



**SAHLGRENKA ACADEMY**

## **Immune Checkpoint Receptor Expression in NK Cells**

Degree Project in Medicine

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# Abstract

## Background

Immunotherapy for cancer has improved overall survival and revolutionized the field of oncology. Blockade of immune checkpoint receptors has reversed cancer-induced inhibition of T cells and natural killer (NK) cells have similar receptors. An attractive approach would be to activate the cytotoxic potential of NK cells using blocking agents to these receptors. NK cells have different combinations of inhibitory NKG2A and KIR receptors which when bound to their cognate ligands both set the functional potential and dampen the cytotoxic behavior of the NK cell, a process called licensing.

## Aim

To develop a method for transcript analyses of genes encoding NK cell immune checkpoint receptors that may be used to gain further knowledge of how to block the inhibition while maintaining the licensed state.

## Method

PCR primers for immune checkpoint receptors (CTLA-4, PD-1, TIM-3, LAG-3 and NKG2A) were designed and tested on IL-2 and IL-15 activated peripheral blood mononuclear cells. NK cells were stimulated with IL-2 and leukemic K562 cells to trigger degranulation.

Fluorescence-activated cell sorting (FACS) was performed to identify responder cells of specific NK cell subsets. cDNA of the transcriptome was generated using reverse transcription and the expression levels of immune checkpoint receptor genes was assayed using q-PCR.

## Results

The primers developed were tested and shown to have good specificity. No consistent expression of PD-1 or CTLA-4 could be shown in any NK cell subsets. The LAG-3 level was notably low in NKG2A+ subsets. TIM-3 was expressed in all subsets.

## Conclusion

The results suggest that the developed methodology may be useful in studying how immune checkpoint transcript phenotypes are related to NK cell function. Studying responses in transcript-positive cells could not be tested, as all KIR+NKG2A- NK cells were negative for NKG2A transcripts. Future experiments will be designed to find the time window where these transition NK cells are present, enabling us to discriminate between the educating role of NKG2A and its role as an activation marker.

Keywords: NK cells; immune checkpoint inhibitors; cancer immunotherapy

# Introduction

## The Immune System

The homeostasis of multicellular organisms is under constant threat by a multitude of pathogens. Through evolution, a line of defense called the immune system has developed to counter this threat. It consists of an array of effector cells and molecules protecting the organism from infectious agents and cancer cells. The less a cancer cell resembles a normal cell, the better the chances are for the immune system to recognize and kill it. In recent years, the concept of immunotherapy has revolutionized the treatment of a wide range of solid and hematologic cancers (1) and the Nobel Prize in Physiology or Medicine 2018 was awarded to James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation."

All blood cells including immune cells originate from a common cell, the hematopoietic stem cell (HSC). In a process called hematopoiesis the HSC proliferates, matures and differentiates through a series of steps into the different types of blood cells as shown in Figure 1 (2).

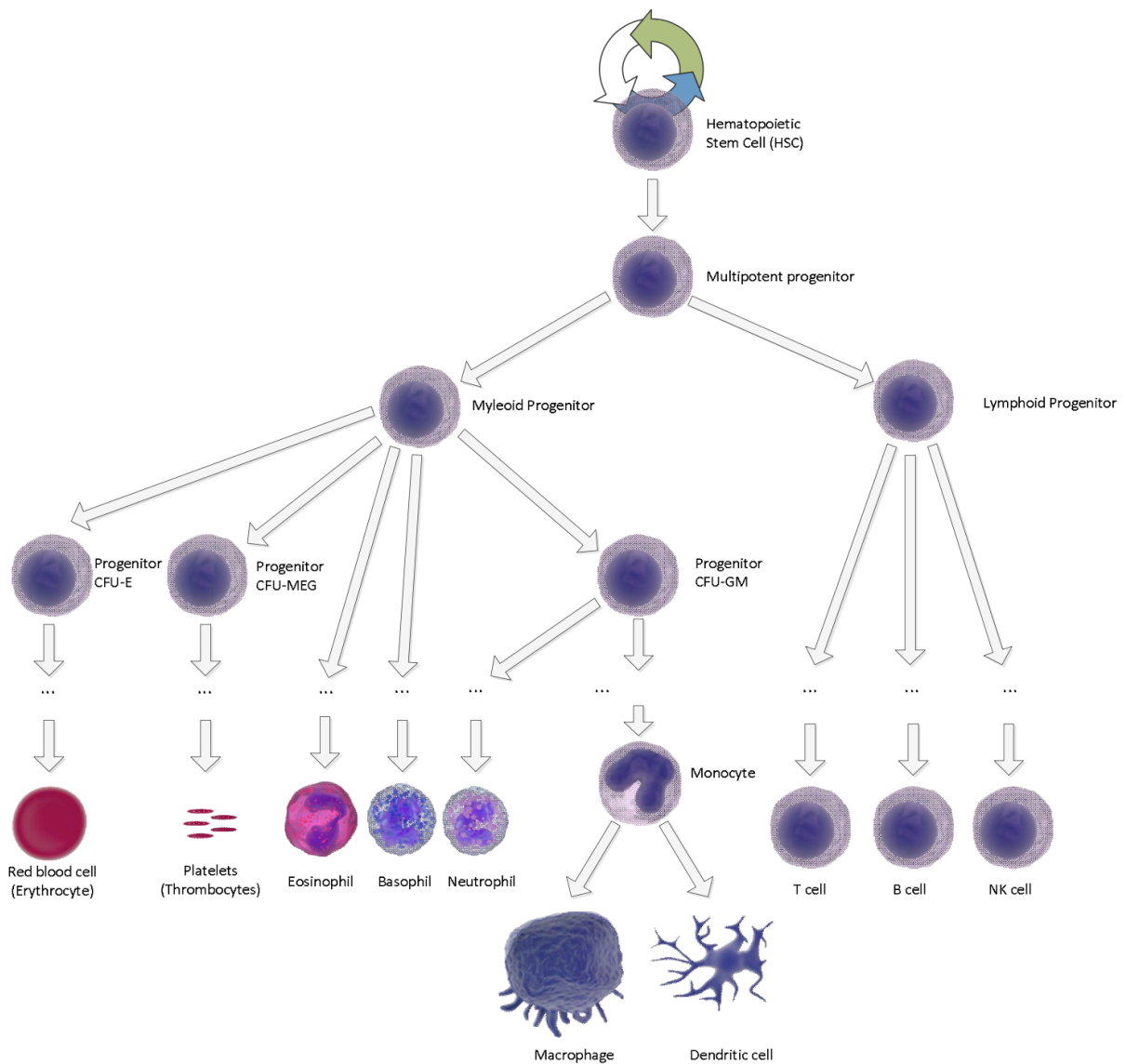


Figure 1 Overview of hematopoiesis (Created using resources from Somersault 18:24 (3))

The blood cells are divided into myeloid and lymphoid cells, derived from common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) cells. Further differentiation of the CMP give rise to erythrocytes, platelets, granulocytes and monocytes. Granulocytes consist of eosinophils, basophils and neutrophils while monocytes can be divided into macrophages and dendritic cells. The CLP cells differentiate into B, T, NK and dendritic cells. Together, lymphocytes, monocytes and macrophages are called peripheral blood



mononuclear cells (PBMC) (2). By filtering away the other cells from a blood sample the PBMC can be extracted.

The immune system can be classified into two subsystems known as the innate and adaptive immune systems.

### **Innate Immune System**

The innate immune system developed first in evolution and can respond quickly to the invasion of an infectious agent. It consists of phagocytic cells (macrophages, eosinophils, basophils and neutrophils), antigen-presenting cells (APCs) including macrophages and dendritic cells (DCs), monocytes and innate lymphoid cells, e.g. natural killer (NK) cells.

When an infectious agent is encountered, inflammatory signals attract innate immune cells, which form a first line of defense. Dendritic cells continuously scanning the extracellular environment for invading agents are also called to the site. They pick up foreign antigens and migrate to lymph nodes where they initiate an adaptive immune response aimed at the infectious agent.

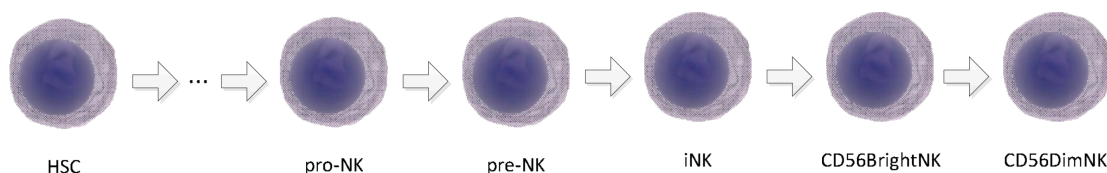
### **Adaptive Immune System**

Albeit slower than the innate immune system, the adaptive immune system complements it by having the ability to direct precise attacks towards the antigens presented by DCs. T cells specific for the antigen undergo clonal expansion and produce cytokines, in turn promoting maturation of B cells producing antigen-specific antibodies. This leads to a highly specific immune response against pathogens and infected cells. After resolution of the infection a small number of memory T and B cells remain, able to quickly respond in case of a reinfection.

The recent finding that some innate cells (macrophages and NK cells) have been shown to develop immunological memory (4–6) has blurred the line between the innate and adaptive immunity.

## Natural Killer Cells

NK cells are part of the innate immune system and are implicated in the defense against viral infection, malignant cells and even physical or chemical damage (7,8). These innate cytotoxic lymphocytes develop from HSCs in a step-wise fashion as depicted in Figure 2.



*Figure 2 NK cell development and maturation (Created using resources from Somersault 18:24 (3))*

The mature CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells differ in the level of expression of CD56 and CD16 but they also have distinct functional features. CD56<sup>bright</sup> NK cells constitute a smaller fraction of the total number of NK cells (9) and have traditionally been considered to modulate the immune reaction by secreting cytokines attracting other immune cells to a site. CD56<sup>dim</sup> cells, on the other hand, make up about 90% of the NK cells and have been ascribed cytotoxic ability to attack and lyse aberrant cells. They can do this without any prior sensitization owing to the presence of germline-encoded activating and inhibitory receptors on their surface (7,10–13). The presence or absence of cognate ligands on the target cell determines in a complex fashion whether the NK cell will release its cytotoxic granules and kill the target cell. CD107a expression on the NK cell surface is a marker for degranulation

and by measuring this using flow cytometry the cytotoxic activity can be assessed at the single-cell level (14).

### ***Integration of activating and inhibitory signals guiding the NK cell behavior***

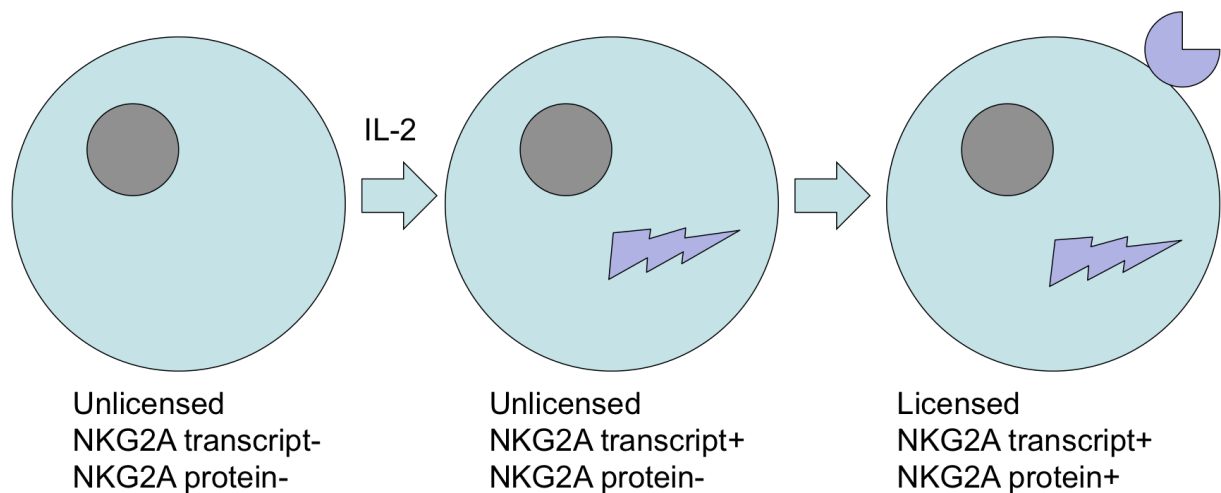
Through surface receptors, NK cells are inhibited by major histocompatibility (MHC) class I molecules expressed on most normal healthy “self” cells. This interaction keeps the NK cells calm in physiological conditions. Virus infected and aberrant cells that do not express “self” MHC class I molecules are recognized and eliminated by NK cells. This is the basis of the “missing self” theory.

A complex intracellular integration of signals from inhibitory and activating receptors determines the magnitude of NK-cell mediated cytotoxicity and cytokine production (11–13). Inhibitory killer-cell immunoglobulin-like receptors (KIRs) constitute a major family of inhibitory receptors. They bind to MHC class I molecules represented by several of the human leukocyte antigen (HLA) class I molecules in humans. Natural Killer Cell Receptor Group 2 Member A (NKG2A) acts together with CD94 as an inhibitory receptor that appears on NK cells and recognizes the non-classical MHC class I molecule, HLA-E. Expression of HLA-E requires expression of the other MHC class I molecules because it presents the leader peptides derived from classical MHC class I molecules. The level of HLA-E thus indicates the expression level of classical MHC class I. Through NKG2A, NK cells can monitor these levels and if they are low the “missing self” response is triggered. Many cancers, including both solid tumors and leukemia/lymphoma, upregulate the expression of HLA-E . By blocking NKG2A pharmacologically, the inhibition of NK cells is lowered and HLA-E is then available for binding to the activating receptor NKG2C, thus increasing the activation of the

NK cell. The monoclonal antibody Monalizumab works this way and has shown efficacy against HLA-E-overexpressing tumor cells in both solid and hematological cancers (16).

In addition to inhibiting NK cell activation, the interaction of inhibitory receptors with their cognate ligands on healthy cells also renders NK cells more responsive to activating signals (17). This process is called licensing. For this to happen at least one inhibitory receptor must be expressed that recognize self-HLA class I molecules. Without this, the NK cell will be unlicensed, i.e. hyporesponsive, under steady state. It will however carry an autoreactive potential that can be activated through perturbations of the immune homeostasis, such as autologous transplantation, treatment with monoclonal antibodies and viral infections (18).

Stimulation of unlicensed NK cells with IL-2 causes transcription of the NKG2A gene and results in expression of NKG2A on the cell surface (Figure 3).



*Figure 3 Steps toward NKG2A expression after IL-2 stimulation of NK cells.*

Transcript-positive unlicensed cells constitute a subset of NK cells that are activated, but not yet expressing any inhibitory receptors. This cell population may be an important effector population during immunotherapy because of its potential cytotoxic power without the

inhibition of licensed cells. Identifying the specific characteristics of this cell population would be advantageous for developing future therapies.

## Cancer Immunotherapy

Malignant tumors are capable of activating negative regulatory pathways, also called checkpoints, associated with immune homeostasis, effectively suppressing immune responses (15). The purpose of immunotherapy is to somehow induce or reactivate the immune system to recognize and eliminate virus infected and malignant cells. One type of immunotherapy called checkpoint inhibition relies on eliciting a dynamic anticancer response aimed not only at a single malignant derangement or specific features of cancer cells. Therefore many of the aberrations distinguishing cancer cells from healthy cells can be targeted simultaneously (19).

One common way for cancer cells to evade the immune system is, like some viruses, to downregulate the expression of MCH class I molecules so that antigens are not presented to cytotoxic T cells, reducing their activation. However, this will allow for NK cell activation because of the lower inhibitory signal they get. For example, chronic myeloid leukemia (CML) patients in remission after treatment with tyrosine kinase inhibitors had a significantly better clinical outcome after discontinuing their medication if they had a high proportion NK cells (20). In non-small cell lung cancer (NSCLC), clear cell renal cancer and colorectal cancer, infiltration of NK cells into tumors is associated with better prognosis (15).

Other immune subversion mechanisms employed by cancers include production of immunosuppressive factors or regulatory cytokines and expression of immune checkpoint molecules. Immune checkpoint molecules have the ability to inhibit immune cells, T cells in particular, from attacking cancer cells. By pharmacologically blocking immune checkpoints

using antibodies the inhibition can be avoided, thus enabling T cells' function to kill cancer cells. The T cell functions have been shown to decrease in association with enhanced expression of immune checkpoints like programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), T cell immunoglobulin and mucin-domain containing 3 (TIM-3), and lymphocyte-activation gene 3 (LAG-3). The roles of these molecules in NK cells have been much less studied (16). That is why these four immune checkpoints, together with inhibitory receptor NKG2A are the focus of this thesis. The current knowledge of these molecules is summarized below.

### **PD-1**

PD-1 is expressed on activated T and B lymphocytes. Its ligands PD-L1 and PD-L2 are expressed on tumors, infected cells and APCs in inflammatory infiltrates (19). It is involved in the termination of immune responses by T and B cells and deficient expression of PD-1 is associated with autoimmunity. Blocking PD-1 during cancer treatment can also cause autoimmune manifestations. PD-1 is upregulated after prolonged T cell receptor (TCR) stimulation and the receptor/ligand interactions hamper the T cell which becomes exhausted. PD-1 has also been shown to be a negative regulator of NK cells and is present most often on CD56<sup>dim</sup>CD16<sup>+</sup> cells. Blocking PD-1 has emerged as a promising treatment of various cancer types although only a minority of patients respond strongly to monotherapy with 10-40% reduction in tumor volume (15). It is considered to rely heavily on the enhanced effector functions of tumor-specific T cells. However, blocking PD-1 also reverts the NK cell function defects induced by PD-1/PD-L1 interactions, indicating that PD-1 is involved in NK cell exhaustion and not a mere marker of activation (16). The phenotypic and functional

characteristics of PD-1<sup>+</sup> NK cells have not been studied in depth, justifying the development of reliable methods for analyzing these cells.

### **CTLA-4**

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) also acts as an immune checkpoint molecule on T cells (19). It is an inhibitory receptor, which is upregulated upon T cell activation during chronic viral infections and tumors. This helps tumor immune escape by hampering effector functions of T cells. Lack of CTLA-4 in animal models is associated with aggressive and fatal autoimmune diseases, indicating its function in regulating immune homeostasis (16).

CTLA-4 has been shown on mouse NK cells activated by IL-2 and in mouse tumors, but the knowledge about CTLA-4 expression on human NK cells is limited. The effect of cancer treatment with anti-CTLA-4 therapy on NK cells would be beneficial to elucidate. By targeting regulatory T (Treg) cells and improving T cell IL-2 production, anti-CTLA-4 treatment could possibly also lead to NK cell anti-tumor immune response (16).

### **TIM-3**

T-cell immunoglobulin and mucin domain 3 (TIM-3) was first described on activated T cells but is also expressed on NK cells. It is a co-inhibitory receptor important in negatively regulating proinflammatory responses to avoid excessive host damage. It also mediates T cell exhaustion during cancer and chronic viral infections. Highly exhausted CD8 T cells express both PD-1 and TIM-3 indicating that these receptors cooperate to inhibit T cell functions. By blocking TIM-3 in tumor models, T cell functions have been revived leading to better control of tumor growth. In advanced melanoma patients, increased expression of TIM-3 on

circulating NK cells has been reported to correlate with exhaustion of these cells. Blocking TIM-3 signaling with monoclonal antibodies in lung adenocarcinoma increases the cytotoxicity and IFN- $\gamma$  (15). On NK cells TIM-3 acts as a marker of maturation and/or activation. The function is however uncertain, showing signs of both activating and inhibitory effects depending on experimental or clinical setting. Because of this, further studies are needed to understand if and how blocking TIM-3 on NK cells can help restore immune surveillance in cancer (16).

### **LAG-3**

Lymphocyte Activation Gene-3 (LAG-3) appears on activated T and NK as well as several other immune cells and is an inhibitory receptor. It binds MHC class II molecules and has diverse biological effects (15). For example, it has been shown to be involved in T cell exhaustion in various cancers and chronic infections. Blocking LAG-3 improves T cells functions against several cancers and chronic infections, especially in combination with PD-1 blockade. The effect of LAG-3 on NK cells has not been thoroughly studied and there is inconclusive data regarding its role in NK cells. Antibodies to LAG-3 and soluble LAG-3 has no effect on NK cell cytotoxic capability but low NK cell expression of LAG-3 is associated with viral control in HIV patients (16).

### **Aim**

The aim of this project was to develop a method for transcript analyses of genes encoding NK cell immune checkpoint receptors and NKG2A which may be used to gain further knowledge of the mechanisms underlying NK cell regulation and licensing.



The aim was further to apply the method to study the response of transcript-positive NK cells with and without prior IL-2 stimulation after exposure to leukemic K562 cells.

## **Material and Methods**

### **Instrumentation**

All cell sorting was performed using a 3-laser FACSAria III flow cytometer (405, 488 and 633 nm; BD Biosciences, San Diego, CA, USA). Reverse transcriptions were performed using a BioRad T100 thermal cycler. The qPCR operations were performed using a BioRad CFX384 Touch™ Real-Time PCR Detection system.

### **qPCR Assay Design**

To find as good PCR primer pairs as possible a number of web applications were used. With the help of the NCBI Gene web page (21) genes with as many verified (NM) and predicted (XM) splice variants as possible were identified. Primer-BLAST (22) was then used to find suggestions of primer pairs for these genes. Finally, the NetPrimer (23) web page was used to calculate the theoretical quality of the primer pairs, i.e. the tendency to form hairpins and self and cross complementarity as well as other parameters.

A pool of cDNA material was created to test the primer pairs. PBMCs had previously been isolated from buffy coat from healthy donors. 10e6 PBMCs were mixed with IL-2 and IL-15 (10 ml of IMDM with 10% P9 medium containing IL-2 at a concentration of 100 U/ml and IL-15 at 10 ng/ml) and incubated for 5 days at 37°C. Using the RNEasy Plus Mini Kit from QIAGEN according to the manufacturer's instructions, the RNA from the cells was purified.

Finally, the cDNA was created by running reverse transcription (RT) on the RNA using the TATAA GrandScript cDNA Synthesis Kit. To the wells of a 96-well plate was added 5  $\mu$ l nuclease free water, 4  $\mu$ l TATAA GrandScript cDNA Synthesis Mix, 1  $\mu$ l TATAA GrandScript RT reverse transcription enzyme and 10  $\mu$ l of the purified RNA. RT enzyme was replaced with nuclease free water in a number of wells. These wells are used as controls and are denoted no reverse transcription (RT-) wells. The plate was then run in the thermal cycler to create cDNA.

The primer pairs with the best theoretical values were evaluated by running qPCR on the cDNA material created. Each primer pair was evaluated on 9 wells with 6  $\mu$ l reactions (3  $\mu$ l of TATAA SYBR GrandMaster Mix, 0.24  $\mu$ l of 10  $\mu$ M primer pair mix, 0.76  $\mu$ l of nuclease free water and 2  $\mu$ l of cDNA material). Three normal wells contained the cDNA material previously created, two no reverse transcript (NRT) control wells contained RT- material and four no template control (NTC) wells contained nuclease free water instead of cDNA material. Quantitation cycle (C<sub>q</sub>) is the qPCR cycle in which fluorescence can be detected. It is inverse to the amount of target nucleic acid and correlates to the number of target copies in the sample (24). The acceptance criteria were a stable signal in the normal wells and a C<sub>q</sub> value over 34 for the NRT and NTC wells. This number represents the presence of a single copy of the target DNA sequence.

To verify the uniqueness and correct length of each product, the contents of the wells were also analyzed using gel electrophoresis.

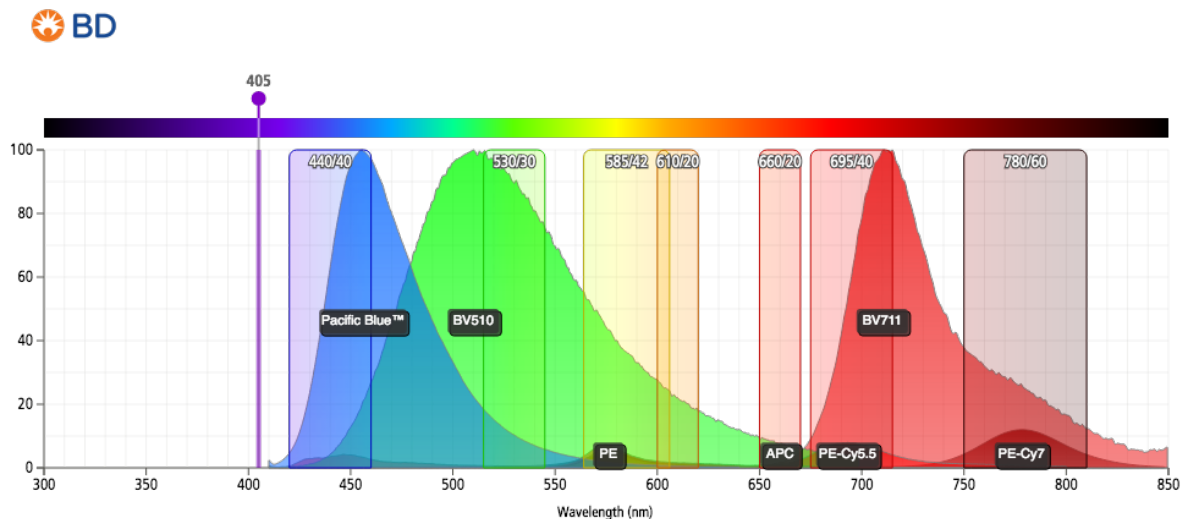
## FACS Panel

Table 1 shows the FACS panel used for sorting the stained cells using the FACSAria III flow cytometer.

*Table 1 FACS panel used for the sorting*

Marker	Comment	Fluorochrome	Laser
<b>CD56</b>	NK cell marker	BV711	Violet 405
<b>NKG2A</b>		PE	Blue 488
<b>KIR2DL2/L3</b>		PeCy5.5	Blue 488
<b>KIR2DL1/S1</b>	CD158	PeCy7	Blue 488
<b>KIR3DL1</b>		APC	Red 633
<b>CD107a</b>	Degranulation	BV510	Violet 405
<b>Cell stain</b>	K562 cells	Cell Trace Violet	Violet 405

For each of the lasers, the overlaps of the fluorochromes were analyzed using the BD Biosciences Spectrum Fluorescence Viewer (Figure 4, Figure 5 and Figure 6).



*Figure 4 FACS panel spectral overlaps for the 405 nm laser*

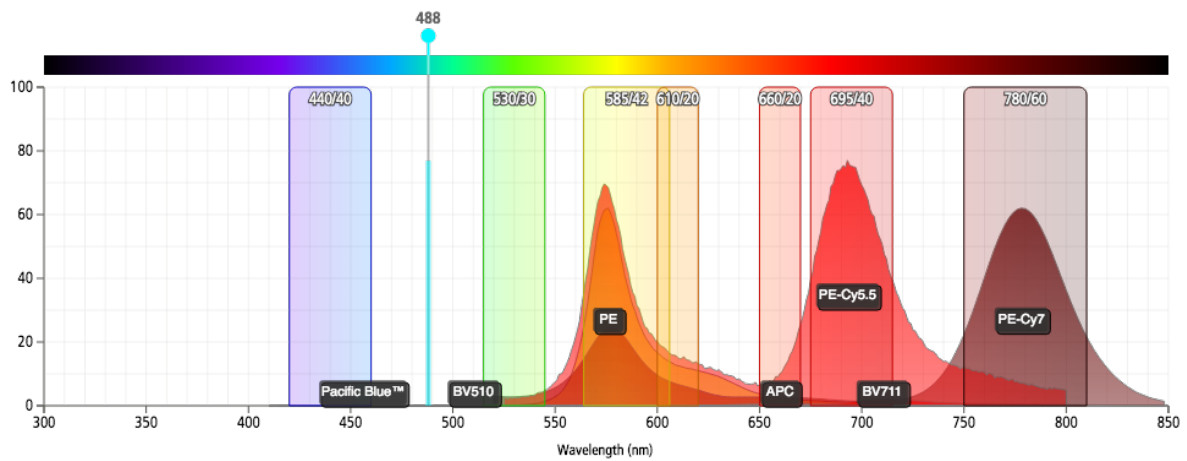


Figure 5 FACS panel spectral overlaps for the 488 nm laser

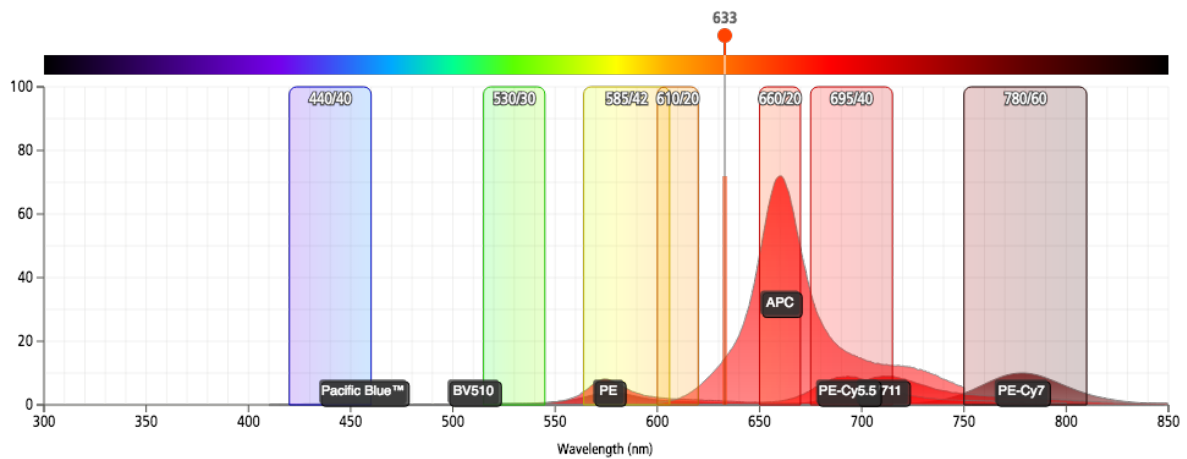


Figure 6 FACS panel spectral overlaps for the 633 nm laser

## Laboratory Measurements

The assays developed were used to measure the difference in immune checkpoint receptor expression between those NK cells having been stimulated by IL-2 and those that have not after being exposed to K562 CML cells.

## **Cell Preparation**

A cryopreserved sample of PBMC isolated from a patient with HLA-typed NK cells was thawed, mixed with pooled human serum (P9) and washed in buffered saline containing 0.5% BSA and 0.1% EDTA (referred to as elutriation buffer, E-buffer). The cells were then dyed for T cell marker CD3 and NK cell marker CD56 and analyzed with an Accuri C6 flow cytometer to determine the NK cell content. Following the protocol of the NK Cell Isolation Kit from Miltenyi Biotec the NK cells were isolated. They were then washed in E-buffer, counted, analyzed for NK cell content like above and washed in medium (IMDM with 1% penicillin and 10% Fetal Calf Serum). On a 96-well plate the NK cells were added to two wells, one of which contained 500 U/ml IL-2. The plate was then incubated at 37°C for 48 hours. Thereafter, cells of the K562 leukemic cell line dyed with Cell Trace Violet was added to both wells in a 1:1 ratio relative to the NK cells and incubated for four hours in presence of CD107a antibody. The plate was centrifuged and the supernatant discarded. Fluorochrome-conjugated antibodies of the FACS panel were added to the wells and incubated for 30 minutes in a fridge. The cells were washed to remove any free antibodies before being resuspended in NaCl and moved to two FACS tubes, one with cells stimulated by IL-2 and one with unstimulated cells.

## **Sorting of Cells**

Two 96-well plates were prepared with 5 µl lysis buffer per well. The IL-2 stimulated and unstimulated cells were sorted to different plates with the same plate layout using the FACSAria III flow cytometer. 100 cells were sorted to each well. Living NK cells with the correct size and granularity were selected using gates on forward scatter, side scatter and CD56.

The K562 cells were gated out. NK cells that had degranulated were identified as cells expressing the granule marker CD107a. 100 NK cells of each subset were sorted by combining gates for NKG2A and the three KIRs using logical gating (illustrated in Figure 7) into the groups shown in Table 2.

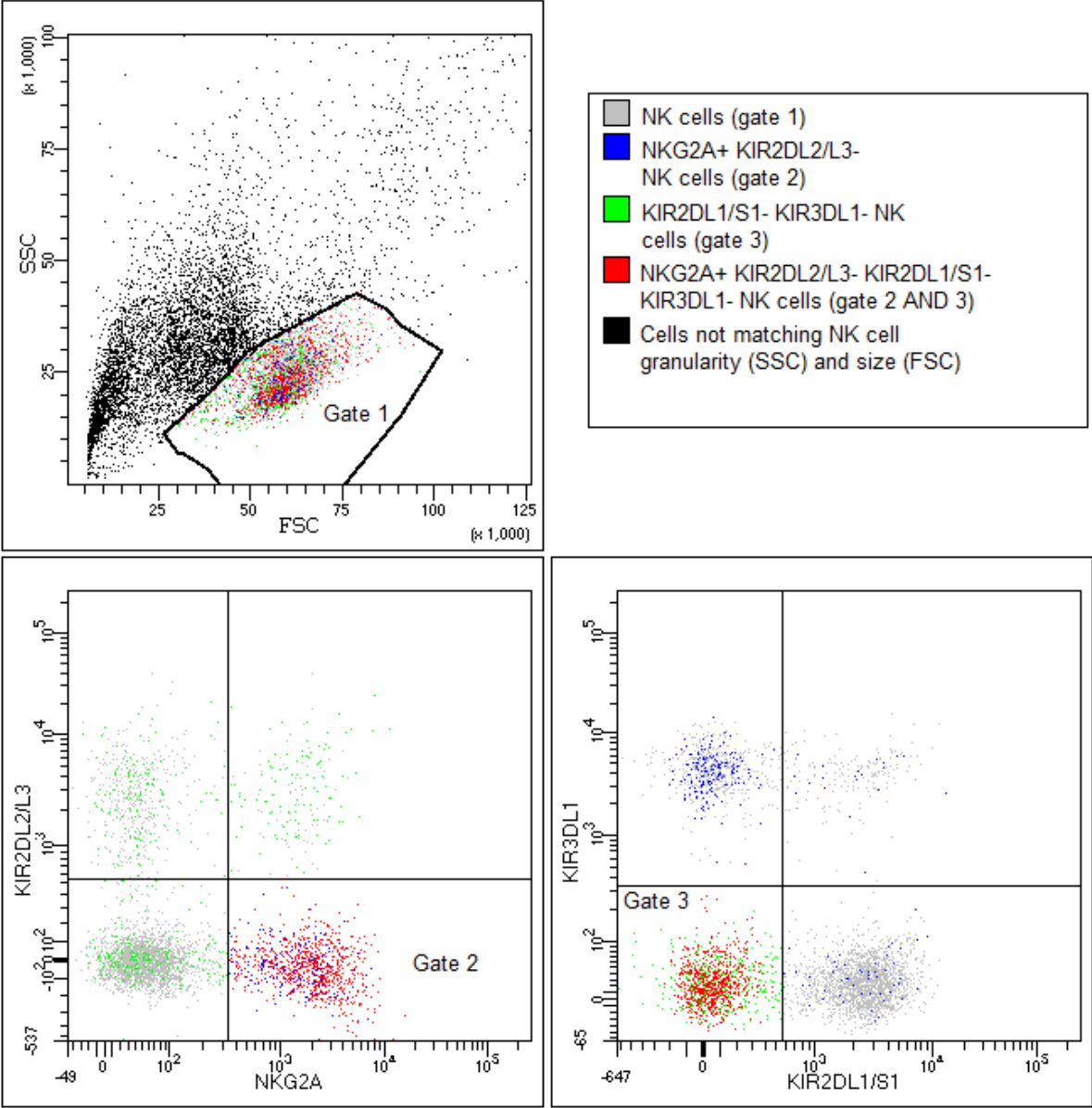


Figure 7 Dot plots illustrating how FACS gates are used for identifying NK cell subsets based on the amount of NKG2A and KIRs on the cell surface. The image shows how the NKG2A<sup>+</sup> KIR<sup>-</sup> NK cell population is identified using logical gating.

Table 2 NK cell subsets studied and the abbreviations used in this thesis

<b>NK cell subsets</b>	<b>Abbreviation</b>
<b>No NKG2A and no KIR</b>	N-K-
<b>NKG2A and no KIR</b>	N+K-
<b>NKG2A and one KIR</b>	N+K1
<b>No NKG2A and one KIR</b>	N-K1
<b>No NKG2A and two KIRs</b>	N-K2

Every combination of subset and CD107a status (CD107a<sup>+</sup> or CD107a<sup>-</sup>) was sorted to four wells each. After sorting the plate was stored in a -80°C freezer.

### **Reverse Transcription**

To be able to analyze the amount of relevant NK cell mRNA it was reverse transcribed into cDNA using the TATAA GrandScript cDNA Synthesis Kit. To each well of the sorted 96-well plate was added 2.5 µl nuclease free water, 2 µl TATAA GrandScript cDNA Synthesis Mix and 0.5 µl TATAA GrandScript RT reverse transcription enzyme. To one of the four wells of each combination of NK cell subset and CD107a status, RT enzyme was replaced with nuclease free water to create NRT wells.

The recommended thermal cycling program from TATAA was run (22°C for 5 min, 42°C for 30 min, 85°C for 5 min and hold at 4°C). Thereafter 20 µl of nuclease free water was added to each well and the plate was stored in a -18°C freezer.

### **Quantitative Polymerase Chain Reaction**

The amount of cDNA for our five immune checkpoints of interest was analyzed using qPCR. Each well of a 384-well plate was prepared with 3 µl TATAA SYBR GrandMaster Mix, 0.24 µl 10 µM forward and reverse primer, 0.76 µl nuclease free water and 2 µl cDNA. The cDNA was replaced with corresponding RT negative material for the NRT wells and with nuclease

free water for the NTC wells. Three cDNA wells and one NRT well were used per cell type and gene and three NTC wells were used per gene. The data was collected using the software Bio-Rad CFX Maestro.

## **Ethics**

All experiments were performed using blood cells collected from blood donors. Blood donors give a general consent to the use of blood cells in research. Since the connection between blood donor and buffy coat is removed, no further ethical review of experiments using blood cells is needed according to Swedish law.

## **Data Collection Procedures**

The qPCR data collected by the qPCR software Bio-Rad CFX Maestro was exported to Excel and analyzed. The Cq value for each assay was calculated as the average of the Cq values of the three wells of the assay. Since the Cq value represents the number of PCR cycles, i.e. the number of doubling of genetic material, a relative value for the amount of product of the gene in question was calculated as  $2^{(40-Cq)}$ .

## **Missing and Extreme Data Values**

Missing or very high Cq values of a single well have been adjusted to 36, indicating low expression, in order not to ruin the average Cq value.

Wells with no melt temperature values, thus indicating a very low or no expression or an error, have been adjusted to Cq value 38. This value represents a very low expression of the gene in question and means that the lowest relative expression marker value is  $2^{(40-38)} = 4$ .



# Results

## qPCR Assay Design

For the assays to be considered acceptable a number of criteria must be met. Preferably only the desired product with its expected length should be amplified during the qPCR process. As little amplification as possible of the primers themselves should occur, a concept called primer dimers. A low C<sub>q</sub> value in the NTC wells may be a sign of this. The NRT wells contain no cDNA from mRNA and a low C<sub>q</sub> value here could indicate that the primers are picking up genomic DNA which is not desirable. In addition the melt temperature should be approximately the same across all wells containing reverse transcribed mRNA material. Table 3 shows results from the qPCR of the primer tests. The primers that were evaluated are listed in the Appendix.

*Table 3 qPCR results of primer tests*

<b>Primer pair</b>	<b>Mean C<sub>q</sub></b>	<b>Min NRT C<sub>q</sub></b>	<b>Min NTC C<sub>q</sub></b>	<b>Mean Melt Temperature</b>	<b>Melt Temperature Spread</b>
<b>CTLA4</b>	24.52	40.97	35.13	77.83	0.50
<b>PD-1v1</b>	30.86	34.21	35.69	84.17	0.50
<b>PD-1v2</b>	31.53	38.47	36.20	85.00	0.00
<b>TIM-3</b>	25.05	47.56	32.77	79.50	0.00
<b>LAG-3v1</b>	23.86	29.66	29.54	82.00	0.00
<b>LAG-3v2</b>	24.86	No target detected	38.61	80.00	0.00
<b>LAG-3v3</b>	24.57	42.91	42.62	82.00	0.00
<b>NKG2Av1</b>	25.06	46.13	39.28	76.00	0.00
<b>NKG2Av2</b>	25.15	33.34	33.49	76.50	0.00
<b>NKG2Av3</b>	22.83	35.61	34.70	76.33	0.50

The results of running the qPCR products on gel electrophoresis can be seen in Figure 8. Both wells for PD-1 v1 show some bands in addition to the expected one. The other primer tests show distinct single bands.

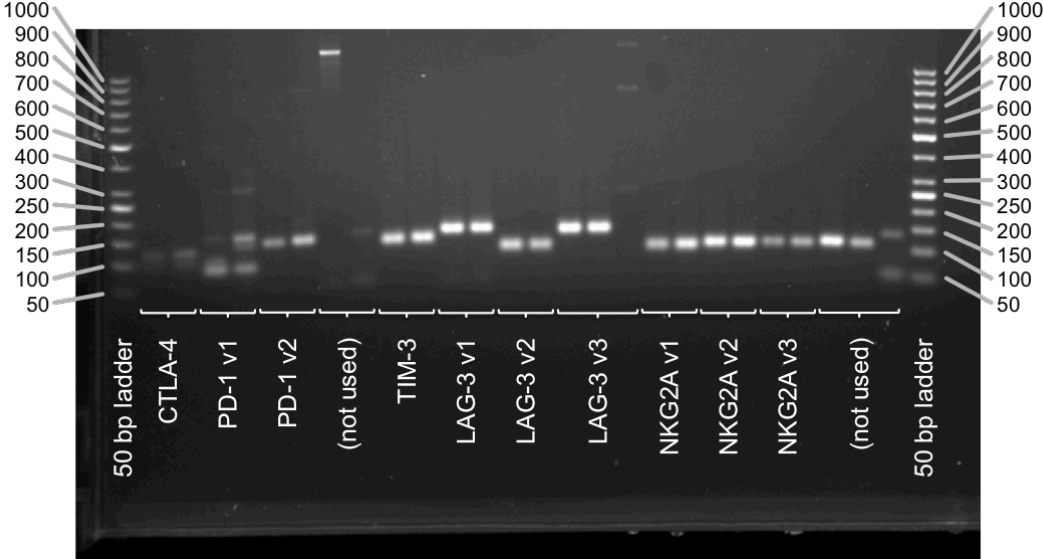


Figure 8 Results of running the qPCR products on gel electrophoresis

The lengths of the DNA segments duplicated by the primer pairs found using gel electrophoresis are shown in Table 4. The observed length is without exception slightly greater than the expected length. On average, the observed length is 23.5% greater, varying between 6.8% and 35.4%.

*Table 4 Observed and expected lengths of qPCR products for the different primer pairs*

<b>Primer pair</b>	<b>Observed length (base pairs)</b>	<b>Expected length (base pairs)</b>
CTLA-4	125	117
PD-1v1	85	77
PD-1v2	150	132
TIM-3	150	121
LAG-3v1	175	140
LAG-3v2	130	101
LAG-3v3	170	136
NKG2Av1	130	96
NKG2Av2	135	100
NKG2Av3	135	103

#### **CTLA-4**

Only one primer pair with promising theoretical properties was found for CTLA-4. The qPCR results are shown in figure 4. PTC wells are all well separated from NRT and NTC regarding cycles. The C<sub>q</sub> values of the NRT and NTC wells are all above the acceptance criteria of 34. The corresponding melt peaks are also distinct (Figure 9). The gel electrophoresis indicates a single product with a length close to the expected (Table 4).

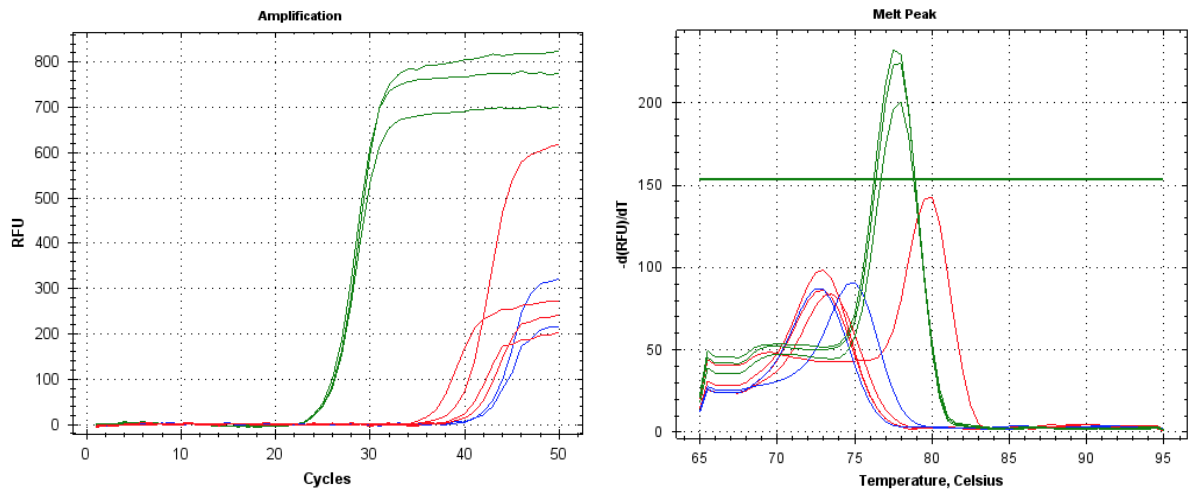


Figure 9 Amplification and melting curves for CTLA-4. cDNA, NTC and NRT lines are green, red and blue, respectively.

## PD-1

Two primer pairs denoted PD-1v1 and PD-1v2 with satisfactory theoretical properties were discovered and their amplification and melting curves are shown in Figure 10 and Figure 11, respectively. The amplification curves for neither PD-1v1 nor PD-1v2 are well separated. All NRT and NTC Cq values are above 34. The PTC melt peaks of PD-1v1 are more distinct than those of PD-1v2, although according to the gel electrophoresis in Figure 8 the product amplified by PD-1v2 is more specific and of the expected length.

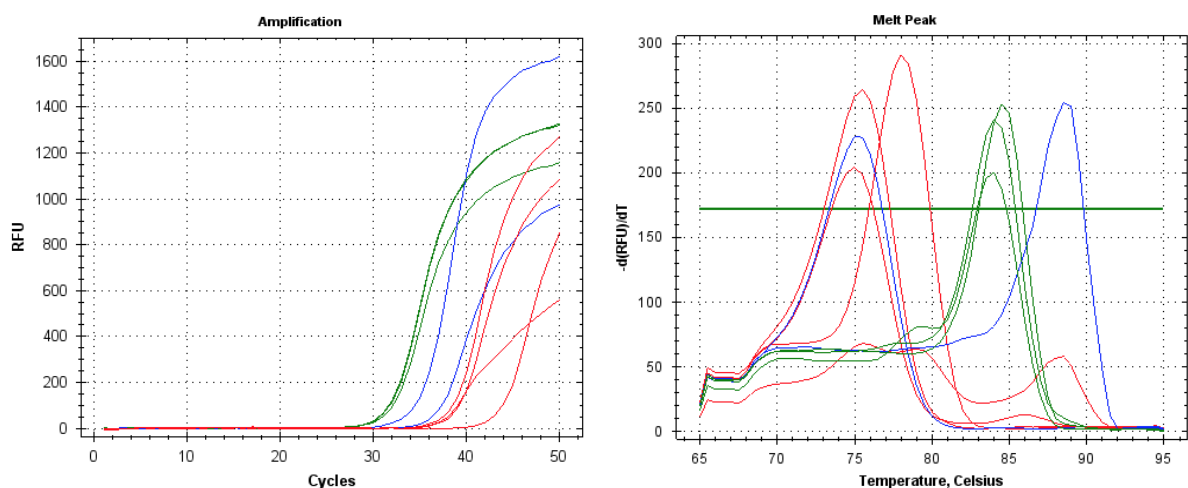


Figure 10 Amplification and melting curves for PD-1v1. cDNA, NTC and NRT lines are green, red and blue, respectively.

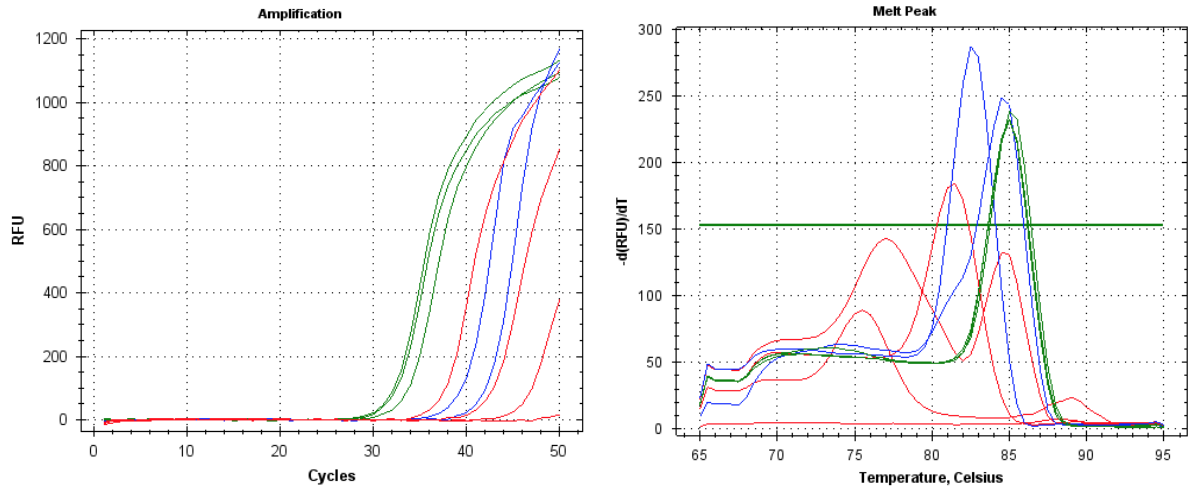


Figure 11 Amplification and melting curves for PD-1v2. cDNA, NTC and NRT lines are green, red and blue, respectively.

### TIM-3

One auspicious primer pair was identified for TIM-3. Cycle separation, melt peaks and gel electrophoresis indicate a clear-cut product with the expected length and low expression in the NRT and NTC wells Figure 12 and Table 4. One NTC Cq value is 32.77, that is below the acceptance criteria. However, the expression in the cDNA wells is far greater with a mean Cq value of 25.05.

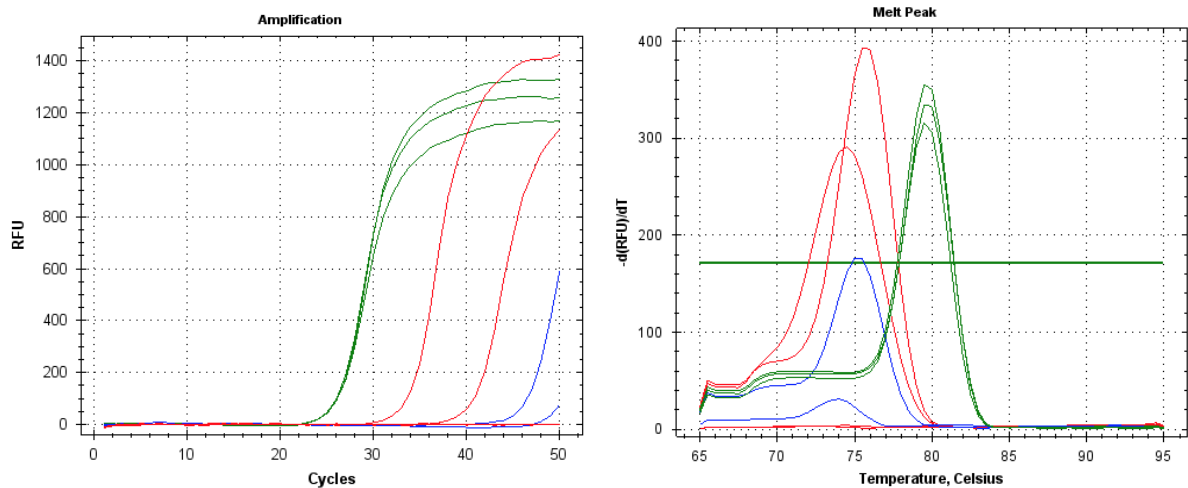


Figure 12 Amplification and melting curves for TIM-3. cDNA, NTC and NRT lines are green, red and blue, respectively.

### LAG-3

Three primer pairs denoted LAG-3v1, LAG-3v2 and LAG-3v3 with promising theoretical properties were found. All three have unambiguous cycle separation, melt peaks and product lengths (Figure 13, Figure 14, Figure 15, Figure 8 and Table 4). The NTC and NRT C<sub>q</sub> values for LAG-3v1 are all below 34 thus not meeting the acceptance criteria.

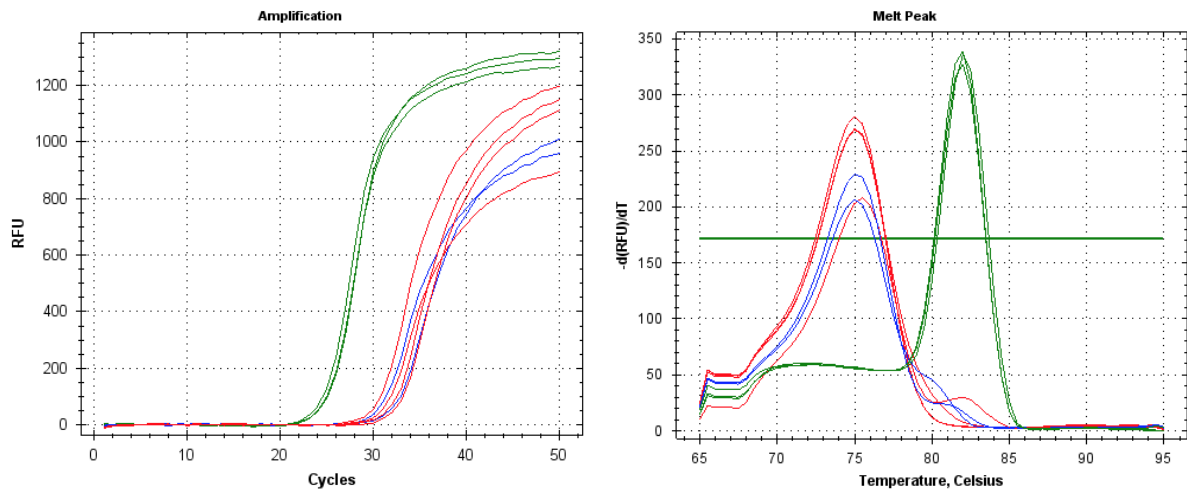


Figure 13 Amplification and melting curves for LAG-3v1. cDNA, NTC and NRT lines are green, red and blue, respectively.

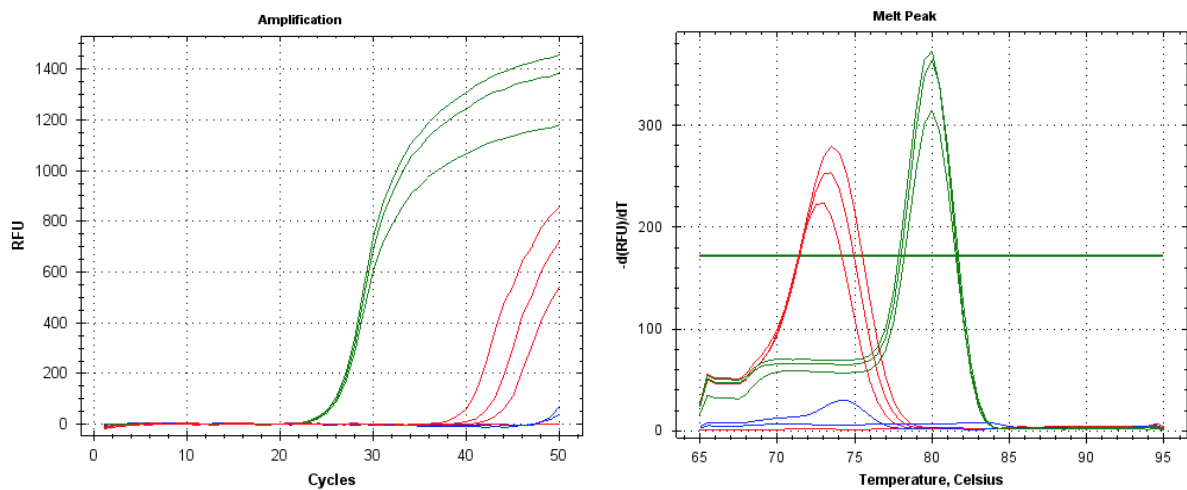


Figure 14 Amplification and melting curves for LAG-3v2. cDNA, NTC and NRT lines are green, red and blue, respectively.

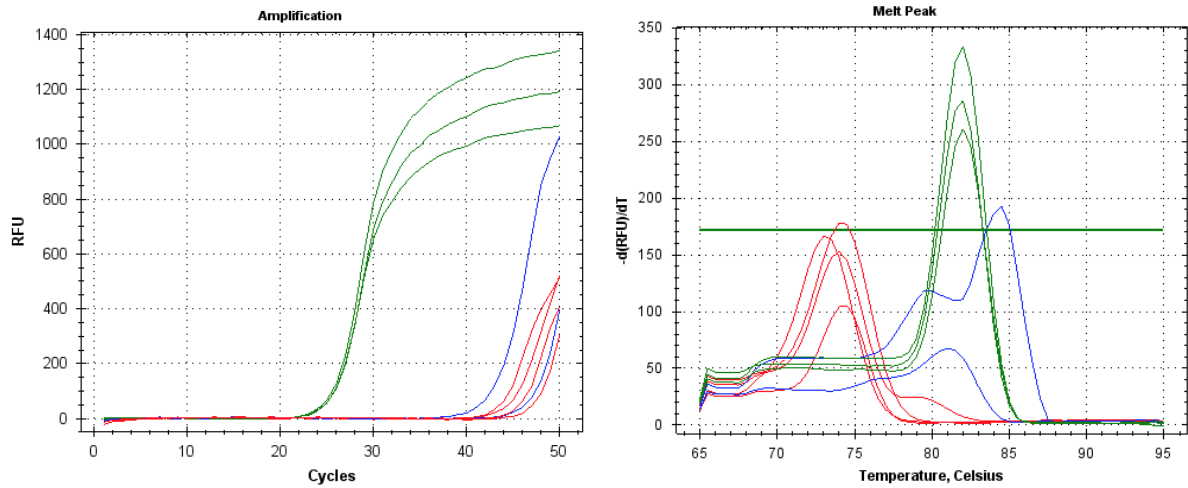


Figure 15 Amplification and melting curves for LAG-3v3. cDNA, NTC and NRT lines are green, red and blue, respectively.

## NKG2A

The theoretical evaluation of potential primers resulted in three candidates. Both NKG2Av1 and NKG2Av2, but not NKG2Av3, were shown to have distinct amplification and melt curves in addition to the expected length. The NTC and NRT Cq values are above 34 for all three primer pairs (Figure 16, Figure 17, Figure 18, Figure 8 and Table 4).

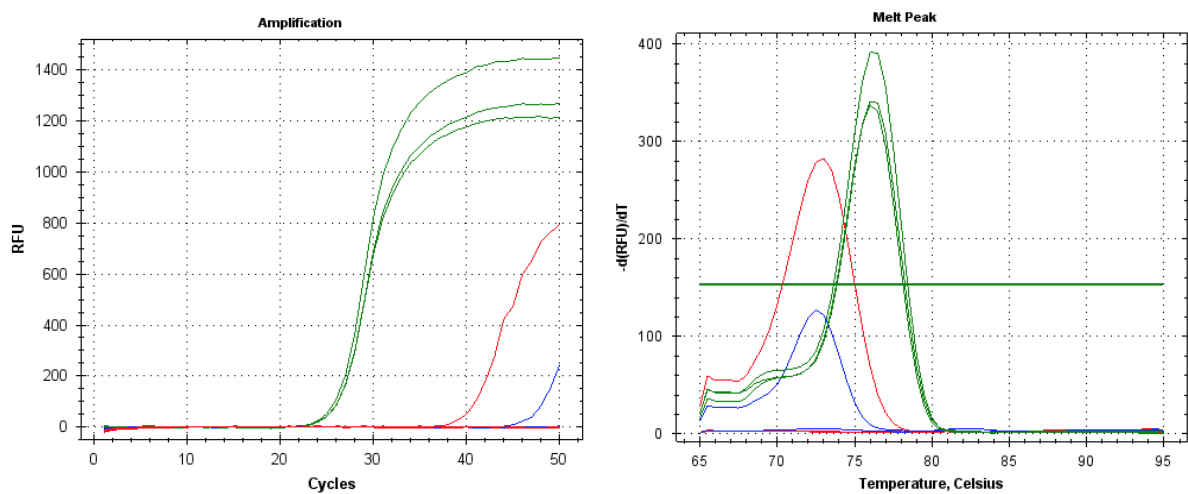


Figure 16 Amplification and melting curves for NKG2Av1. cDNA, NTC and NRT lines are green, red and blue, respectively.

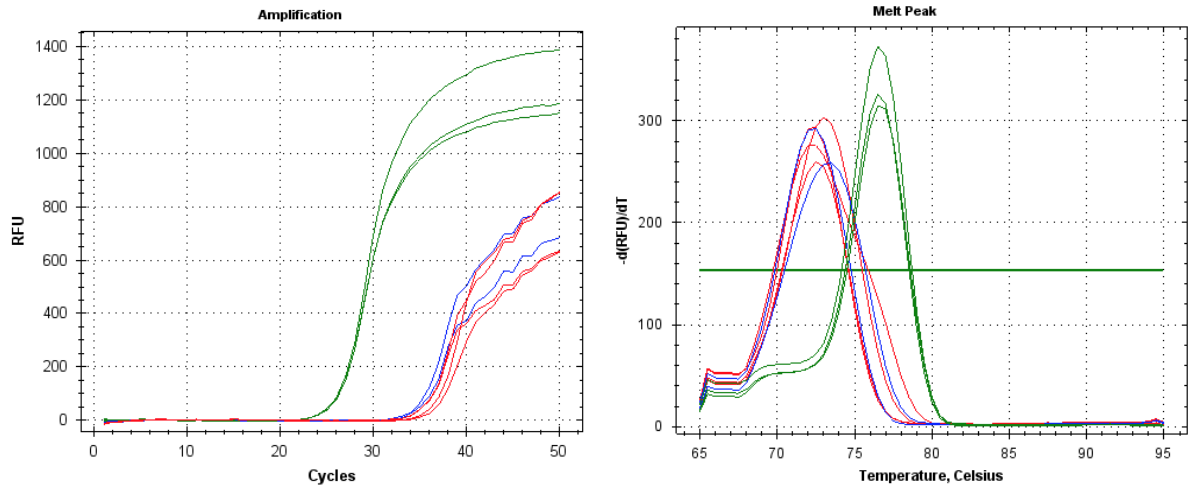


Figure 17 Amplification and melting curves for NKG2Av2. cDNA, NTC and NRT lines are green, red and blue, respectively.

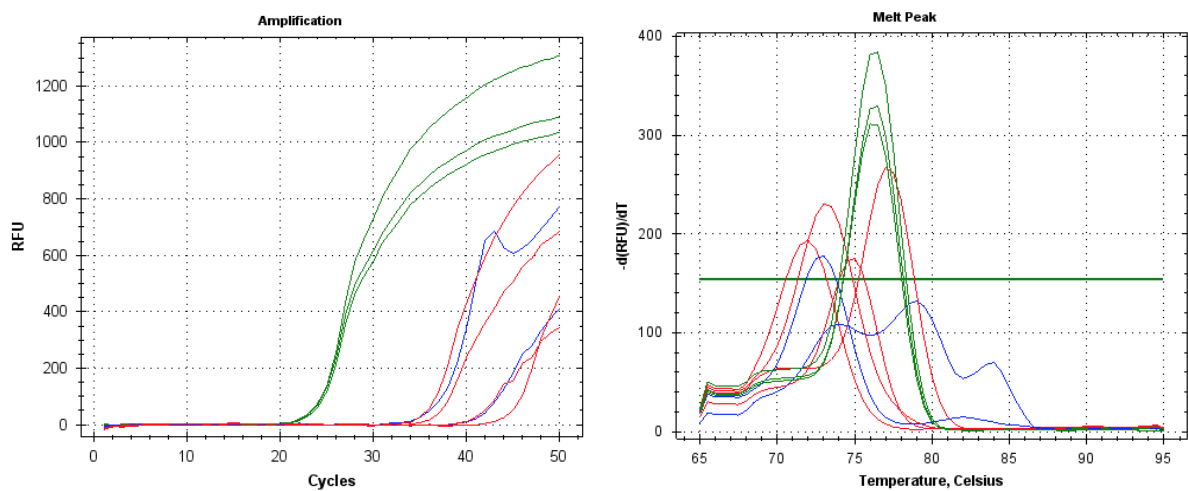


Figure 18 Amplification and melting curves for NKG2Av3. cDNA, NTC and NRT lines are green, red and blue, respectively.

## Analysis of immune checkpoint expression on CML-exposed NK cells

The qPCR assay design in the first part of the project resulted in verified methods for analysing the transcript expression of genes for all four checkpoint inhibitor as well as NKG2A. In this second part of the project those methods were applied to NK cells exposed to leukemic K562 cells to study the reaction and identify patterns of checkpoint inhibitor and NKG2A transcript expression in different NK cells subsets.



## FACS Analysis Results

The relative amount of product for each immune checkpoint receptor is presented graphically in respective bar chart. The fraction of NK cells that degranulated after exposure to K562 cells was 44.6% and 37.1% with and without IL-2 treatment, respectively. The NK cell fraction expressing NKG2A increased from 27.9% to 31.2% with IL-2 treatment and the shift towards NKG2A<sup>+</sup> subtypes is shown in Figure 19.

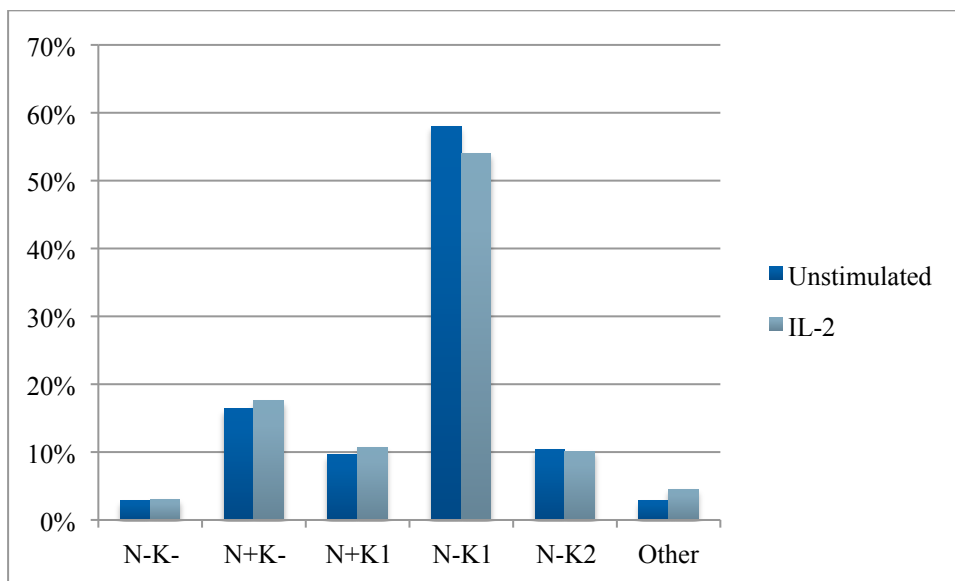


Figure 19 Distribution of NK cell subsets after exposure to K562 cells depending on IL-2 stimulation.

## qPCR Results

### CTLA-4

No or very low expression of CTLA-4 could be shown in all of the NK cell subsets regardless of IL-2 stimulation or NKG2A/KIR expression (Figure 20). Unstimulated cells possibly express slightly more CTLA-4 transcript than IL-2 stimulated cells. Similarly, cells that degranulate express slightly more than non-degranulating cells. The differences are however miniscule.

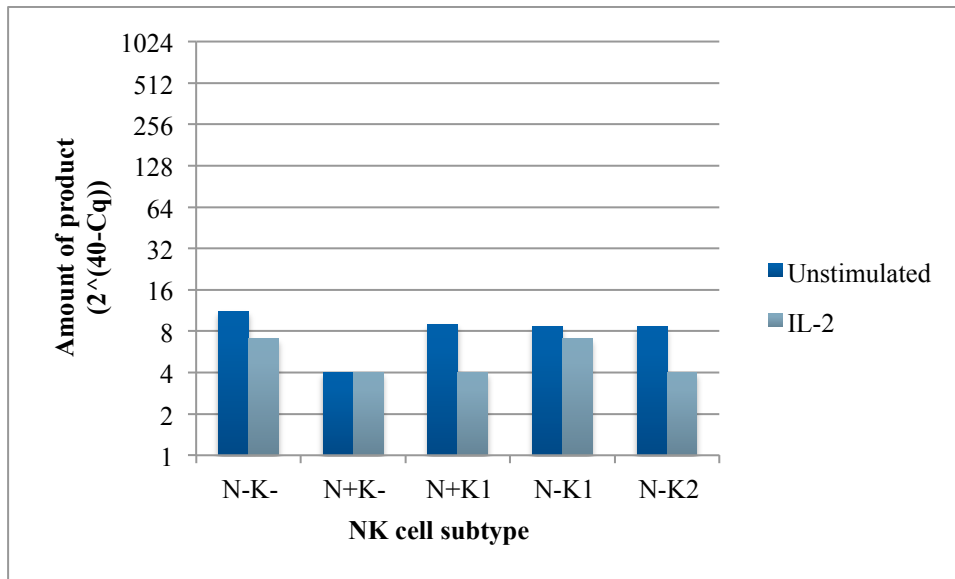


Figure 20 Relative amount of CTLA-4 expression of NK cells after exposure to K562 cells.

### PD-1

No expression of PD-1 could be shown in any of the NK cell subsets regardless of IL-2 stimulation or NKG2A/KIR expression (Figure 21).

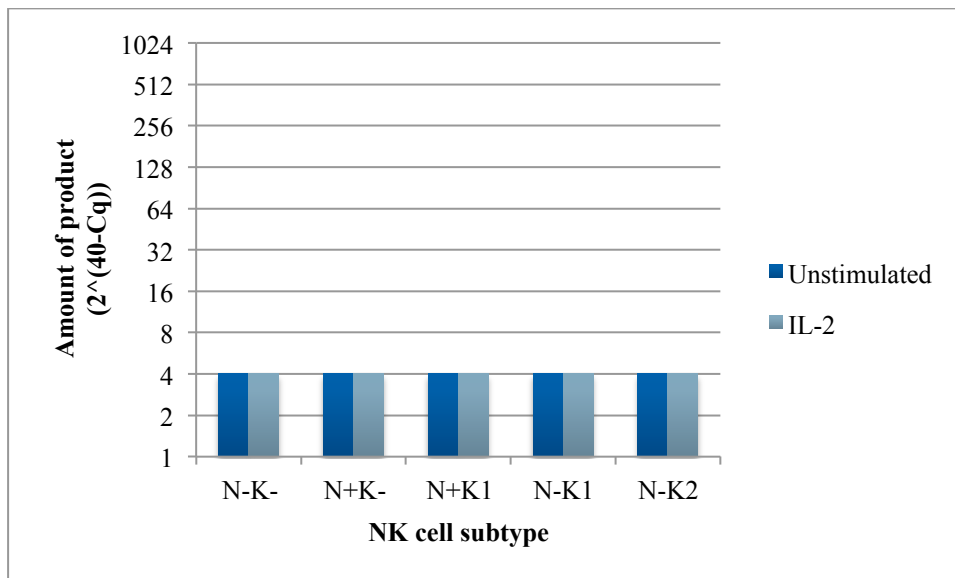


Figure 21 Relative amount of PD-1 expression of NK cells after exposure to K562 cells.

### TIM-3

All NK cell subsets contain transcript for TIM-3. In all subsets but NKG2A<sup>-</sup> KIR<sup>-</sup> cells the effect of IL-2 treatment is an increased expression of TIM-3. Among the IL-2 stimulated cells, the immature NKG2A<sup>-</sup> KIR<sup>-</sup> subset shows the lowest level of expression (Figure 22).

There is no apparent correlation between degranulation status and the level of TIM-3 transcript (Figure 23).

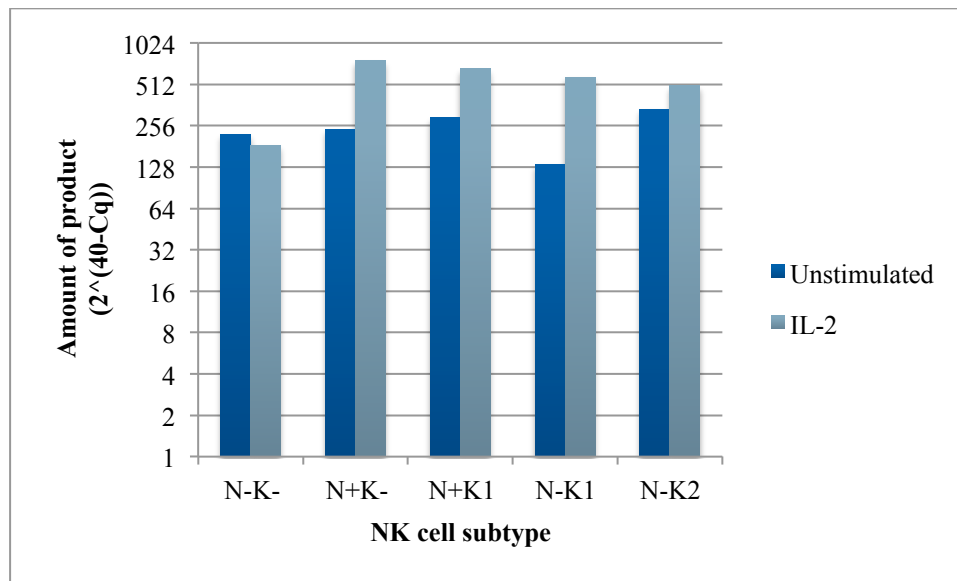


Figure 22 Relative amount of TIM-3 expression of NK cells after exposure to K562 cells depending on IL-2 stimulation.

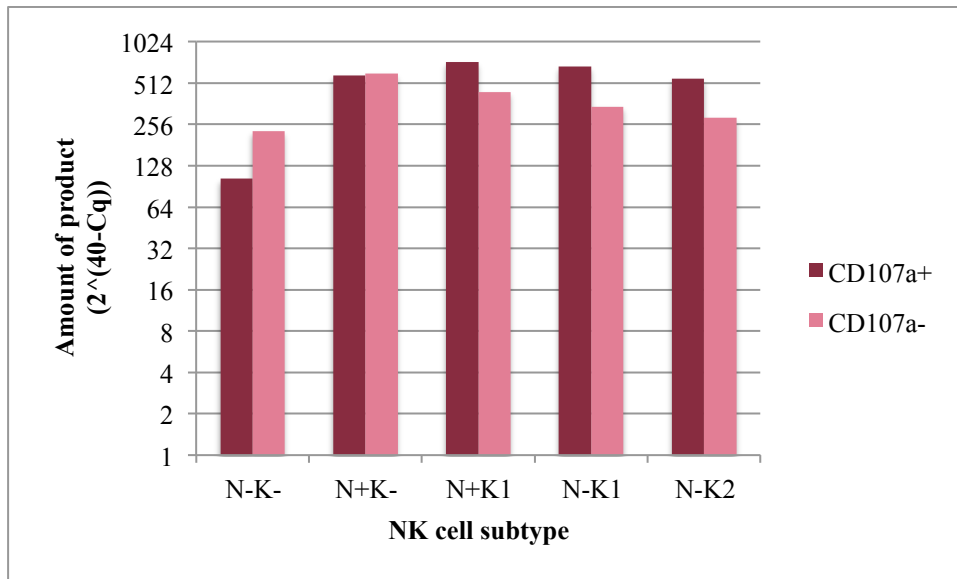


Figure 23 Relative amount of TIM-3 expression of NK cells after exposure to K562 cells depending on degranulation status. CD107a is a marker for degranulation.

### LAG-3

The transcript level of LAG-3 varies considerably between NK cell subsets. The more mature KIR<sup>+</sup> subsets express more transcript than the KIR<sup>-</sup>. There is a clear difference between unstimulated and IL-2 exposed NK cells in almost all subsets and IL-2 favors LAG-3 expression (Figure 24). Cells that degranulate tend to have a lower level of expression than cells that do not (Figure 25).

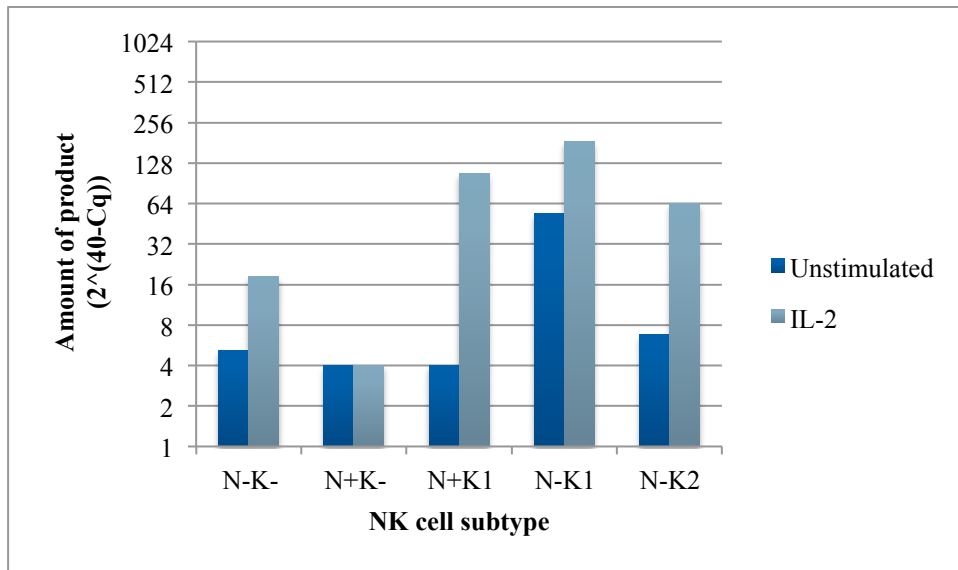


Figure 24 Relative amount of LAG-3 of NK cells exposed to K562 cells depending on IL-2 stimulation

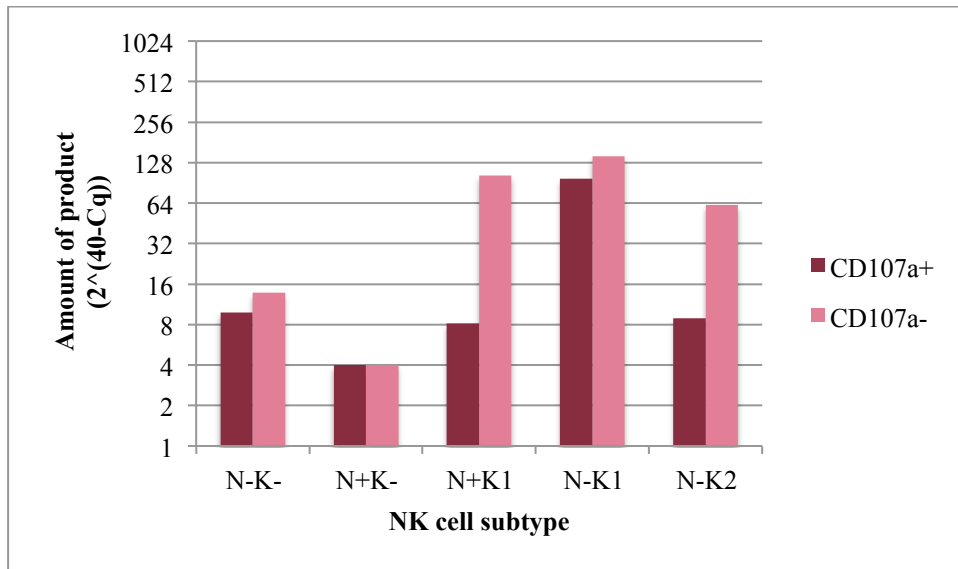


Figure 25 Relative amount of LAG-3 of NK cells exposed to K562 cells depending on degranulation status. CD107a is a marker for degranulation.

## NKG2A

The NKG2A transcript could only be markedly shown in cells already expressing NKG2A protein on their surface. The analysis also suggests that these cells have a slightly higher NKG2A transcript level if they are CD107a<sup>-</sup>, i.e. those cells that have not degranulated upon

exposure to the K562 cells (Figure 26). IL-2 had no clear effect on the level of NKG2A expression per cell subset. However, as noted above the NKG2A<sup>+</sup> fraction of NK cells increases with IL-2 stimulation.

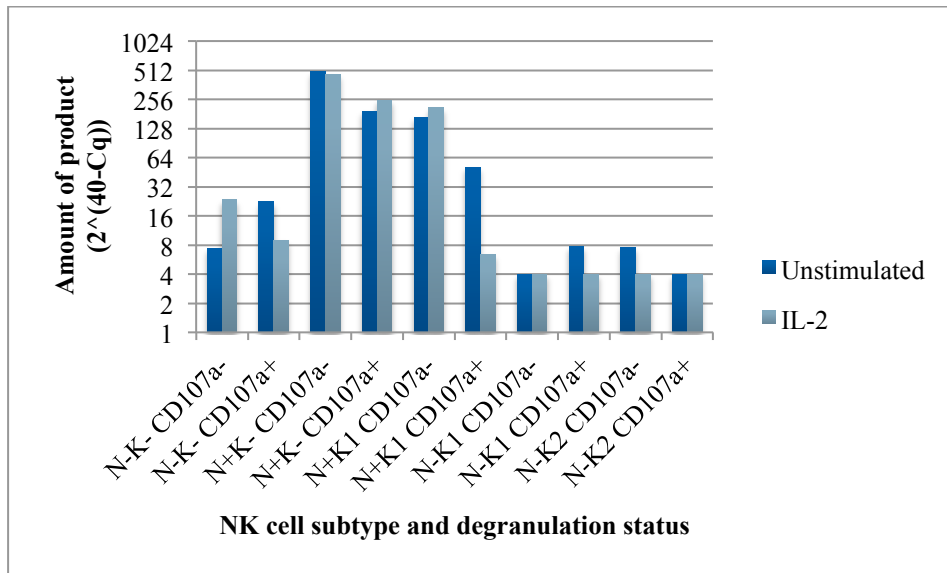


Figure 26 Relative amount of NKG2A of NK cells exposed to K562 cells depending on degranulation status. CD107a is a marker for degranulation.

## Discussion with Conclusions and Implications

The aim of this project was to develop a method for studying the transcript expression levels of four checkpoint inhibitor genes and NKG2A in NK cells and to apply this method in a pilot experiment to study this expression in NK cells and their response after exposure to leukemic K562 cells.

Our initial hypothesis to use this method for studying responses in cells transcript-positive but protein-negative for NKG2A could not be tested, as all KIR<sup>+</sup>NKG2A<sup>-</sup> NK cells, i.e. NKG2A protein-negative, were also negative for NKG2A transcripts. In this study we incubated NK

cells for 48 hours both with and without 500 U/ml IL-2. Even though there was an expected shift towards more NKG2A expression with IL-2 stimulation (30), there was no obvious difference in transcript expression in each NK cell subtype depending on the presence or absence of stimulation. All extra NKG2A expression induced by IL-2 thus seems to have not only reached the transcript stage but also the protein stage after 48 hours. It is possible that a stronger stimulus given during at shorter time period would generate more transcript-positive cells that are not yet expressing the cell surface protein.

CTLA-4 was not shown to be clearly expressed in any NK cell subsets and data on it is scarce in the literature (16). If anti-CTLA-4 therapy improves NK cell contribution to anti-tumor immune response, it is likely to be indirectly, for example by improving IL-2 production by T cells which may unleash NK cells from their suppressive effects (25,26).

The yield of the PD-1 assays was lower than for the other assays during the primer tests and effectively zero when NK cells were analyzed. This can of course be because the amount of PD-1 mRNA in the primer test material was low and that none of the NK cells subsets express PD-1. Previous studies have shown that depending on the clinical setting, varying NK cell subsets express PD-1 (27,28) However, there is also a possibility that the quality of the primer pair was low leading to low sensitivity in the qPCR experiments. Further evaluation of this primer pair would be desirable to verify its value.

In line with previous findings in the literature, TIM-3 was found to be expressed in all NK cell subsets regardless of degranulation status and it has been assigned both activating and inhibitory effects (16). Of the IL-2 stimulated cells the level was lowest for the immature NKG2A- KIR- subset. This is in line with it being constitutively expressed in functional and

more mature NK cells (15). Since no PD-1 expression was detected in any of the subsets, it seems like TIM-3 is not coexpressed with PD-1 on NK cells like it is on T cells in some other cancers (15).

The expression of LAG-3 transcript was found to be low mainly in the NKG2A<sup>+</sup> and high in the NKG2A<sup>-</sup> NK cells. This is the opposite of the NKG2A transcript expression and suggests a shift from LAG-3 and inhibition by MHC class II in immature NK cells to NKG2A and inhibition by HLA-E as the cells mature. Later maturation seems to reverse the shift, once again in favor of LAG-3, when NKG2A levels decrease. Further studies are required to determine if the LAG-3 transcript is translated to protein expressed on the cell surface, if there really is a causal connection between LAG-3 and NKG2A and the implications thereof.

LAG-3 is an inhibitory receptor binding MHC class II molecules. It has been well established that K562 cells do not express MHC class II on the cell surface (29). The observed tendency for NK cells with low a level of LAG-3 transcripts to degranulate to a greater degree thus likely does not necessarily involve LAG-3 directly. Instead, LAG-3 may in certain circumstances constitute a marker for less cytotoxic NK cells.

In conclusion, a methodology was developed that may be useful in studying how immune checkpoint transcript phenotypes are related to NK cell function. Since no cells transcript-positive and protein-negative for NKG2A were found, future experiments will be designed to find the time window where these transition NK cells are present, enabling us to discriminate between the educating role of NKG2A and its role as an activation marker.

The presence of LAG-3 on NK cells has not previously been thoroughly studied and our finding that the gene transcript is present in different amounts in the various NK cell subsets



as well as the seemingly inverse relationship with NKG2A calls for further investigation with the hope of finding new targets for treatment of malignant disease.

## Strengths and Weaknesses

A method for studying transcript levels of five NK cell receptor genes was successfully developed. The primer test assays for CTLA-4, TIM-3, LAG-3 and NKG2A showed good specificity for the desired transcript of the correct length without picking up genomic DNA or producing primer-dimers in disturbing amounts. However, during the verification of the primer pairs there was a recurring discrepancy between the observed and expected length of the DNA copies on the gel electrophoresis. The fact that the observed length on average was 23.5% (varying between 6.8% and 35.4%) greater than the expected length implies a systematic error in the gel electrophoresis. Investigating the cause of this and possible structural differences between the DNA ladder and the amplified DNA would be desirable to be able to determine if there really is a length difference.

The method developed was tested on 100 cells, which means that the results represent the average of those cells. Important patterns may be obscured by the noise from this large number of cells. Future experiments will focus on scaling it down to single cells for more detailed analysis.

The PCR analysis was only run once per checkpoint receptor and NK cell subtype which means that no standard deviation or other measures could be calculated to indicate the statistical significance of the findings. This shortcoming could be overcome by repeating the experiments multiple times.

# Populärvetenskaplig sammanfattning på svenska

## Förekomst av “immune checkpoint”-receptorer hos NK-celler

Den mänskliga kroppen är uppbyggd av flera miljarder celler. Dessa kommunicerar med varandra för att reglera antalet celler och vilken typ av celler som behövs. Fel i denna reglering kan leda till ohämmad celltillväxt vilket är karaktäristiskt för de sjukdomar som benämns cancer. För att skydda kroppen mot infektioner och cancer finns immunförsvaret bestående av celler som har förmågan att attackera främmande mikroorganismer och cancerceller. Vissa cancerceller har förmågan att påverka immunförsvarets celler genom att stimulera sensorer på deras yta, s.k. immune checkpoint-receptorer. Detta får till effekt att immunförsvaret bromsas och cancercellerna undkommer attack. En relativt ny och mycket lovande medicinsk behandlingsmetod för cancer kallas immunterapi och innebär att immune checkpoint-receptorer på immunförsvarets s.k. T-celler blockeras så att cancercellerna inte kan bromsa immunförsvaret.

En annan typ av immunförsvarets celler är naturliga mördarceller, även kallade NK-celler. Vi vet inte lika mycket om immune checkpoint-receptorer på dessa celler men vi vet att deras attackbeteende kontrolleras av aktiverande och hämmande receptorer. Ett syfte med detta projekt var att studera hur mycket av fyra immune checkpoint-receptorer, kända från T-celler, som finns i olika undergrupper av NK-celler. Detta som ett steg mot att hitta nya sätt att på medicinsk väg kunna stimulera NK-cellers cancerdödande förmåga.

Under vissa omständigheter har NK-celler den hämmande receptorn NKG2A och reagerar då på två sätt vid stimulering. Dels stärks deras celldödande förmåga, men samtidigt hämmas deras attackbeteende. Genom att lära oss mer om NK-celler som är i färd med att skaffa sig

NKG2A hoppas vi kunna utnyttja att deras celldödande förmåga stärkts, men innan deras attackbeteende hämmas, till att behandla cancer.

En metod togs fram för att mäta mängden NKG2A samt de fyra immune checkpoint-receptorerna PD-1, CTLA-4, TIM-3 och LAG-3. NK-celler som fått interagera med en viss typ av blodcancer celler sorterades till olika undergrupper och analyserades med den framtagna metoden.

Resultaten visade att den framtagna metoden fungerade väl. Två av de undersökta receptorerna, PD-1 och CTLA-4, kunde inte påvisas i NK-cellerna. Däremot kunde vi se TIM-3 och LAG-3 vilket inger förhoppning om att dessa kan utnyttjas för immunterapi. Betydelsen av detta kommer visa sig i framtida studier och kan eventuellt leda till nya behandlingar av cancersjukdomar. Tyvärr kunde inga NK-celler i färd med att skaffa NKG2A hittas men med förfinad metodik kommer framtida studier lära oss mer om dessa celler och deras terapeutiska potential.

## **Acknowledgement**

I would like to thank my supervisor Fredrik Bergh Thorén for invaluable guidance throughout this project. I would also like to thank Malin Nilsson, Alexander Hallner and Johan Gustafsson for teaching me about the procedures at the lab and help with all practical issues. Thanks to Hana Komic for helping me keep the structure during the most intense lab sessions. To all members of the TIMM lab, thanks for letting me feel like a part of the group and for encouraging support whenever needed. Last but not least, love and thanks to my family for their support and understanding during my work with this thesis.

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Figure 26 Relative amount of NKG2A of NK cells exposed to K562 cells depending on degranulation status. CD107a is a marker for degranulation. .... 38

## Appendix – PCR Primers

Table A 1 lists the primer pairs that were designed, evaluated and used in the analysis of checkpoint inhibitor transcript expression. Table A 2 lists the primer pairs that were designed and evaluated but not considered adequate for the analysis.

*Table A 1 Primer pairs used in the analysis*

Gene	Primer label	Ensembl number	Left Primer 5'-3'	Right primer 5'-3'	T-anneal	Length
CTLA-4	CTLA4	ENSG00000163599	GCAGCAGTTAGTTCGGGG	TGGGGGCATTTTCACATAGACC	58.11/60.62	117
PD-1	PDCD1v2	ENSG00000188389	TCGTCTGGGCGGTGCTA	AGGTGAAGGTGGCGTTGTC	60.43/60.23	132
TIM-3	HAVCR2	ENSG00000135077	GTGTCTCTGACTTTTCTTCTGC	CGACCTCCGCTCTGTATTCC	59.50/59.97	121
LAG-3	LAG3v3	ENSG00000089692	TTCGACTAGAGGATGTGAGCC	GATCCAGGTGACCCAAAGGATT	58.97/60.02	136
NKG2A	KLRC1v1	ENSG00000134545	AACGATAGTTGTTATCCCTCTACA	GGACAATGGCCACAATGACG	57.59/59.83	96

*Table A 2 Primer pairs designed but not used in the analysis*

Gene	Primer label	Ensembl number	Left Primer 5'-3'	Right primer 5'-3'	T-anneal	Length
PD-1	PDCD1v1	ENSG00000188389	GCACGAGGGACAATAGGAGC	ACAGAGAACACAGGCACGG	60.53/59.93	77
LAG-3	LAG3v1	ENSG00000089692	AGGCTGGGACCTACACCTG	TGGAGTCACCTCACAAAGCAG	60.61/60.20	140
LAG-3	LAG3v2	ENSG00000089692	TCATCACAGTGACTCCCAAATCC	GCTCCACACAAAGCGTTCTT	60.31/59.34	101
NKG2A	KLRC1v2	ENSG00000134545	ACGATAGTTGTTATCCCTCTACAT	CCTCAGGACAATGGCCACAA	57.40/60.25	100
NKG2A	KLRC1v3	ENSG00000134545	TTGGGAAGAGATTTGCTGG	TGAGGATGGTAAATGATGGAC	57.72/58.11	103