGGCAGCAGCCGCGCTACCAAGATCCTTCTGTGCCTCCCGCTTCTGCTCCTGCTGTCC 66
            |||
Sbjct 1      ATGGCAGCAGCCGCGCTACCAAGATCCTTCTGTGCCTCCCGCTTCTGCTCCTGCTGTCC 60

Query 67     GGCTGGTCCCGGGCTGGGGCAGCCGACCTCACTCTCTTTGCTATGACATCACCGTCATC 126
            |||
Sbjct 61     GGCTGGTCCCGGGCTGGGGCAGCCGACCTCACTCTCTTTGCTATGACATCACCGTCATC 120

Query 127    CCTAAGTTCAGACCTGGACCACGGTGGTGTGCGGTTCAAGGCCAGGTGGATGAAAAGACT 186
            |||
Sbjct 121    CCTAAGTTCAGACCTGGACCACGGTGGTGTGCGGTTCAAGGCCAGGTGGATGAAAAGACT 180

Query 187    TTTCTTCACTATGACTGTGGCAACAAGACAGTCACACCTGTCAGTCCCCTGGGGAAGAAA 246
            |||
Sbjct 181    TTTCTTCACTATGACTGTGGCAACAAGACAGTCACACCTGTCAGTCCCCTGGGGAAGAAA 240

Query 247    CTAATGTCAACAACGGCCTGGAAAGCACAGAACCCAGTACTGAGAGAGGTGGTGGACATA 306
            |||
Sbjct 241    CTAATGTCAACAACGGCCTGGAAAGCACAGAACCCAGTACTGAGAGAGGTGGTGGACATA 300

Query 307    CTTACAGAGCAACTGCGTGACATTCAGCTGGAGAATTACACACCCAAGGAACCCCTCACC 366
            |||
Sbjct 301    CTTACAGAGCAACTGCGTGACATTCAGCTGGAGAATTACACACCCAAGGAACCCCTCACC 360

Query 367    CTGCAGGCCAGGATGTCTTTGTGAGCAGAAAGCTGAAGGACACAGCAGTGGATCTTGGCAG 426
            |||
Sbjct 361    CTGCAGGCCAGGATGTCTTTGTGAGCAGAAAGCTGAAGGACACAGCAGTGGATCTTGGCAG 420

Query 427    TTCAGTTTCGATGGGCAGATCTTCCCTCTTTGACTCAGAGAAGAGAATGTGGACAACG 486
            |||
Sbjct 421    TTCAGTTTCGATGGGCAGATCTTCCCTCTTTGACTCAGAGAAGAGAATGTGGACAACG 480

Query 487    GTTCATCCTGGAGCCAGAAAGATGAAAGAAAAGTGGGAGAATGACAAGGTTGTGGCCATG 546
            |||
Sbjct 481    GTTCATCCTGGAGCCAGAAAGATGAAAGAAAAGTGGGAGAATGACAAGGTTGTGGCCATG 540

Query 547    TCCTTCCATTACTTCTCAATGGGAGACTGTATAGGATGGCTTGAGGACTTCTTGATGGGC 606
            |||
Sbjct 541    TCCTTCCATTACTTCTCAATGGGAGACTGTATAGGATGGCTTGAGGACTTCTTGATGGGC 600

Query 607    ATGGACAGCCCCAGAGGGCCACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCT 666
            |||
Sbjct 601    ATGGACAGCCCCAGAGGGCCACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCT 660
                                     BspHI

Query 667    AACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAGATCAAGGATGTACTCATG 726
            |||
Sbjct 661    AACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAGATCAAGGATGTACTCATG 720

Query 727    ATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGAT 786
            |||
Sbjct 721    ATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGAT 780

Query 787    GTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCAT 846
            |||
Sbjct 781    GTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCAT 840

Query 847    AGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACCAGGAC 906
            |||
Sbjct 841    AGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACCAGGAC 900

Query 907    TGGATGAGTGGCAAGGAGTCAAATGCAAGGTCAACAACAAGACCTCCCAGCGCCCATC 966
            |||
Sbjct 901    TGGATGAGTGGCAAGGAGTCAAATGCAAGGTCAACAACAAGACCTCCCAGCGCCCATC 960

Query 967    GAGAGAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTCC 1007
            |||
Sbjct 961    GAGAGAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTCC 1001

```

#### 9.4.2 Recombinant hULBP2-mFc2 sequence

ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACCTTGTACGAATTCGTGTGCGGTTCAAGGCCAGGTGGATGAAAAGACTTTTCTTCACTATGACTGTGGCAACAAGACAGTCACACCTGTCAGTCCCCTGGGGAAGAACTAAATGTCACAACGGCCTGGAAAGCACAGAACCAGTACTGAGAGAGTGGTGGACATACTTACAGAGCAACTGCGTGACATTCAGCTGGAGAATTACACACCCAAGGAACCCCTCACCTGCAGGCCAGGATGTCTTGTGAGCAGAAAGCTGAAGGACACAGCAGTGGATCTTGGCAGTTCAGTTTCGATGGGCAGATCTTCTCCTCTTTGACTCAGAGAAGAGAATGTGGACAACGGTTCATCCTGGAGCCAGAAAGATGAAAGAAAAGTGGGAGAATGACAAGGTTGTGGCCATGTCCTTCCATTACTTCTCAATGGGAGACTGTATAGGATGGCTTGAGGACTTCTTGATGGGCATGGACAGCCCCAGAGGGCCACAATCAAGCCCTGTCCTCCATGCAAATGCCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTTCAGTGCCTCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCATCGAGAGAACCATCTCAAAA CCCAAAGGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAA CAGGTCACTCTGACCTGCATGGTTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGGACCAACAAC GGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCTGGACTCTGATGGTTCTTACTTCATGTAC AGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAGAAATAGTACTCTGTTCAGTGGTCCACGAG GGTCTGCACAATCACCACACGACTAAGAGCTTCTCCCGACTCCGGGTAAA (1221 bp)

Green: hIL-2 leader sequence = 60 bp

Red: Sequence encoding for extracellular portion of ULBP2 protein = 458

Blue: Sequence encoding for Fc region of mIgG2A = 699 bp

#### Translation

MYRQLLSCIALSLALVTNSCAVQGVDEKTFLLHYDCGNKTVTPVSPLGKKNLNVTTAWKAQNPVLRVVDILTEQLRDIQLENYTPKEPLTLQARMSCEQKAEGHSSGSWQFSFDGQIFLLFDSEKRMWTTVHPGARKMKEKWENDKVVAMSFHYFSMGDCIGWLEDFLMGMDSPRGPTIKPCPPCKCPAPNLLGGPSVFIFFPKIKDVLMLISLSPIVTCVVVDVSEDDPDVQISWVFNNEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPEEEMTKKQVTLTCMVTFDFMPEDIYVEWTTNNGKTELNYKNTEPVLDSGDSYFMYSKLRVEKKNWVERNSYSVSVHEGLHNNHHTTKSFSRTPGK

Green: IL-2 Leader protein sequence

Red: Extracellular portion of ULBP2 protein

Blue: Fc portion of mIgG2A

Molecular weight = 46106.91 Daltons

#### 9.4.2.1 Alignment of the cloned nucleotide sequence to hULBP2-mFc2 sequence

#### Results of sequencing

NNTNNNNNTGTGACCGGCGCCTACCTGAGATCACCGGCGAAGGAGGGCCACCATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACCTTGTACGAATTCGTGTGCGGTTCAAGGCCAGGTGGATGAAAGACTTTTCTTCACTATGACTGTGGCAACAAGACAGTCACACCTGTCAGTCCCCTGGGGAAGAACTAAATGTCACAACGGCCTGGAAAGCACAGAACCAGTACTGAGAGAGGTGGTGGACATACTTACAGAGCAACTGCGTGACATTCAGCTGGAGAATTACACACCCAAGGAACCCCTCACCTGCAGGCCAGGATGTCTTGTGAGCAGAAAGCTGAAGGACACAGCAGTGGATCTTGGCAGTTCAGTTTCGATGGGCAGATCTTCTCCTCTTTGACTCAGAGAAGAGAATGTGGACAACGGTTCATCCTGGAGCCAGAAAAGATGAAAGAAAAGTGGGA GAATGACAAGGTTGTGGCCATGTCCTTCCATTACTTCTCAATGGGAGACTGTATAGGATGGCTTGAGGACTTCTTGATGGGCATGGACAGCCCCAGAGGGCCACAATCAAGCCCTGTCTCCATGCAAATGCCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCTGAGCCCCATAGTCACATGTGTGNTGG

GAATTC: EcoRI restriction site

TCATGA: BspHI restriction site

## Alignment

			EcoRI	
Query	53	ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCAC <b>TAATTCG</b>		112
Sbjct	1	ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACGAAATTCG		60
Query	113	TGTGCGGTTCAAGGCCAGGTGGATGAAAAGACTTTTCTTCACTATGACTGTGGCAACAAG		172
Sbjct	61	TGTGCGGTTCAAGGCCAGGTGGATGAAAAGACTTTTCTTCACTATGACTGTGGCAACAAG		120
Query	173	ACAGTCACACCTGTCAGTCCCCTGGGAAGAACTAAATGTCACAACGGCCTGGAAAGCA		232
Sbjct	121	ACAGTCACACCTGTCAGTCCCCTGGGAAGAACTAAATGTCACAACGGCCTGGAAAGCA		180
Query	233	CAGAACCCAGTACTGAGAGAGGTGGTGGACATACTTACAGAGCAACTGCGTGACATTCAG		292
Sbjct	181	CAGAACCCAGTACTGAGAGAGGTGGTGGACATACTTACAGAGCAACTGCGTGACATTCAG		240
Query	293	CTGGAGAATTACACACCCAAGGAACCCCTCACCTGCAGGCCAGGATGTCTTGTGAGCAG		352
Sbjct	241	CTGGAGAATTACACACCCAAGGAACCCCTCACCTGCAGGCCAGGATGTCTTGTGAGCAG		300
Query	353	AAAGCTGAAGGACACAGCAGTGGATCTTGGCAGTTTTCAGTTTCGATGGGCAGATCTTCCTC		412
Sbjct	301	AAAGCTGAAGGACACAGCAGTGGATCTTGGCAGTTTTCAGTTTCGATGGGCAGATCTTCCTC		360
Query	413	CTCTTTGACTCAGAGAAGAGAATGTGGACAACGGTTCATCCTGGAGCCAGAAAGATGAAA		472
Sbjct	361	CTCTTTGACTCAGAGAAGAGAATGTGGACAACGGTTCATCCTGGAGCCAGAAAGATGAAA		420
Query	473	GAAAAGTGGGAGAATGACAAGGTTGTGGCCATGTCCCTCCATTACTTCTCAATGGGAGAC		532
Sbjct	421	GAAAAGTGGGAGAATGACAAGGTTGTGGCCATGTCCCTCCATTACTTCTCAATGGGAGAC		480
Query	533	TGTATAGGATGGCTTGAGGACTTCTTGATGGGCATGGACAGCCCCAGAGGGCCCAACATC		592
Sbjct	481	TGTATAGGATGGCTTGAGGACTTCTTGATGGGCATGGACAGCCCCAGAGGGCCCAACATC		540
Query	593	AAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC		652
Sbjct	541	AAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC		600
Query	653	ATCTTCCCTCCAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGT		712
Sbjct	601	ATCTTCCCTCCAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGT		660
Query	713	GTG <b>N</b> TGG 719		
Sbjct	661	GTG <b>C</b> TGG 667		

## 9.5 Human ULBP3 sequence

The human ULBP3 sequence (NCBI Reference Sequence: NM\_024518.1) is presented below. It consists of a leader peptide (L), extracellular segment ( $\alpha 1$  and  $\alpha 2$  domains) and GPI anchor signal (Cosman et al, 2001). The sequence encoding for extracellular region includes L,  $\alpha 1$  and  $\alpha 2$  and it is 612 bp in length (Cosman et al, 2001). It starts from position 1 bp and ends at position 612.

```

ATGGCAGCGG CCGCCAGCCC CGCGATCCTT CCGCGCCTCG CGATTCCTCC GTACCTGCTA
TTCGACTGGT CCGGGACGGG GCGGGCCGAC GCTCACTCTC TCTGGTATAA CTTCACCATC
ATTCAATTTGC CCAGACATGG GCAACAGTGG TGTGAGGTCC AGAGCCAGGT GGATCAGAAG
AATTTTCTCT CCTATGACTG TGGCAGTGAC AAGGTCTTAT CTATGGGTCA CCTAGAAGAG
CAGCTGTATG CCACAGATGC CTGGGGAAAA CAACTGGAAA TGCTGAGAGA GGTGGGGCAG
AGGCTCAGAC TGGAACTGGC TGACACTGAG CTGGAGGATT TCACACCCAG TGGACCCCTC
ACGCTGCAGG TCAGGATGTC TTGTGAGTGT GAAGCCGATG GATACATCCG TGGATCTTGG
CAGTTCAGT TCGATGGACG GAAGTTCCTC CTCTTTGACT CAAACAACAG AAAGTGGACA
GTGGTTCACG CTCGAGCCAG GCGGATGAAA GAGAAGTGGG AGAAGGATAG CGGACTGACC
ACCTTCTTCA AGATGGTCTC AATGAGAGAC TGCAAGAGCT GGCTTAGGGA CTTCTGATG
CACAGGAAGA AGAGGCTGGA ACCACAGCA CCACCCACCA TGGCCCCAGG CTTAGCTCAA
CCCAAAGCCA TAGCCACCAC CCTCAGTCCC TGGAGCTTCC TCATCATCCT CTGCTTCATC
CTCCCTGGCA TCTGA (735 bp)

```

Red: Leader sequence = 150 nucleotides

Blue: Alpha 1 domain = 310 nucleotides

Green: Alpha 2 domain = 152 nucleotides

### 9.5.1 Recombinant hULBP3-mFc1 sequence

```
ATG GCAGCGGCCGCCAGCCCCGCGATCCTTCCGCGCCTCGCGATTCTTCCGTACCTGCTATTCGACTGG
TCCGGGACGGGGCGGGCCGACGCTCACTCTCTCTGGTATAACTTCACCATCATTTCATTTGCCAGACAT
GGGCAACAGTGGTGTGAGGTCCAGAGCCAGGTGGATCAGAAGAATTTCTCTCCTATGACTGTGGCAGT
GACAAGGTCTTATCTATGGGTACCTAGAAGAGCAGCTGTATGCCACAGATGCCTGGGGAAAAACAATG
GAAATGCTGAGAGAGGTGGGGCAGAGGCTCAGACTGGAAGTGGCTGACACTGAGCTGGAGGATTTACA
CCCAGTGGACCCCTCACGCTGCAGGTGAGGATGTCTTGTGAGTGTGAAGCCGATGGATACATCCGTGGA
TCTTGGCAGTTCAGCTTCGATGGACGGAAGTTCCTCCTCTTTGACTCAAACAACAGAAAAGTGGACAGTG
GTTACGCTGGAGCCAGGCGGATGAAAGAGAAGTGGGAGAAGGATAGCGGACTGACCACCTTCTTCAAG
ATGGTCTCAATGAGAGACTGCAAGAGCTGGCTTAGGGACTTCTGATGCACAGGAAGAAGCCCAGAGGG
CCCACAATCAAGCCCTGTCCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC
ATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTG
GATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCT
CAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCAC
CAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAGACCTCCCAGCGCCCATCGAG
AGAACCATCTCAAACCCAAAGGGTCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAGAAGAA
GAGATGACTAAGAAACAGGTCACTCTGACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTG
GAGTGGACCAACAACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCTGGACTCTGATGGT
TCTTACTTCATGTACAGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAAGAAATAGCTACTCCTGT
TCAGTGGTCCACGAGGGTCTGCACAATCACCACACGACTAAGAGCTTCTCCCGGACTCCGGGTAAA
```

(1311 bp)

Black: Leader sequence = 150 bp

Red: Ssequence encoding for the extracellular portion of hULBP3 protein = 462 bp

Blue: Sequence encoding for Fc region of mIgG2A = 699 bp

TCATGA: BspHI restriction site

### Translation

```
MAAAASPAILPRLAILPYLLFDWSGTGRADAHSLWYNFTIIHLPRHGQQWCEVQSQVDQKNFLSYDCGSD
KVL SMGHLEEQLYATDAWGKQLEMLREVGQRLRLELADTELEDFTPSGPLTLQVRMSCECEADGYIRGSW
QFSFDGRKFLLFDSNNRKWTVVHAGARRMKEKWEKDSGLTTFFKMVS MRDCKSWLRDFLMHRKKPRGPTI
KPCPPCKCPAPNLLGGPSVFI FPPKIKDVLMI SLSPIVTCVVVDVSEDDPDVQISWVNNVEVHTAQTQT
HREDYNSTLRVVSALPIQHQDWMMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPEEEMTKK
QVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSGDSYFMYSKLRVEKKNWVERNSYSCSVVHEG
LHNHHTTKSFSRTPGK
```

Black: Leader protein sequence

Red: Extracellular portion of ULBP3 protein

Blue: Fc portion of mIgG2A

Molecular weight = 49966.2 Daltons

### 9.5.1.1 Alignment of the cloned nucleotide sequence to hULBP3-mFc1 sequence

#### Results of sequencing

NAATTGAANCGGGTGCNNNNNNNGGTNGGCNNNGGGGTAAANTGGAAAGTGANGTNGNGTACNGNNCNNNTTTTTNN  
CNNGGGTGGGGNNNNACCGTATATAAGTGCAGTAGTCGCCGTGAACGTCTTTTTTCGCAACGNGTTTGCCGCCAGA  
ACACAGCTGAAGCTTCGAGNNGCTCGCATNTNTCTTACACGCCCGCCGCCCTACCTGAGGCCCCANCCACGCCG  
GTTGAGTCGCGTTTCGCCGCTCCCCGNTGTGGTGCCTCNTGAAC TCGTCCGCCGTCTAGGTAAGTTTAAAGCTCA  
GGTCGAGACCGGGCTTTGTCCGGCCTCCCTTGAGCCTACCTAGACTCAGCCGGCTCNCACGCTTTGCCTGACC  
CTGCTTGCTCAACTCTACGTCTTTGTTTCGTTTTCTGTTCTGCGCCGTACAGATNCAAGCTGTGACCGCGCCTAC  
CTGAGATCACCGGTGAATTCATGGCAGCGGGCCAGCCCGCGATCCTTCCGCGCTCGCGATTCTTCGGTACCTG  
CTATTCGACTGGTCCGGGACGGGGCGGGCCAGCCTCACTCTCTCTGGTATAACTTCACCATCATTCATTGCCAG  
ACATGGGAACAGTGGTGTGAGGTCCAGAGCNNNTGGATNNNNN  
NNATGGNNAANNNTNNNNNNGTNNNNNAGAGCCAGGTGGATCAGAAGATTTTCTCTCTATGACTGTGCCAGTGACA  
AGGTCTTATCTATGGGTACCTAGAAGAGCAGCTGTATGCCACAGATGCCCTGGGGAAAAACAAGTGAATGCTGAGA  
GAGGTGGGGCAGAGGCTCAGACTGGAAGTGGCTGACTGAGCTGGAGGATTTACACCCAGTGGACCCCTCACGCT  
GCAGGTGAGGATGTCTTGTGAGTGTGAAGCCGATGGATACATCCGTGGATCTTGGCAGTTCAGCTTCGATGGACGGA  
AGTTCCTCCTCTTTGACTCAAACAACAGAAAGTGGACAGTGGTTACGCTGGAGCCAGCGGATGAAAGAGAAGTGG  
GAGAAGGATAGCGGACTGACCACCTTCTTCAAGATGGTCTCAATGAGAGACTGCAAGAGCTGGCTTAGGGACTTCT  
GATGCACAGGAAGAAGCCCAGAGGGCCACAATCAAGCCCTGTCTCCATGCAAATGCCAGCACCTAACCTCTTG  
GTGGACCATCCGTCTCATCTTCCCTCCAAGATCAAGGATGTACTCATGATCTCCNNANCCNANNNNNNNNN

#### Alignment

```
Query 483 ATGGCAGCGGCCGCCAGCCCGCGATCCTTCCGCGCTCGCGATTCTTCCGTACCTGCTA 542
          |||
Sbjct 1 ATGGCAGCGGCCGCCAGCCCGCGATCCTTCCGCGCTCGCGATTCTTCCGTACCTGCTA 60

Query 543 TTCGACTGGTCCGGGACGGGGCGGGCCAGCCTCACTCTCTCTGGTATAACTTCACCATC 602
          |||
Sbjct 61 TTCGACTGGTCCGGGACGGGGCGGGCCAGCCTCACTCTCTCTGGTATAACTTCACCATC 120

Query 603 ATTCATTTGCCAGACATGGGCAACAGTGGTGTGAGGTCCAGAGCCAGGTGGATCAGAAG 662
          |||
Sbjct 121 ATTCATTTGCCAGACATGGGCAACAGTGGTGTGAGGTCCAGAGCCAGGTGGATCAGAAG 180

Query 663 AATTTTCTCTCCTATGACTGTGGCAGTGACAAGGTCTTATCTATGGGTACCTAGAAGAG 722
          |||
Sbjct 181 AATTTTCTCTCCTATGACTGTGGCAGTGACAAGGTCTTATCTATGGGTACCTAGAAGAG 240

Query 723 CAGCTGTATGCCACAGATGCCTGGGGAAAAACAAGTGAATGCTGAGAGAGGTGGGGCAG 782
          |||
Sbjct 241 CAGCTGTATGCCACAGATGCCTGGGGAAAAACAAGTGAATGCTGAGAGAGGTGGGGCAG 300

Query 783 AGGCTCAGACTGGAAGTGGCTGACTGAGCTGGAGGATTTACACCCAGTGGACCCCTC 842
          |||
Sbjct 301 AGGCTCAGACTGGAAGTGGCTGACTGAGCTGGAGGATTTACACCCAGTGGACCCCTC 360

Query 843 ACGCTGCAGGTGAGGATGTCTTGTGAGTGTGAAGCCGATGGATACATCCGTGGATCTTGG 902
          |||
Sbjct 361 ACGCTGCAGGTGAGGATGTCTTGTGAGTGTGAAGCCGATGGATACATCCGTGGATCTTGG 420

Query 903 CAGTTCAGCTTCGATGGACGGAAGTTCCTCCTCTTTGACTCAAACAACAGAAAGTGGACA 962
          |||
Sbjct 421 CAGTTCAGCTTCGATGGACGGAAGTTCCTCCTCTTTGACTCAAACAACAGAAAGTGGACA 480

Query 963 GTGGTTACGCTGGAGCCAGGCGGATGAAAGAGAAGTGGGAGAAGGATAGCGGACTGACC 1022
          |||
Sbjct 481 GTGGTTACGCTGGAGCCAGGCGGATGAAAGAGAAGTGGGAGAAGGATAGCGGACTGACC 540

Query 1023 ACCTTCTTCAAGATGGTCTCAATGAGAGACTGCAAGAGCTGGCTTAGGGACTTCTCTGATG 1082
          |||
Sbjct 541 ACCTTCTTCAAGATGGTCTCAATGAGAGACTGCAAGAGCTGGCTTAGGGACTTCTCTGATG 600

Query 1083 CACAGGAAGAAGCCAGAGGGCCCAATCAAGCCCTGTCTCCATGCAAATGCCAGCA 1142
          |||
Sbjct 601 CACAGGAAGAAGCCAGAGGGCCCAATCAAGCCCTGTCTCCATGCAAATGCCAGCA 660

Query 1143 CCTAACCTCTTGGGTGGACCATCCGCTTTCATCTTCCCTCCAAGATCAAGGATGTACTC 1202
          |||
Sbjct 661 CCTAACCTCTTGGGTGGACCATCCGCTTTCATCTTCCCTCCAAGATCAAGGATGTACTC 720
```

**BspHI**

Query	1203	<b>ATGA</b> FCTCC	1211
Sbjct	721	ATGATCTCC	729

### 9.5.2 Recombinant hULBP3-mFc2 sequence

**ATG**TACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCAC**GAATTC**G**TGT**  
 GAGGTCCAGAGCCAGGTGGATCAGAAGAATTTCTCTCCTATGACTGTGGCAGTGACAAGGCTTATCT  
 ATGGGTCACCTAGAAGAGCAGCT**GATG**CCACAGATGCCTGGGGAAAACAAGTGGAAATGCTGAGAGAG  
 GTGGGGCAGAGGCTCAGACTGGAAGTGGCTGACACTGAGCTGGAGGATTTACACCCAGTGGACCCCTC  
 ACGCTGCAGGTGAGGATGTCTTGTGAGTGTGAAGCCGATGGATACATCCGTGGATCTTGGCAGTTCAGC  
 TTCGATGGACGGAAGTTCCTCCTTTGACTCAAACAACAGAAAGTGGACAGTGGTTCACGCTGGAGCC  
 AGGCGGATGAAAGAGAAGTGGGAGAAGGATAGCGGACTGACCACCTTCTTCAAGATGGTCTCAATGAGA  
 GACTGCAAGAGCTGGCTTAGGGACTTCTGATGCACAGGAAGAAG**CCC**AGAGGGCCCAATCAAGCCC  
 TGTCCTCCATGCAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAG  
 ATCAAGGATGTAC**TCATGA**TCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGAT  
 GACCCAGATGTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCAT  
 AGAGAGGATTACAACAGTACTCTCCGGGTGGTCAAGTGCCTCCCATCCAGCACCAGGACTGGATGAGT  
 GGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCATCGAGAGAACCATCTCAAAA  
 CCCAAAGGGTCAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAA  
 CAGGTCACCTCTGACCTGCATGGTACAGACTTCATGCCTGAAGACATTTACGTGGAGTGGACCAACAAC  
 GGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCCCTGGACTCTGATGGTCTTACTTCATGTAC  
 AGCAAGCTGAGAGTGGAAAAGAAGAACTGGGTGGAAAGAAATAGCTACTCCTGTTCAAGTGGTCCACGAG  
 GGTCTGCACAATCACCACACGACTAAGAGCTTCTCCCGGACTCCGGGTAA**AG**

Green: hIL-2 leader sequence = 60 bp.

Red: Sequence encodes for the extracellular portion of ULBP3 protein = 462

Blue: Sequence encoding for Fc region of mIgG2A = 699 bp.

**GAATTC**: EcoRI restriction site

**TCATGA**: BspHI restriction site

### Translation

**MYR**MQLL**SCIALSLALVTNS**CEVQSQVDQKNFLSYDCGSDKVL**SMGHLEE**QLYATDAWGK**QLEMLREV**  
**QRLRLELADTELEDFTPSGPLTLQVRMSCECEADGYIRGSWQFSFDGRKFL**LFD**SNNR**KWTVVHAGARR  
**MKEKWEKDSGLTTF**FKM**VS**MRDCK**SWLRD**FL**MHR**KK**PRGPT**IKPCPPCKCPAPNLLGGPSVFI**FP**PKIK  
 DVL**MISL**SPIVTCVVVDVSEDDPDVQISW**FN**NVEVHTAQ**TQ**THREDY**N**STLRVVSALPIQH**Q**DWMSGK  
 EFKCKV**NNKDL**PAPIERTISK**PKGS**VRAPQVYVLPPEEEMTK**Q**VTLTCMVTD**F**MPEDIYVEWT**NN**GK  
 TELNYKNTEPVLDSDGSY**F**MYSK**L**RVEK**N**WVERNSYSCSVV**HEGL**HNHHTTKS**F**S**R**TPGK

Green: hIL-2 Leader protein sequence

Red: Extracellular portion of ULBP3 protein

Blue: Fc portion of mIgG2A

Molecular weight = 46546.94 Daltons

### 9.5.2.1 Alignment of the cloned nucleotide sequence to hULBP3-mFc2 sequence

#### Results of sequencing

AAGCTGTGACCGGCGCCTACCTGAGATCACCGGCGAAGGAGGGCCACCATGTACAGGATGCAACTCCTG  
TCTTGCATTGCACTAAGTCTTGCACCTTGTACGAATTCGTGTGAGGTCCAGAGCCAGGTGGATCAGAAG  
AATTTTCTCTCCTNNNNNGTGNNNN  
AATTTTCTCTCCTATGACTGTGGCAGTGACAAGGTCTTATCTATGGGTCACCTAGAAGAGCAGCTGTAT  
GCCACAGATGCCTGGGAAAACAACCTGGAATGCTGAGAGAGGTGGGGCAGAGGCTCAGACTGGAACCTG  
GCTGACACTGAGCTGGAGGATTTACACCCAGTGGACCCCTCACGCTGCAGGTCAGGATGTCTTGTGAG  
TGTGAAGCCGATGGATACATCCGTGGATCTTGGCAGTTCAGCTTCGATGGACGGAAGTTCCTCCTCTTT  
GACTCAAACAACAGAAAGTGGACAGTGGTTCACGCTGGAGCCAGGCGGATGAAAGAGAAGTGGGAGAAG  
GATAGCGGACTGACCACCTTCTTCAAGATGGTCTCAATGAGAGACTGCAAGAGCTGGCTTAGGGACTTC  
CTGATGCACAGGAAGAAGCCCAGAGGGCCACAATCAAGCCCTGTCTCCATGCAAAATGCCAGCACCT  
AACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTG  
AGCCCCATAGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTT  
GTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGG  
GTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAAC  
AACAAAGACCT

#### Alignment

			EcoRI	
Query	49	ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACCTGTGCACGAATTC	GAATTC	108
Sbjct	1	ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACCTGTGCACGAATTCG		60
Query	109	TGTGAGGTCCAGAGCCAGGTGGATCAGAAGAATTTTCTCTCCTATGACTGTGGCAGTGAC		168
Sbjct	61	TGTGAGGTCCAGAGCCAGGTGGATCAGAAGAATTTTCTCTCCTATGACTGTGGCAGTGAC		120
Query	169	AAGGTCTTATCTATGGGTACCTAGAAGAGCAGCTGTATGCCACAGATGCCTGGGAAAA		228
Sbjct	121	AAGGTCTTATCTATGGGTACCTAGAAGAGCAGCTGTATGCCACAGATGCCTGGGAAAA		180
Query	229	CAACTGGAAATGCTGAGAGAGGTGGGGCAGAGGCTCAGACTGGAAGTGGCTGACACTGAG		288
Sbjct	181	CAACTGGAAATGCTGAGAGAGGTGGGGCAGAGGCTCAGACTGGAAGTGGCTGACACTGAG		240
Query	289	CTGGAGGATTTACACCCAGTGGACCCCTCACGCTGCAGGTCAGGATGTCTTGTGAGTGT		348
Sbjct	241	CTGGAGGATTTACACCCAGTGGACCCCTCACGCTGCAGGTCAGGATGTCTTGTGAGTGT		300
Query	349	GAAGCCGATGGATACATCCGTGGATCTTGGCAGTTCAGCTTCGATGGACGGAAGTTCCTC		408
Sbjct	301	GAAGCCGATGGATACATCCGTGGATCTTGGCAGTTCAGCTTCGATGGACGGAAGTTCCTC		360
Query	409	CTCTTTGACTCAAACAACAGAAAGTGGACAGTGGTTCACGCTGGAGCCAGGCGGATGAAA		468
Sbjct	361	CTCTTTGACTCAAACAACAGAAAGTGGACAGTGGTTCACGCTGGAGCCAGGCGGATGAAA		420
Query	469	GAGAAGTGGGAGAAGGATAGCGGACTGACCACCTTCTTCAAGATGGTCTCAATGAGAGAC		528
Sbjct	421	GAGAAGTGGGAGAAGGATAGCGGACTGACCACCTTCTTCAAGATGGTCTCAATGAGAGAC		480
Query	529	TGCAAGAGCTGGCTTAGGGACTTCTGATGCACAGGAAGAAGCCCAGAGGGCCACAATC		588
Sbjct	481	TGCAAGAGCTGGCTTAGGGACTTCTGATGCACAGGAAGAAGCCCAGAGGGCCACAATC		540
Query	589	AAGCCCTGTCTCCATGCAAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC		648
Sbjct	541	AAGCCCTGTCTCCATGCAAAATGCCAGCACCTAACCTCTTGGGTGGACCATCCGTCTTC		600
Query	649	ATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGT	BspHI     	708
Sbjct	601	ATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGT		660
Query	709	GTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAAC		768
Sbjct	661	GTGGTGGTGGATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAAC		720

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Query 769 GTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGG 828
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Sbjct 721 GTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCGG 780

Query 829 GTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGC 888
          |||
Sbjct 781 GTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCAAATGC 840

Query 889 AAGGTCAACAACAAAGACCT 908
          |||
Sbjct 841 AAGGTCAACAACAAAGACCT 860

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