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Analysis of ligands for NK cell receptors in cells and exosomes from acute leukemia patients

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Abstract

Natural Killer (NK) cells are lymphocytes that function to clear infected and transformed cells. They contribute to tumor immune surveillance, and lack of NK cells lead to spontaneous development of tumors. Targeting of tumors is mediated by recognition of stress-induced proteins on the tumor cells that serves as ligands for numerous activating NK cell receptors. These stress-induced proteins are not normally found on healthy cells. The recognition of these ligands by NK leads to lysis of the target cell. Here we investigated the expression of ligands for the NK cell activating receptors NKG2D, DNAM-1, and NKp30 on acute leukemia cells from pediatric patients by quantitative PCR. The acute leukemia cells, particularly leukemic cells from the myeloid lineage, were observed to express several of the ligands for NKG2D, DNAM-1, and NKp30 receptors. Tumor cells have been shown to shed ligands for activating NK cell receptors either in a soluble form or in small vesicles called exosomes. All cells are thought to be capable of secreting exosomes, which are cargo-containing vesicles that can be found in plasma as well as in most body fluids. The exosome cargo can consist of small RNAs, metabolites, and several proteins from their cell of origin. A protocol to separate exosomes from plasma was developed, aided by mass spectrometric analysis of known exosomal markers and electron microscopy. The exosomal proteome of pediatric patients with acute leukemia compared to exosomes from healthy children controls was characterized by mass spectrometry. We were able to demonstrate presence of the immunosuppressive molecules TGF-β1, pyruvate kinase, CD99, and CD13 specifically in the patients. This indicates that leukemia-derived exosomes may be part of a tumor evasion mechanism to suppress immune responses against the malignant cells.

Keywords/phrases: NK cell, NKG2D, DNAM-1, NKp30, exosomes, immunisurveillance, tumor evasion, pre-B ALL, T-ALL, AML.
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# Table of contents

1 Introduction
   1.1 – The immune system
      1.1.1 – Lymphocytes
   1.2 – NK cells
      1.2.1 – NK cell recognition
      1.2.2 – NK cell functions
      1.2.3 – Activating receptors
   1.3 – Leukemia
      1.3.1 – Leukemia classification
      1.3.2 – Tumor immune surveillance and immune evasion
   1.4 – Exosomes
      1.4.1 – Biogenesis
      1.4.2 – Exosomal cargo
      1.4.3 – Exosomes as biomarkers
      1.4.4 – NK cells and exosomes
   1.5 – Soluble ligands
      1.5.1 – Tumor cells release soluble ligands
      1.5.2 – Effects of soluble ligands on NK cells
   1.6 – Aims of the study

2 Materials
   2.1 – Buffers
   2.2 – Cell lines
      2.2.1 – Jurkat
      2.2.2 – K562
   2.3 – Patients and healthy controls
      2.3.1 – Patients
      2.3.2 – Healthy controls

3.2 Methods
   3.2.1 – Lymphoprep
   3.2.2 – Freezing and thawing cells
      3.2.2.1 – Freezing cells
      3.2.2.2 – Thawing cells
   3.2.3 – RNA isolation
      3.2.3.1 – RNA isolation from cell lines
      3.2.3.2 – RNA isolation of patient samples
      3.2.3.3 – Analysis of RNA quality on agarose gel
      3.2.3.4 – Measurement of RNA concentration by NanoDrop
   3.2.4 – cDNA generation
   3.2.5 – Polymerase chain reaction (PCR)
      3.2.5.1 – Primer design
      3.2.5.2 – Primer preparation
      3.2.5.3 – Semi-quantitative PCR
      3.2.5.4 – Agarose gel electrophoresis
      3.2.5.5 – Quantitative PCR (qPCR)
   3.2.6 – Exosome isolation
3.2.6.1 – Differential Ultracentrifugation
3.2.6.2 – Total Exosome Isolation from plasma
3.2.6.3 – Size Exclusion Chromatography (SEC)
3.2.7 – Isolation of leukemic blast-derived exosomes
3.2.7.1 – MACS beads
3.2.7.2 – Dynabeads
3.2.8 – Preparation of protein lysates
3.2.8.1 – Lysate of exosomes with SDS lysis buffer
3.2.8.2 – Lysate of exosomes with NP-40 lysis buffer
3.2.9 – Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
3.2.10 – Coomassie Blue staining
3.2.11 – Western blotting
3.2.12 – Protein concentration measurement
3.2.12.1 – Infrared spectrometer
3.2.12.2 – Pierce BCA Protein Assay Kit
3.2.13 – Mass spectrometry
3.2.14 – Electron microscopy

4 Results
4.1 Test runs of potential reference genes for qPCR analysis
4.2 qPCR analysis of NKG2D, DNAM-1 and NKp30 receptor ligands
4.2.1 – Expression of ligands for NKG2D in pre-B ALL, T-ALL, and AML patients
4.2.2 – Expression of ligands for DNAM-1 in pre-B ALL, T-ALL, and AML patients
4.2.3 – Expression of ligands for NKp30 in pre-B ALL, T-ALL, and AML patients
4.3 – Establishing a protocol for isolation of exosomes from plasma
4.3.1 – Exosome isolation by differential ultracentrifugation
4.3.2 – Total Exosome Isolation kit
4.3.2.1 – Electron microscopy images of exosomes isolated with kit
4.3.2.2 – Protein content in exosomes isolated with kit
4.3.3 – Exosome isolation by Size Exclusion Chromatography (SEC)
4.3.3.1 – Protein distribution of the fractions
4.3.3.2 – Electron microscopy images of isolated exosomes by SEC
4.3.3.3 – Proteomic analysis of exosomes
4.4 Measurement of the protein concentration from isolated exosomes
4.4.1 – Protein concentration of exosomes isolated with kit
4.4.2 – Protein concentration of fractions from SEC isolation

5 Discussion
5.1 qPCR analysis of ligands for activating NK cell receptors
5.1.1 Healthy controls used in the qPCR analysis
5.1.2 Patient samples used for qPCR analysis
5.1.3 Melt curves for ligands and reference genes
5.1.4 Ligands for NK cell activating receptors
5.2 Methodological consideration
5.2.1 Reference genes for normalization of the qPCR measurement
5.2.2 The use of SYBR green instead of TaqMan for qPCR analysis
5.2.3 Exosome isolation
5.2.4 Isolation of leukemia-derived exosomes
5.2.5 Methods to measure protein concentration of isolated exosomes
5.2.6 Exosome markers
5.3 Findings

5.3.1 Ligand expression for NKG2D, DNAM-1, and NKp30 in acute leukemia cells

5.3.2 Detection of immusupressive molecules by mass spectrometry analyses

5.3.3 Fractions containing exosomes from SEC

5.4 Conclusions

6 Future perspectives

References

Appendix 1: Primers

Appendix 2: Patient and healthy control information

Appendix 3: Buffers

Appendix 4: Ladders

Appendix 5: Melt curves of genes used for qPCR analysis

Appendix 6: Images of PCR products

Appendix 7: Abbreviations
1 Introduction

In this thesis, the expression of ligands for activating NK cell receptors in acute leukemic cells was investigated. Ligand expression was assessed both in the leukemic cells, and as soluble ligands released in plasma. Further, plasma-derived exosomes from acute leukemic patients were analyzed as a source for released ligands from the leukemic cells. This introduction gives a short description of the immune system, followed by NK cells and their function against virus and cancer. The next sections will give a general background of leukemia, and finally a more in-depth introduction of exosomes.

1.1 The immune system

The immune system is the body’s defense mechanism against foreign invaders, primarily infection-causing organisms such as bacteria, viruses and parasites. It also recognizes abnormal cells like tumorigenic cells. The immune system is made up of a network of cells, tissues, and organs.

The immune system is divided into the innate and the adaptive immune system. As a first line of defense are epithelial barriers. These barriers protect against microbes from external environment. The skin, gastrointestinal, respiratory tracts are lined by continuous epithelial. In addition the body produce tears, mucus, and saliva containing lysozymes ([2] and [3]). Commensal bacterias are found on the surface of the skin, upper respiratory and gastrointestinal tracts were they live in symbioses with the host and protect against invasive pathogens ([4]). The innate immune system provides the first line of cellular defense. Cells of the innate immune system, such as granulocytes and macrophages, react broadly to any microorganism or products of tissue damage. As the innate immune system, the adaptive immune system has the ability to recognize and react to both microbial and non-microbial structures. The main components found in the adaptive immune system are lymphocytes, B cells and T cells, and their secreted products. B and T cells have the unique property of reacting specifically to one particular structure, to clonally expand reactive cells after encountering an antigen, and to generate memory cells that will respond swiftly upon a second encounter with the same antigen. This makes the adaptive immune system unique by having both memory and specificity ([2] and [3]).

1.1.1 Lymphocytes

Lymphocytes are a type of white blood cell, which are developed from stem cells in the bone marrow. The multipotent hematopoietic stem cell differentiates into common lymphoid stem cell progenitors. These lymphoid progenitors can develop further into B cells, T cells, or NK cells/innate lymphoid cells, see figure 1. The bone marrow and the thymus are considered the primary lymphoid organs where development of lymphocytes occurs. The T cell develops in the thymus, while B cells and NK cells develops in the bone marrow [2].
**Natural Killer (NK) cells**

NK cells belong to the innate immune system and are found in all lymphoid organs as well as in certain peripheral tissues such as intestines, lung, liver, and uterus. They defend us against several types of microbial infections and tumors ([2] and [3]). More details on NK cells and their involvement in the immune system will follow below.

**B cells**

B cells are the only cells capable of producing antibodies. Each B cell is programmed to make one specific antibody. They are equipped with a B-cell receptor, which is a membrane-bound immunoglobulin (antibody) molecule that binds to antigens, and of the same specificity as the antibodies that are produced [2].

**T cells**

Peptide antigens from intracellular pathogens (e.g. virus) that multiply in the cytoplasm, are carried to the cell surface by MHC class I and presented to CD8\(^+\) T cells. Peptide antigens derived from ingested extracellular bacteria and toxins, are carried to the surface by MHC class II and presented to CD4\(^+\) T cells. CD8\(^+\) T cells are cytotoxic cells that kill the target cells. CD4 T cells may differentiate into distinct T helper subsets (T\(_{H1}\), T\(_{H2}\), T\(_{H17}\), T regulatory cells) that are important for initiating and controlling the T- and B-cell responses (effector cells) ([2] and [3]).
1.2 NK cells

NK cells make up to 5-20% of the circulating lymphocyte population in humans. These cells are cytotoxic, like CD8⁺ T cells. In addition, they produce important cytokines that indirectly but potently influence both innate and adaptive immune responses. The function of NK cells, as the name suggest, is to kill virus-infected and malignant cells, along with some bacteria-infected cells ([3] and [5]). In humans, NK cells are defined by the surface expression of CD56, CD16, and absence of the T cell receptor CD3 [6].

NK cells do not express antigen specific receptors like B- and T-cells. Instead, NK cells generally recognize stressed cells (due to infection or malignant transformation). NK cell receptors are divided in two groups based on their functions: inhibitory receptors or activating receptors. Inhibitory receptors generally bind to MHC class I and suppress NK cell activity, while activating receptors binds to ligands that trigger NK cell cytotoxicity. Healthy cells normally express MHC class I and no ligands for activating receptors. During cellular stress, activating ligands are up-regulated, and this may shift the balance from inhibition towards activation ([3], [7] and [8]). Therefore it is important to notice that the activation of NK cells is regulated by both activating and inhibitory signals. The main activating receptors on NK cells will be introduced later in this chapter.

1.2.1 NK cell recognition

MHC class I are expressed on almost all nucleated cells of the body and NK cells base their decision to kill on whether there is any MHC class I or not on the surface of the interacted target cell. This ability makes them unique from B cells and T cells, which base their recognition on foreign antigens. Since many virus-infected and tumour cells decrease the expression of MHC, NK cells detect the absence of MHC and are able kill them. In contrast, B cells and T cells neglect to recognize these cells as a threat. CD8⁺ T cells recognizes the peptide presented by the MHC class I, if e.g a tumour cell lack MHC class I, CD8⁺ T cell will not consider the cell as a threat ([3],[9] and [10]). For the NK cells, lack of MHC interaction with inhibitory NK cell receptors is not to enough to induce target cell killing. Also, the activating receptors have to bind to a ligand expressed by the target cell.

1.2.2 NK cell function

NK cells are activated through activating NK cell receptors responding to ligands up-regulated on infected or malignant cells. The ligand – activating receptor interaction induces intracellular signaling pathways leading to a functional response as well as gene transcription.

NK cell activity is further enhanced by cytokines, such as IL-12, IL-15, and IL-18. These are produced from dendritic cells and macrophages [11]. In addition, IL-2 produced by T cells induces proliferation and enhanced cytotoxic function [12].

Once activated, NK cells may kill the target cell. Killing of target cells are initiated via different pathways depending on the target cells. The major mechanism is secretion of specialized lytic granules containing the proteins perforin and serine proteases called granzymes. Perforins create pores in the target cell membrane, allowing granzymes to enter and start the apoptotic pathway by inducing cascade activity ([13],[14] and [15]). In addition, cytotoxicity may be induced via transmembrane death receptors belonging to the tumor necrosis factor (TNF) family, such as tumor necrosis factor (TNF)- related apoptosis-inducing ligand (TRAIL) or Fas ligand (FasL) on NK cells. These molecules bind to TRAIL receptors or Fas, respectively, expressed by the target cell and induces apoptosis.
([16],[17],[18] and [19]). In addition, antibody dependent cellular cytotoxicity (ADCC) is induced when the Fc receptor (CD16) on NK cells interact with the Fc-part of antibodies coating a target cell ([20], [21] and [18]).

Beside their function to kill target cells, NK cells are also important as modulators of both adaptive and innate immune responses by producing and secreting cytokines. The most prominent cytokines secreted by NK cells are the inflammatory cytokines interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and macrophage inflammatory protein 1β (MIP-1β), but they may also secrete cytokines such as IL-5, IL-10, and GM-CSF ([11]and [22]).

1.2.3 Activating receptors
Both activating and inhibitory receptors fall under two structural categories, lectin-like extracellular regions and immunoglobulin-like extracellular regions. Therefore it is the intracellular regions that decide the receptor’s function [3]. The intracellular domain of the receptors contains either an ITAM (immunoreceptor tyrosine-based activation motif) or an ITIM (immunoreceptor tyrosine-based inhibitory motif). The activating receptors associate with small adapter molecules, such as CD3ζ or DAP12 that contain ITAM motifs, while the inhibitory receptors contain ITIM motifs [23]. ITAM consist of the consensus sequence YXXM, while ITIM consists of the consensus sequence (I/VxYxx(L/V) [24]. As this thesis is concentrated on ligands for activating receptors, only the activating receptors will be introduced in detail.

**NKG2D**
NKG2D is expressed on all NK cells, in addition to CD8⁺ T cells, NKT cells, and γδ T cells. In humans the ligands for NKG2D are the stress-inducible surface glycoproteins, MICA and MICB, and the ULBP (UL16 binding protein) protein family (ULBP1-6). The NKG2D receptor forms a complex with the adaptor protein DAP10. When ligand binds to NKG2D, DAP10 initiates intracellular signaling by recruiting and activating PI3K (phosphoinositide 3-kinase) ([25], [26] and [27]).

**NKG2C**
NKG2C belongs to a family of receptors that forms a heterodimer with the C-type lectin-like receptor CD94, and binds to HLA-E. NKG2C signals via CD94 that form a disulphide-bond homodimer with the adaptor molecule DAP12. When the NKG2C/CD94/DAP12 receptor complex is bound by ligands, activating signals are transmitted through the ITAM motifs contained in DAP12 ([28] and [29]).

**NCRs (Natural cytotoxicity receptors)**
NKp30 is one of three natural cytotoxicity receptors [30]. The NCRs are type I membrane proteins. NCRs belong to the immunoglobulin-like family and are among the major activating NKRs (natural killer receptors). Besides NKp30, the NCRs include the activating receptors NKp46 and NKp44, for which the ligands are unknown. NKp30 recognizes BAG6 and B7-H6 ([31]). NKp30 and NKp46 are expressed on resting cells and are up-regulated on activated NK cells, whereas NKp44 is expressed only on activated cells ([32]).

**CD16**
The CD16 receptor on NK cells binds to the Fc portion of Immunoglobulin G (IgG), and upon ligation a potent series of signals are induced, resulting in both cytokine production and cytotoxicity activity via ADCC ([31]).
**DNAX Accessory Molecule – 1 (DNAM-1)**

DNAM-1 is expressed on all NK cells, CD8 T⁺ cells, CD4⁺ T cells, and monocytes. So far only two ligands are known for this receptor, CD155 and CD112 ([33] and [26]). DNAM-1 transmits activating signals through a motif in the cytoplasmic domain that recruits tyrosine kinase Fyn and serine-threonine kinase PKC (Protein kinase C), when interacting with ligand. The DNAM-1 – ligand interaction triggers cell-mediated cytotoxicity and cytokine production [34].

**Table 1: Overview of NK cell activating receptors and their ligands.**

<table>
<thead>
<tr>
<th>Activating receptors</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKG2D</td>
<td>MICA, MICB, ULBP1-6</td>
</tr>
<tr>
<td>NKG2C</td>
<td>HLA-E</td>
</tr>
<tr>
<td>Nkp30</td>
<td>BAG6 (BAT3) and B7-H6</td>
</tr>
<tr>
<td>Nkp44</td>
<td>Unknown</td>
</tr>
<tr>
<td>Nkp46</td>
<td>Unknown</td>
</tr>
<tr>
<td>CD16</td>
<td>Antibodies</td>
</tr>
<tr>
<td>DNAM-1</td>
<td>CD155 and CD112</td>
</tr>
</tbody>
</table>

**1.3 Leukemia**

Cancer being a worldwide health problem is one of the most important causes of mortality in both children and adults. Cancer arises from uncontrolled proliferation and invades other host tissues, and may metastasize to other sites [2].

**1.3.1 Leukemia classification**

Overall leukemia incidence has increased slowly over decades. In contrast to incidence, death rates for leukemia have dropped. Leukemia is a cancer of the bone marrow, which quickly spreads to the blood, resulting in a spread of leukemia throughout the body. Leukemia is classified into four main groups (fig.2), according to cell type (myeloid or lymphoid) and their rate of growth. Acute leukemia usually develops quickly, while chronic leukemia develops slowly.

Therefore the four main groups are:
- Acute lymphoblastic leukemia (ALL)
- Acute myeloid leukemia (AML)
- Chronic lymphoblastic leukemia (CLL)
- Chronic myeloid leukemia (CML)

Leukemia includes 30% of all childhood cancers, where 75% of the cases are ALL in children from 0 to 19 years [35]. The acute lymphoblastic leukemia (ALL) is the most common malignancy of childhood cancers, and the underlying disease mechanisms leading to leukemia are still incompletely understood [36]. Since the acute leukemia is the focus of this thesis, ALL and AML will be introduced in more detail.
Acute lymphoblastic leukemia (ALL)
ALL is characterized by malignant proliferation, and accumulation of immature lymphoid cells within the bone marrow, blood, and lymphoid organs [37]. ALL being the most common form of leukemia in children, is the cause of one quarter of all deaths from childhood cancers [38]. The acute lymphoblastic leukemia is further classified into the type of lymphocyte (B cell or T cell) the leukemia cells arise from, and how mature these leukemia cells are. T-cell ALL is an aggressive malignancy of thymocytes, representing 15% of pediatric and 25% of adult ALL cases [37]. B-cell ALL are classified in several subtypes:

- Early precursor B cell (early pre-B ALL/pro-B ALL)
- Common ALL
- Pre-B cell ALL
- Mature B-cell ALL (also called Burkitt leukemia)

The pre-B acute lymphoblastic leukemia (pre-B ALL) is the most common neoplasm in children and young adults [39].

Acute myeloid leukemia (AML)
AML affects early (immature) bone marrow cells as myeloblasts, erythroblasts, and megakaryoblasts (see fig. 1). In contrast to ALL, which is the most common leukemia in childhood, AML is the most common acute leukemia in adults. It is more common in males than females, and the incidence increases with age [40]. AML accounts for 15-20% of all acute leukemias in children, making it the second most common leukemia group during childhood [41].

1.3.2 Tumor immune surveillance and immune evasion
The immune system can specifically identify and eliminate tumor cells on the basis of their expression of tumor-specific antigens or molecules induced by cellular stress. As this thesis focuses on NK cells in tumor immune surveillance, the concept of tumor immune surveillance will be introduced in this chapter.

The immune system has several mechanisms that contribute to host anti-tumor immunity by the means of destroying transformed cells or creating an environment that suppresses tumor growth. In vitro studies have shown NK cell cytotoxicity to be essential for inducing tumor
cell apoptosis ([42] and [43]). The NK cell-mediated immune surveillance interpret signals on tumor cells, either reduced MHC-I expression or molecules induced by cellular stress. If activation is induced by any of these tumor signals, the target is eliminated (e.g. via cytotoxic granules, FasL, or TRAIL) [44].

Although the immune system, including NK cells, is capable of elimination of tumor cells, the tumor cells have evolved strategies to circumvent attack by the immune system. Immunoediting represent a dynamic process consisting of 3 phases; elimination of the tumor by the immune system, equilibrium between immune system and the residual tumor, and escape from immunosurveillance and development of the tumor. As fig. 3 [40] illustrates, if the elimination phase is incomplete an equilibrium is temporarily established. In this equilibrium phase, the tumor cells can accumulate further changes, e.g. DNA mutations or changes in gene expression. If the immune system is not successful in elimination of tumor cells at this stage, the next phase starts; the escape. As a result of the immune system failing to eliminate tumor cells, only the variant of tumor cells able to resist, avoid, or suppress the antitumor immune response, are able to escape [45].

To counteract tumor immune surveillance, tumor cells have developed strategies to suppress the immune system to support its continued growth. There are several mechanisms of tumor escape that are involved. For instance, the anti-inflammatory cytokine transforming growth factor (TFG)-β interferes with immune responses through inhibition of maturation and antigen presentation by dendritic cells, and by inhibiting activation of T and NK cells. TGF-β also influences the pro-invasive functions of tumour cells, and influences the activity of matrix metalloproteinases (MMPs) by endothelial cells and tumor cells, favors angiogenesis.

Figure 3: The immunoediting phases; elimination, equilibrium, and escape.
MMPs are proteolytic enzymes that shape the cellular microenvironment [46]. It is also known that tumor cells shed ligands for activating receptors of the immune system, this is described in more details for NK cells below (see section 1.6). Something that is a very recent discovery is the tumor cells ability to use exosomes as an escape mechanism. The tumor-derived exosomes relationship with NK cells is covered in section 1.4.2 and 1.4.4.

1.4 Exosomes
Exosomes are small (30 to 100 nm) cargo-containing vesicles secreted by all cell types and found in most body fluids, including blood, urine, saliva, and breast milk ([47] and [48]). Electron microscopy has demonstrated that the exosomes has a cup shaped morphology, and this has been used as a hallmark for morphologic identification of exosomes [49]. The exosome content in plasma and other body fluids have been observed to increase in diseases compared to healthy donors, and the amount of exosomes depends on the disease ([50] and [48]).

The functions of exosomes are not well known, but hypothesis on their functions have been reflected by their proteomic composition and cellular origins. They were originally associated with the function of removing unwanted proteins or molecules from the cell, but numerous studies the last decade have shown that it is more to this small vesicle. Exosomes can exchange materials between cells, and have thus a role in intercellular communication and possibly in propagation of pathogens. They also seem to have the ability to activate or suppress the immune system [51]. An example of their stimulatory effect on the immune system is exosomal expression of the NKp30 ligand BAT3 on tumor-derived exosomes (human fibroblast kidney, colon carcinoma, and myeloma cell lines were used). It stimulates the NK cell activation via NKp30 [52]. Overall exosomes function may depend on both the cell they origin from and the context when they are secreted [51].

1.4.1 Biogenesis
The biogenesis of exosomes begins with endocytosis, and it is therefore important to have an understanding of this process to be able to understand how exosomes are formed. The origin of the cells, exosomes are secreted from indicates their content. This can be useful to discover other aspects of immune evasion in cancer.

Endocytic vesicles are formed by plasma membrane budding inward and then pinched off. The endocytotic vesicles mature to early endosomes, which either recycle proteins back to the plasma membrane or further mature to late endosome or multi-vesicular bodies (MVBs). During this maturation process, internal vesicles within the endosomes termed intraluminal vesicles (ILVs) are formed by inward budding of the MVBs [53]. A general overview is shown in fig. 4 [54]. There are two options for MVB cargo, lysosomal degradation or secretion through exocytosis [47]. The vesicles inside MVBs with mannose-6-phosphate receptors are delivered to lysosomes, while vesicles lacking this receptor are released from the cells through exocytosis. These vesicles are called exosomes [55].
1.4.2 Exosomal cargo
The exosomal cargo is dependent on the cell type they originate from. Generally, proteins from the plasma membrane, the membrane fusion machinery, the MVB formation machinery, and heat shock proteins are found in exosomes [56] and [47] (fig. 5 [55]). Exosomes can also contain RNA, such as mRNA and miRNA. Therefore tumor-derived exosomes can transfer proteins and RNAs with oncogenic activity to recipient cells [57] and [58]. Classical exosome markers are several tetraspanin proteins including CD9, CD53, CD63, CD81, and CD82 [59]. CD63, in addition to CD9, are the most commonly used markers for exosomes [60].
1.4.3 Exosomes as biomarkers
An ideal biomarker should have high sensitivity and specificity, with the possibility to analyze it easily, and be minimally invasive or non – invasive [61]. Exosomes, isolated from either plasma or urine, seem to have the qualities for becoming a diagnostic biomarker.

Exosomes have shown to be stable under different conditions, and therefore the cargo they contain with proteins and RNAs are protected against degradation and denaturation in the extracellular environment [58]. Exosomes being easily accessed via blood samples or other fluid is an advantage. Since their cargo depends on the cell they originated from, the proteins and RNA can be used as biomarkers for diseases, including cancer.

The tumor- derived exosomes have been shown to contain miRNA, which may be an important form of cellular communication. A study by Chung E. P., et al, revealed that exosomes from Kaposi’s sarcoma (KS) patient plasma contains oncogenic and viral miRNA [62]. This shows the potential of exosomes, to become a novel and stable biomarker for several diseases. There have also numerous other studies identifying miRNAs, for other cancer types. A good example is the study by Iorio V. M et al., on miRNA signature in ovarian cancer. They showed that the overall miRNA expression could clearly separate normal vs. cancer tissues. [63].

1.4.4 NK cells and exosomes
Investigation on whether exosomes contain ligands for NK cell receptors has been done for different cancer types. A previous study by a Dutch group claims that ligands for NKG2D, but not DNAM-1, can be detected on exosome derived from epithelial ovarian cancer. The NKG2D ligands found on the exosome surface were MICA, MICB and ULBP1-3 [49]. Others have investigated the tumor-derived exosome effects on NK cells. Their data suggest that exosomes isolated from prostate and AML patients are likely involved in down-regulation of NK receptors such as NKG2D. This was shown when lymphocytes from healthy donors were incubated with exosomes isolated from cancer patients, resulting in downregulation of NKG2D expression in the lymphocytes ([64], [65] and [66]). This makes exosomes a part of the tumor evasion, whereby tumor cells are able to escape NK cell cytotoxicity by secreting exosomes with ligands for NK cell activating receptors.

1.5 Soluble ligands
1.5.1 Tumor cells release soluble ligands
Numerous reports have shown that human tumor cells spontaneously release ligands for activating NK cell receptors as a mechanism for immune evasion. This conclusion was drawn from observations of high levels of soluble ligands in cancer patient sera. There seem to be different mechanisms for the release of different ligands. The release can be caused by proteolytic cleavage by metalloproteinases, specifically family members of ADAM (metalloprotease domain) [66], and also as mentioned earlier exosomes could contain soluble ligands.

NKG2D ligands have received most attention compared to ligands for other activating receptors. A recent study showed that MICA was released by matrix metalloproteinases (MMP) in patients with gastrointestinal malignancies. For MICB, ADAM17 is involved in its shedding and the regions of the membrane enriched in cholesterol and sphingolipids (lipid raft) is an important area for this proteolysis ([67],[68] and [69]). Another study focusing on
sULBP1-3, found that ULBP2 is mainly shed by metalloproteases, while ULBP3 is released in exosomes [70].

1.5.2 Effects of soluble ligands on NK cells
Several studies have shown that soluble ligands for NK cell receptors are released by tumor cells. Upon interaction of NK cells with the soluble ligands, the soluble ligands have been shown to down-regulate the expression of surface receptors, e.g. soluble MICA and soluble ULBPs for NKG2D. A study done on the soluble ULBPs, showed that there is a functionally relevant difference in the biochemistry of ULBP1-3. The exosomal ULBP3 is more potent for down modulation of NKG2D, than ULBP2. The same effect on NKG2D is seen with exosomes containing MICA ([66] and [70]). Surprisingly a team of scientist discovered something, which is the opposite of what the literature suggests. Numerous studies on the effect of soluble ligands have been done on NKG2D, but this group (Deng W. et al..) found an increase of NKG2D expression levels [71]. Another example is the enhancement of the NK cell activating receptor NKp30. BAT3- enriched supernatant derived from tumor cells (several tumor cell lines were used) stimulated NK cell-mediated cytokine release. Since purified, recombinant protein acted in a suppressive manner, it was hypothesized that the stimulation observed with supernatants was due to exosomal release of BAT3 [52].
1.6 Aims of the study

NK cell activating receptors recognize stress-induced molecules that are not normally found on healthy cells, but up-regulated on infected or malignant cells. The ligand-receptor binding initiates the NK cells’ cytolytic function. The expression of ligands for different activating receptors varies between different cancer types and also between patients with the same cancer. The ligands for the NK cell activating receptors NKG2D, DNAM-1, and NKp30 are well characterized. In this thesis acute leukemia patients sample will be investigated for expression of ligands for these three receptors. This will be accomplished by examining both the gene expression of the ligands, and testing for presence of ligands in acute leukemia- derived exosomes. The cancer cells have several mechanisms to escape recognition of immune cells, including NK cells. Exosomes have been hypothesized to be one of the mechanism used by tumors for immune evasion, and several studies done on tumors have confirmed that some tumors uses exosomes to escape immunosurveillance. Therefore the goal of this thesis is also to analyze the leukemia–derived exosome proteome for immunosuppressive molecules.

The aims of this thesis are:

• To analyze gene expression of ligands for NKG2D, DNAM-1, and NKp30 from paediatric patients diagnosed with acute leukemia, and from plasma-derived exosomes.
• Establish a protocol for isolating exosomes from plasma with minimal contamination of plasma proteins
• To analyse the proteome of acute leukemia–derived exosomes to characterize possible immunosuppressive molecules.
2.1 MATERIALS

2.1.1 Buffers

See Appendix 3 for buffer recipes

- Blocking buffer for Western blotting (5% skimmed milk)
- Elution buffer (PBS w/ 0.32% trisodiumcitrate)
- Lysis buffer NP-40 for exosomes
- Red blood cell lysis buffer
- PBS
- PBS+2%FBS
- SDS lysis buffer for exosomes
- SDS running buffer
- TAE buffer
- TBS (tris-buffered saline) buffer
- TBS-T

2.1.2 Cell lines

2.1.2.1 Jurkat

Jurkat (ATCC® TIB-152™) is a cell line derived from the peripheral blood of a 14 year old boy with acute T cell leukemia. This cell line is widely used as a model for T cells. Jurkat cells were used as positive control for expression of ligands for activating NK cell receptors.

2.1.2.2 K562

The K562 cell line (ATCC® CCL-243™) originates from a 53-year old female with chronic myelogenous leukemia (CML). The K562 cells were used as positive control for expression of ligands for activating NK cell receptors.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Organism</th>
<th>Cell type</th>
<th>Tissue</th>
<th>Morphology</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>Homo sapiens, human</td>
<td>Myeloid cells</td>
<td>Bone marrow</td>
<td>Lymphoblast</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Homo sapiens, human</td>
<td>T lymphocyte</td>
<td>Peripheral blood</td>
<td>Lymphoblast</td>
<td>Acute T cell leukemia</td>
</tr>
</tbody>
</table>

2.1.3 Patients and healthy controls

2.1.3.1 Patients

Peripheral blood from pediatric patients diagnosed with acute leukemia was obtained from Department of Children at Rikshospitalet, Oslo University Hospital. The blood was collected as part of routine blood sampling in connection with their admission for diagnosis, and written informed consent from parents were obtained in accordance with the Declaration of Helsinki. The study is approved by the Regional Ethical Committee (REK 2013-1866).

2.1.3.2 Healthy controls

Peripheral blood was collected from adult, healthy controls after informed consent, and the study approved by the Regional Ethical Committee (REK 2012-1452).
3.2 METHODS

3.2.1 Lymphoprep
Lymphoprep is a medium used to isolate mononuclear leukocytes from peripheral blood, cord blood, and bone marrow. The lymphoprep is a density gradient medium and therefore uses the difference in cell density to isolate the mononuclear cells. Mononuclear cells (monocytes and lymphocytes) have a lower buoyant density than the erythrocytes and the granulocytes. The vast majority of mononuclear have a density below 1.007 g/mL and are therefore centrifuged on an isosmotic medium with a density of 1.077 g/mL. This allows the erythrocytes and granulocytes to sediment through the medium while retaining the mononuclear cells at the interface of sample/Lymphoprep medium.

For this thesis, Lymphoprep was used to isolate mononuclear cells and plasma from the blood of pediatric patients diagnosed with acute leukemia and from healthy donors. The mononuclear cells were used to isolate RNA and make cDNA for gene expression analysis, while the plasma was collected to isolate exosomes.

Reagents:
Lymphoprep (Axis-Shield)
PBS
PBS + 2% FBS
Red blood cell lysis buffer

Procedure
Freshly drawn full blood was added an equal amount of PBS in a 50 mL tube. Diluted blood was carefully layered onto 10 mL Lymphoprep solution in a new 50 ml tube. The tube was centrifuged with the settings below:
Time = 20 min
Temperature = 20 °C
Acceleration = 4
Deceleration = 0 (without breaks)
Speed = 650g
After centrifugation, the blood was separated in different layers (fig.7). The mononuclear cells and the plasma were collected separately with a pipette. The layer with mononuclear cells was pipetted carefully without disrupting the other layers and transferred to another tube, while plasma was collected and stored at -80 °C for later exosome isolation. The mononuclear cells were added 40 mL PBS+2%FBS and centrifuged at 350g for 10 min at room temperature. The supernatant was removed, and the pellet resuspended in PBS + 2% FBS to a total volume of 20 mL. The cells were spun again for 10 min at 350g, and the supernatant was discarded. To remove contaminating red blood cells, the cell pellet was resuspended in 5 mL red blood cell lysis buffer (see Appendix 3) + 50 µl KHCO₃. After 3 min incubation at room temperature 25 ml PBS+2% FBS was added, and cells centrifuged at 300g for 8 min. This method is based on that red blood cells are more sensitive to osmotic shock than white blood cells. Therefore the red blood cells will be selectively lysed. After centrifugation, the supernatant containing the lysed red blood cells was discarded, and the mononuclear leukocytes were frozen at -80°C to be used for RNA isolation.

3.2.2 Freezing and thawing cells

3.2.2.1. Freezing cells

Cells can be stored in liquid N₂ for several years. For freezing and thawing to be successful, it is important that cells are in good condition at time of freezing. The freezing process must also occur slowly to avoid formation of ice crystals inside the cells.

Reagents:
Cryotubes (1.0 or 1.8 ml from GIBCO)
Medium A: 50% FBS in RPMI 1640
Medium B: 20% DMSO + 20% FBS in RPMI 1640

Figure 7: Overview of the Lymphoprep separation principle. Blood is diluted 1:1 with PBS, and centrifuged at 650g for 20 min. After the centrifugation, the first layer, plasma, and the second layer, mononuclear cells were collected.
**Procedure:**
The cells were counted, and the number of tubes to be frozen down estimated (1x10^7 cells/tube). The empty cryotubes were cooled on ice or at -20°C, while medium A and B were put on ice. The cells were resuspended medium A to a concentration of 2 x 10^7 /ml, and an equal volume of medium B was added drop by drop to the cell suspension. DMSO in medium B is used as cryopreservant, and is added to cell media to reduce ice formation and thereby prevent cell death during the freezing. The cells were transferred to the cold cryotubes on ice, and immediately transferred to -80°C. The tubes were transferred into N2 tank after two weeks.

### 3.2.2.2 Thawing cells

**Reagents:**
cRPMI medium
PBS +2%FBS

**Procedure**
All the samples used in the thesis are stored long-term in liquid nitrogen at Institute of Immunology, OUS. The samples (cell lines, patient, and control samples) were thawed in water bath at 37°C until a small clump of ice remained, then transferred to a 50 ml tube, and 10 ml cRPMI medium was added slowly to the tube wall to let the cells adjust to the new osmolarity. Further, the cells were centrifuged at 200g for 5 min, and the supernatant was discarded. The pellet was resuspended in 10 ml cPRMI medium. Cell lines were transferred to a T125 culture flask (Thermo Scientific™, Waltham, Massachusetts), and incubated in a cell incubator (Forma Scientific) holding 37°C, 5 % CO2, and a humid atmosphere. Patient and control samples were used directly after thawing. Both K562 and Jurkats were maintained by splitting the cultures 1:10 every second day with fresh cRPMI. After ~25 passages (2 months) the cells were replaced by new batches of cells.

### 3.2.3 RNA isolation

RNA was isolated from the cell lines K562 and Jurkat, as well as from mononuclear cells isolated from the blood of pediatric patients diagnosed with acute leukemia and from healthy donors (Table 3 and 4, Appendix 2). The purpose of isolating RNA was to make cDNA. RNA from cell lines was isolated by TRI reagent, a reagent that allows isolation of total RNA (3.2.3.1). To isolate RNA from mononuclear cells, a kit specifically optimized for increased yield of microRNA, in addition to other RNA species, was used. This approach was chosen to allow for potential later analysis of microRNA expression in the patient samples.

RNA is easily degraded by ribonucleases (RNase) and therefore gloves were used at all time under the isolation procedure.
3.2.3.1 RNA isolation from cell lines

Reagents:
- TRI reagent (Sigma-Aldrich, St. Louis, Missouri)
- Chloroform (Sigma-Aldrich, St. Louis, Missouri)
- 2-propanol (Sigma-Aldrich, St. Louis, Missouri)
- 75% ethanol (VWR, Thermo Scientific™, Waltham, Massachusetts)
- DEPC (Diethylpyrocarbonate) H₂O (mirVana™ miRNA, Isolation kit)
- PBS

Procedure
Ten million K562 or Jurkat cells were harvested from viable cultures, pelleted at 300 g for 10 min, and washed two times with PBS. The pellet was lysed in 0.5 mL TRI reagent by pipetting, and the samples were incubated for 5 min at room temperature. Afterwards the samples were added 0.1 mL chloroform and the tube was shook for 15 sec before the samples were incubated for 15 min at room temperature. Next, the samples were centrifuged for 15 min, 4 °C, at 12,000 g. At this stage, 3 phases were visual. The colorless top layer was transferred to a new tube, and added 0.25 mL 2-propanol. The samples were incubated for 10 min at room temperature to allow precipitation of the RNA, before centrifugation at 4°C for 10 min at 12,000 g. The supernatant was removed, and the remaining pellet was washed by adding 0.5 mL 75% ethanol and centrifuged at 4°C for 5 min at 7,500 g. The ethanol was removed and any remaining ethanol left after pipetting was removed by letting it air dry shortly, taking care not to let the pellet dry out. As the final step, 20 μl DEPC- H₂O was added. The RNA isolated was further analyzed for a quality check (see section 3.2.3.3)

3.2.3.2 RNA isolation of patient samples

Reagents:
- RNA Homogenate additive (mirVana™ miRNA, Isolation kit)
- Acid – Phenol- Chloroform (Ambion®, Thermo Scientific™)
- 100% ethanol (VWR, Thermo Scientific™, Waltham, Massachusetts)
- Filter cartridge tube (mirVana™ miRNA, Isolation kit)
- miRNA wash solution 1 (mirVana™ miRNA, Isolation kit)
- miRNA wash solution 2/3 (mirVana™ miRNA, Isolation kit)
- DEPC –H₂O (mirVana™ miRNA, Isolation kit)

Procedure
PBMC from patients or healthy donors were thawed from -80°C or from liquid nitrogen as described (section 3.2.2.2). Thawed cells were washed two times with PBS, and spun at 300g for 10 min. The pellets were added 30 μl RNA Homogenate additive, and mixed by vortexing before incubation on ice for 10 min. Thereafter 300 μl Acid-Phenol- Chloroform was added with care, the samples were vortexed for 30-60 sec, and centrifuged for 5 min at 10,000 g. After the centrifugation the sample contained two layers: an upper aqueous phase and a lower phenol phase. The upper layer was removed carefully without disturbing the phenol phase into a new tube. 100% ethanol was added to the aqueous phase and the mixture was transferred to a filter cartridge tube. The filter cartridge tube was centrifuged for 15 sec at 10,000 g. The flow through was discarded and 700 μl miRNA wash solution 1 was added to the
filter, and centrifuged for 10 sec at 10 000g. The flow through was discarded after centrifugation. 500 μl miRNA wash solution 2/3 was added to the filter and centrifuged for 10 sec at 10 000g, flow through was discarded, and the whole step was repeated. After discarding the last flow through from the final wash, the empty filter cartridge tube was centrifuged for 1 min at 10 000g to remove any residual fluid and to dry the filter. The filter was transferred to a new cartridge tube, and 100 μl pre-heated DEPC – H₂O (95°C) was added to the center of the filter, and centrifuged at max speed for 30 sec to recover RNA.

3.2.3.3 Analysis of RNA quality on agarose gel
The isolated RNA was run on an agarose gel for quality analysis before it was used to generate cDNA.

Reagents:
- TAE
- Agarose (Sigma-Aldrich, St. Louis, Missouri)
- SYBR® Safe DNA gel stain (ThermoScientific)
- RNA samples
- 6x MassRuler™ Loading Dye solution (Fermentas, Thermo Scientific™, Waltham, Massachusetts)

Procedure:
A 1.5 % agarose gel solution was first made by mixing 3g agarose with 200 ml TAE buffer, and the mixture was boiled for 2 min in a microwave oven until it was dissolved. The soluble agarose was chilled to ~50°C, and 40 ml was mixed with 4 μl SYBR® Safe DNA gel stain. Next the soluble agarose gel was added to a casting tray with combs. When the gel was solidified, the comb was removed, and the gel placed in a gel chamber and covered with TAE buffer. The RNA samples, 1 μl, were mixed with 1 μl 6x MassRuler™ Loading Dye solution and 4 μl DEPC-H₂O prior to loading into wells (total volume 6 μl). The dye provides color to the samples to ease loading, and allow visual inspection of how far the samples are run into the gel. Moreover, the buffer contains high glycerol content, which makes the sample heavier than the surrounding TAE buffer, and will allow settling of the sample to the bottom of the wells. The voltage source was set at 70 voltages, 150 mA for 1 hour.

3.2.3.4. Measurement of RNA concentration by NanoDrop
Concentration and purity of RNA can be determined by measuring the absorbance at different wavelengths. Nucleic acids (both DNA and RNA) are traditionally quantified using UV absorption by a spectrophotometer, and the absorbance is measured at 260 nm and 280 nm. The concentration can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration.

To measure the concentration of isolated RNA, the NanoDrop 1000 Spectrophotometer (Thermo Scientific™) was used.

Reagents:
- 1μl RNA
- DEPC –H₂O
- NanoDrop 1000 Spectrophotometer (Thermo Scientific™, Waltham, Massachusetts)
- NanoDrop1000 3.7.1
To measure the concentration of the isolated RNA, both from cell lines and from PBMCs, 1 µl RNA was used. As a blank DEPC - H₂O was used, since DEPC - H₂O was used to resuspend the RNA after isolation. The program, NanoDrop1000 3.7.1, was set to nucleic acid and then RNA.

3.2.4 cDNA generation

RNA can be converted to DNA. A good example is the virus HIV, which have an RNA genome that is converted into DNA by the enzyme reverse transcriptase. This principle is used by scientists to convert mRNA into complementary DNA (cDNA) using reverse transcriptase. RNA is very easily degraded by RNases, therefore it is more convenient to use the more stable cDNA for analysis of mRNA. To convert the isolated RNA into cDNA, random hexamer primers were chosen. Random hexamer primers give the possibility to produce cDNA from all RNA species without knowing their sequence. The use of random hexamer primers is based on a work by Feinberg and Vogelstein (1983), and these primers consist of only 6 oligonucleotides (hexamer). These hexamers have every possible combination of bases, which in total makes 4⁶ different combinations and therefore potential to bind to any section of RNA. The DNA polymerase used in this thesis was the M-MLV Reverse Transcriptase, which is an RNA-dependent DNA polymerase purified from *E. coli* expressing the *pol* gene of M-MLV on a plasmid.

Reagents:

RNA
Random hexamer primers (Thermo Scientific™, Waltham, Massachusetts)
dH₂O

dNTP (10 µM) (Thermo Scientific™, Waltham, Massachusetts)
M-MLV RT 5x buffer (PROMEGA, Madison, WI)
DTT (0,1M) (Sigma-Aldrich, St. Louis, Missouri)
RNAsin R® Plus RNasin inhibitor (PROMEGA, Madison, WI)
M-MLV Reverse Transcriptase (PROMEGA, Madison, WI)
Thermal Cycler (Applied Biosystems, Foster city, California)

Procedure

1 µg RNA was used to make cDNA. The RNA concentration was measured by NanoDrop (section 3.2.3.3). RNA was mixed with 2 µl random hexamer primers and dH₂O up to a total volume of 15 µl. This mixture of 15 µl was incubated in a Thermal Cycler (Applied Biosystems) for 10 min at 70°C, and immediately afterwards cooled down on ice.

Further, the mixture was added 5 µl M-MLV RT 5x buffer, 2 µl dNTP, 1 µl DTT, 1 µl RNAsin inhibitor, and 1 µl M-MLV Reverse Transcriptase. This mixture of total 25 µl was incubated for 1 hour at 37 °C in the Thermal Cycler.

3.2.5 Polymerase chain reaction (PCR)

3.2.5.1 Primer design

A primer design tool, Primer-BLAST; provided by the National Centre for Biotechnology Information (NCBI) was used to design all the primers used in this thesis.
(http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Nucleotide sequences of each gene were also found in the NCBI database (Appendix 1 for accession numbers). All the primers were designed to have a GC-content over 50%, Tm between 57-63°C, if possible yielding a product of less than 200 bp, and if possible spanning an exon/interon site. Primers were ordered from Eurofins Genomics, Germany, Ebersberg.

3.2.5.2 Primer preparation

Primers were delivered as lyophilized powders in tubes, and these were spun for 10 sec at 13.000g to collect the primers at the bottom of the tube. The primers were solubilized in dH₂O to yield a concentration of 100 pmol/µl, and mixed thoroughly. A working solution of at 10 pmol/µl of both forward and reverse primer was made by diluting 10 µl forward and 10 µl reverse primer with 80 µl dH₂O. Primers were stored at –20°C.

3.2.5.3 Semi-quantitative PCR

Polymerase chain reaction (PCR) is a common method used to amplify both short DNA or RNA sequences. The technique is based on the natural DNA replication. To run a PCR, a gene specific primer is needed (both forward and reverse), a high tolerance polymerase (e.g. Taq DNA polymerase), the four deoxynucleotides (dNTP), and a suitable buffer.

The PCR goes through 3 steps to amplify DNA:

1. Denaturation
2. Annealing
3. Extension

During the denaturation step, the temperature is heated to above 90°C, causing a separation of the double-stranded DNA into two separate strands. The next step is annealing, a specific sequence is targeted by primers (both forward and reverse) when they anneal on each side of the targeted DNA sequence. The annealing temperature depends on the specific primer used for the DNA sequence. In the last step, extension, the temperature is increased to 72°C and the polymerase synthesizes new strands of DNA complementary to the target sequence. The enzyme adds nucleotides to the annealed primers, and as a result two identical copies of the original DNA are formed. With an increase in the amount of cycles, the amount of identical DNA copies will increase exponentially (per cycle exponential increase), and by the time 30 cycles is reached, over a million copies are made.

The purpose of using PCR in this thesis was to test whether the primers amplified the DNA sequences of predicted lengths.

Reagents:

dH₂O
5x Colorless GoTaq® Reaction Buffer (Promega, Madison, WI)
MgCl₂ (25mM) (Promega, Madison, WI)
dNTP (10 µM) (Thermo Scientific™, Waltham, Massachusetts)
cDNA
Primers (forward + reverse (10pmol/µl)) EuroFins Genomics
GoTaq® G2 DNA Polymerase (Promega, Madison, WI)
Thermal Cycler (Applied Biosystems by Life technology, Foster city, California)
**Procedure**

A PCR master mix containing 3 μl 5x Colorless GoTaq ® Reaction Buffer, 1.3 μl MgCl₂, 0.24 μl dNTP, cDNA, and dH₂O were made. The amount of dH₂O depended on the amount of cDNA added in the mix. The amount of cDNA differs because of the variable RNA concentration between samples. After using dH₂O to adjust the volume, the total volume of the PCR master mix was 14 μl. The master mix was aliquoted to PCR tubes, and was added 1 μl of the reverse and forward primer mix. The PCR tubes were inserted in the Thermal cycler.

An enzyme mix containing 3.9 μl dH₂O, 0.1 μl GoTaq ® G2 Polymerase and 1.0 μl 5X Colorless GoTaq R Reaction Buffer was made. This enzyme mix was added to the PCR tubes after the initial 3 min, as illustrated in the PCR program chart below.

The total amount in every PCR tube was 20 μl:
- PCR master mix = 14 μl
- Primer (forward and reverse) = 1 μl
- Enzyme mix = 5 μl
- Total: 20 μl

**PCR program:**

The PCR program varied depending on the annealing temperature of the primer (see Appendix 1). A touchdown* cycle was added as an initial step to increase the binding specificity of the primer with its intended target. An example is shown in fig.8. Also the amount of cycles varied depending on the primers, see Appendix 1 for details for each primer. *Touchdown was always set 5°C higher then the primers annealing temperature and was reduced with -1°C for every cycle (in total 5 cycles).

![PCR program chart](image)

**Figure 8: The PCR program used for the experiment.** The enzyme mix was added 3 min after starting the PCR program, and the annealing temperature was dependent on the primer used. *Touchdown was for 5 cycles, and started always 5°C higher than the annealing temperature.

### 3.2.5.4. Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA fragments of varying sizes. After separation, the DNA molecules can be visualized under UV light after staining with an appropriate DNA-binding dye. The dye used in this thesis was SYBR® Safe DNA gel stain. This dye has reduced mutagenicity compared to the commonly used ethidium bromide.
Reagents:
DNA ladder Fastruler™ – low range (50 – 1500 bp) (Thermo Scientific™, Waltham, Massachusetts)
TAE
Agarose (Sigma-Aldrich, St. Louis, Missouri)
SYBR® Safe DNA gel stain (Invitrogen, Thermo Scientific™, Waltham, Massachusetts)
DNA samples
6x MassRuler™ Loading Dye solution (Fermentas, Thermo Scientific™, Waltham, Massachusetts)

Procedure
A 1.5 % agarose gel solution was first made by mixing 3g agarose with 200 ml TAE buffer, and mixture was boiled for 2 min in a microwave till it was dissolved. The soluble agarose was chilled to ~50°C, and 40 ml was mixed with 4 µl SYBR® Safe DNA gel stain. Next the soluble agarose gel was added to a casting tray with combs. When the gel was solidified, the comb was removed, and the gel placed in a gel chamber and covered with TAE buffer. 10 µl of the DNA ladder Fastruler™ was added to one well, and the DNA samples was mixed with 1 µl 6x MassRuler™ Loading Dye solution prior to loading into wells. The voltage source was set at 70 voltages, 150 mA for 1 hour.

3.2.5.5 Quantitative PCR (qPCR)
The measurement of gene expression is important for understanding the pathogenesis of diseases and to study diverse biological processes. Quantitative PCR (qPCR) is a rapid and reliable method for quantifying gene expression in a given sample. For this reason qPCR was chosen for measuring the gene expression from cDNA. All RNA used in this thesis was extracted from mononuclear cells, from both patients diagnosed with acute leukemia and healthy donors. For normalization, the TBP references gene was chosen. TBP was selected as the preferential reference gene because of low variations in Ct values between different samples, and because of modest expression levels. Analysis of several potential references genes, and the selection process of a suitable reference gene can be found in the results (see section 4.1).

Fast SYBR® Green Master Mix contains buffer, dNTPs, DNA polymerase and SYBR Green dye. The dye is used as a fluorescent reporter, it binds to the minor groove of double stranded DNA (dsDNA) and emits light when bound to dsDNA.

The melt curve produced by qPCR is a used to assess whether it has produced a single, specific product, or not. One peak in the melt curve indicates one amplicon, more than one peak can sometimes indicate more than one amplicon, but not necessary always. Gel electrophoresis can be done as a follow up to ensure that only one amplicon was produced, when two peaks appears.

Ct (threshold cycle) is defined as the number of cycles required for the fluorescent signal to cross the threshold. The Ct levels are inversely proportional to the amount of the target nucleic acid in the sample.

Reagents:
Fast SYBR® Green Master Mix (Applied Biosystems™ by Life technology, Foster city,
3.1.6 Exosome isolation
One of the aims for this thesis was to analyze exosomes for detection of ligands for activating NK cell receptors and for detection of immunosuppressive molecules. For this reason we established a protocol to isolate exosomes from plasma with minimal plasma protein contamination.

3.2.6.1 Differential ultracentrifugation
The most commonly technique used for isolation of exosomes is ultracentrifugation, which relies on their physical properties. This technique is considered the "gold standard" for separating exosomes from cell cultures, but there exist currently no standardized protocols for separating exosomes from body fluids such as plasma. As a consequence, laboratories use different protocols. This technique is based on low-speed centrifugation to remove cells and large vesicles, followed by high-speed ultracentrifugation to pellet exosomes.
Reagents:
PBS
Lysis buffer NP-40 for exosomes (see Appendix 3)
AIM V® medium (1x) (Gibco, Thermo Scientific™, Waltham, Massachusetts)

Ultracentrifugation was tested on culture supernatants from the cell lines K562 and Jurkat. These cells are cultured in medium containing serum (fetal calf serum), which naturally contains exosomes from calf. Therefore, K562 and Jurkat cells were grown in AIM-V® serum-free medium for 24 hrs prior to exosome separation from the culture medium. These cells were washed two times in 40 ml PBS at 300g for 8 min. The cell pellet was added 20 mL of AIM V® medium and the cells were transferred to a culture flask, and placed in the cell incubator.

After overnight culture in AIM-V, the cell culture was transferred to a 50 ml tube and centrifuged at 300g for 10 min (fig.10). The supernatant containing exosomes were transferred to a new tube, taking care not to aspirate cells. The supernatant was re-centrifuged at 2000 g for 10 min. The supernatant was collected and centrifuged at 10 000 g for 30 min. The supernatant was transferred to an ultracentrifugation tube, and the tube was filled with PBS, making sure all the ultracentrifugation tubes weighed the same for balanced centrifugation. Next, the tubes were placed in the XL-90 ultracentrifuge, and the samples were centrifuged at 100 000g for 70 min. The supernatant was discarded from the ultracentrifugation tubes, leaving only the pellet in the tubes. The pellet potentially containing the exosomes was resuspended in PBS, and re-centrifuged at 100,000g for 70 min. The supernatant was discarded. The pellet was resuspended in 100 µl lysis buffer NP-40 for exosomes (see section 3.2.8.2 and Appendix 3) and stored at -80°C for later analysis.

Figure 10: Flow chart for differential centrifugation. The flow chart gives an overview over the order and steps to isolate exosomes by differential centrifugation.
3.2.6.2 – Total Exosome Isolation from plasma

As a second alternative for isolating exosomes, we utilized an exosome precipitation reagent developed by Thermo Scientific. This reagent is optimized for isolation of exosomes from plasma, and is designed to forces less soluble components (as exosomes) out of the solution, thereby allowing them to be collected by low-speed centrifugation.

Reagents:
PBS
Total Exosome Isolation Kit (from plasma) (Thermo Scientific™, Waltham, Massachusetts)

Procedure
Preparation of plasma samples

Frozen plasma samples (1 ml) were thawed in a water bath (37°C, ap. 2 min) and placed on ice. The plasma was transferred to an Eppendorf tube, and centrifuged at 2000g for 20 min at room. The supernatant was transferred to a new Eppendorf tube without disturbing the pellet (which could contain contaminating of cells and cell debris), and centrifuged at 10,000g for 20 min at room temperature. The supernatant was transferred to a new Eppendorf tube.

Isolation of exosomes

The prepared plasma was added 0.5 volumes of PBS and mixed by vortexing for 5 sec, and 0.2 volumes of the Exosome Precipitation Reagent were added to the sample. The sample was again mixed by vortexing until the solution was homogenous.

The sample was separated into 3 Eppendorf tubes:
a) 120 µl for total exosome isolation of RNA (for later miRNA analysis; not part of this thesis).
b) 320 µl for total exosome isolation for mass spectrometry or western blotting.
c) 1 mL for leukemic blast-specific exosome isolation.

All samples were incubated at room temperature for 10 min, after the separation, followed by centrifugation at 10,000 g for 5 min at room temperature.

The 3 exosome pellets were centrifuged again for 30 sec at 10,000 g to collect any residual reagent. Any residual supernatant was discarded carefully with a pipette, and the pellets containing exosomes resuspended in:

a) 200 µl RNALater (for later RNA separation, not part of this thesis)
b) Exosome pellets for mass spectrometry were lysed with 100 µl SDS lysis buffer – see Appendix 3 and section 3.2.8.1.
c) 200 µl PBS were added, exosomes resuspended, and magnet beads coated with antibodies directed at leukemic blast markers were added – see section 3.2.7.
3.2.6.3 Size Exclusion Chromatography (SEC)
A third strategy used to separate exosomes, was size exclusion chromatography (SEC). This is a one-step method that can be used to isolate exosomes from plasma, with reportedly less contamination by plasma proteins and thus a purer exosome fraction [72].

Reagents:
Sepharose CL-4B (Sigma-Aldrich, St. Louis, Missouri)
PBS with 0.32% trisodiumcitrate
Non – pyrogenic sterile- R filter (0.22 µm) (Sarstedt)
Syringe (2 mL) (B. BRAUN)
BD Luer-Lock<sup>TM</sup> 10 cm (BD Biosciences, San Jose, CA)
dH<sub>2</sub>O
Syringe (10 ml) (B. BRAUN)

Procedure
A SEC column was prepared by removing the plunger from a 10 ml syringe, and inserting a filter at the bottom of the syringe. The Luer – Lock<sup>TM</sup> was inserted onto the syringe to avoid leakage of fluid. Sepharose CL-4B, 10 ml, was carefully poured into the column, and another filter added on top. Since the sepharose were delivered in 20% ethanol, it was washed once with one column volume (10 ml) of filtered dH<sub>2</sub>O. A running buffer consisting of PBS with 0.32 % trisodiumcitrate (pH 7.4) was filtered through a 0.22 µm filter prior to use (0.22 µm filter). Then, 30 mL of the running buffer was applied to equilibrate the column. 1 mL filtered plasma (through 0.22 µm filters) were added to the equilibrated SEC column, followed by addition of 12 ml running buffer after the plasma had entered the sepharose. 26 fractions à 0.5 ml were collected.

After separation, the SEC column was washed with 30 mL filtered dH<sub>2</sub>O, then 30 mL 20% ethanol, and stored at 4 ºC. The 20% ethanol was added to prevent any microbial growth in the column.

3.2.7 Isolation of leukemic blast-derived exosomes
To isolate leukemic blast – derived exosomes. Magnetic beads coated with antibodies directed at the leukemia were used. Dynabeads and MACS beads are used for separation, by coupling the beads with antibody for the target. The beads respond to a magnetic field, allowing bound material to be rapidly and efficiently separated from the rest of the samples. Unbound material is simply removed by aspiration, and the bead-bound target washed by the use of the magnet.

3.2.7.1 – MACS beads

Reagent:
CD19 MACS beads (Miltenyi Biotec, Berisch, Gladbach Germany)
SDS lysis buffer
MS magnetic column (Miltenyi Biotec, Berisch, Gladbach Germany)

Procedure
100 µl CD19 MACS beads were added to the Eppendorf tube with isolated exosomes and incubated overnight on Eppendorf rotator in the cold room (4ºC).
The next day, 500 µl PBS was added to the tube and placed on ice. The separation of CD19⁺ exosomes was done by using a magnetic MS column. The MS column was placed on the magnet and washed by adding 500 µl PBS to the column and let it drip through, to the waste tube. After washing the column, the exosomes were added to the column, and the flow through was collected in a separate tube, this tube contained now CD19 negative exosomes. The MS column was washed again 3x with 500 µl PBS. The first wash was collected in the tube with the CD19/CD34-negative flow through, the other 2 washes were collected in the waste tube. To collect the CD19 positive exosomes, the column was removed from the magnet and 1 ml PBS was added, and the column flushed with a piston to collect the exosomes.

Next the CD19⁺ exosomal samples were centrifuged at max g-force for 15 min, and the supernatant was discarded while the pellet was kept. To lyse the CD19⁺ exosomes 100 µl SDS lysis buffer (see section 3.2.8.1) was added to the pellet and incubated at 95°C on the heat block for 7 min. After incubation, the lysed exosomes were centrifuged for 5 min at max g-force, and the supernatant was transferred to a new Eppendorf tube, which was frozen at -20°C, for later mass spectrometry analysis.

3.2.7.2 – Dynabeads

Reagents:
Pan Mouse IgG Dynabeads (Thermo Scientific™, Waltham, Massachusetts)
CD3 antibody (clone OKT3, produced from a hybridoma in the lab)
CD14 antibody (provided by F. Lund-Johansen, Institute of Immunology, OUS)
PBS

100 µl of the Pan Mouse IgG Dynabeads were added to an Eppendorf tube on the sterile bench, the Eppendorf was placed on the magnetic rack. The solution was removed and the beads were washed with 500 µl PBS + 2%FBS by removing the tube from the magnetic rack. Thereafter the tube was placed back on the magnetic rack and the buffer removed. The washing was repeated one more time, before 100 µl PBS + 2%FBS was added with 100 µl CD14 antibody or 3.12 µl of CD3 antibody. The tube was incubated for 30 min in order to coat the beads with the antibody. Afterwards, the beads were washed twice with PBS, and the beads finally resuspended in 100 µl PBS. From this tube, 10 µl was added to the isolated exosomes from ultracentrifugation. The exosomes with the Dynabeads was placed in a cold room overnight.

The next day, the tube was placed on the magnetic rack for 3 min, and the supernatant was removed. The tube was removed from the magnetic racket and washed twice by PBS. After the last wash the exosomes were lysed by lysis buffer NP-40 (see section 3.2.87.2 for protocol)
3.2.8 Preparation of protein lysates
To be able to analyze the protein content in the exosomes and in the exosome membrane the exosomes were lysed after isolation.

3.2.8.1 – Lysing exosomes with SDS lysis buffer

Reagents:
SDS lysis buffer (See Appendix 3)

Procedure
100 µl SDS lysis buffer was added to the exosome pellet, and the samples placed on a heat-block at 95°C for 7 min. Then the samples were centrifuged at 13,000 g for 5 min, and the supernatants were collected into a new Eppendorf tube and stored at -20°C for later analysis. This protocol was used for Western blot analysis.

3.2.8.2 – Lysate of exosomes with NP-40 lysis buffer

Reagents:
NP-40 lysis buffer (See Appendix 3)

20 µl NP-40 lysis buffer was added to the exosome pellet isolated after and incubated on ice for 20 min before centrifugation at 10,000 g for 10 min (4°C). Next the supernatant was transferred to a new Eppendorf tube and stored at -20°C for later analysis.

3.2.9 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a method widely used to separates proteins according to their molecular weight, based on differences in their electrophoretic mobility (a function of the length of a polypeptide chain and its charge). SDS is an anionic detergent applied to protein samples to denature and thereby linearize proteins and to impart a negative charge to the linearized proteins. In most proteins, the binding of SDS to the protein imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Depending on their size, each protein will move differently through the gel: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty. The acrylamide concentration of the gel can be varied, generally in the range from 5% to 25%. Lower percentage gels are better for resolving very high molecular weight proteins, while high percentages are needed to resolve smaller proteins. Here, we utilized a 4-20 % gradient gel. The SDS sample buffer added to the protein samples contains 2-mercaptoethanol that breaks down protein-protein disulphide bonds. SDS is also a component of the SDS running buffer, so the proteins stay negative charged and linear throughout the run.

Reagents:
4-20 % Tris-Glycine gel (Criterion™ TGX Precast Gels) (Bio-Rad, Hercules, California)
SDS running buffer
Precision Plus Protein™ Dual Color Standards (Bio-Rad, Hercules, California)
4x SDS sample buffer (see Appendix 3)
**Procedure**
The protein samples were added 4xSDS sample buffer, incubated at 95°C for 7 min, and thereafter centrifuged for 30 sec at 13,000g and cooled down on ice. The Criterion gel was rinsed briefly in dH2O, then inserted into an electrophoresis chamber (Criterion™ Vertical Electrophoresis Cell, BioRad). The upper and lower chamber was filled with SDS running buffer. The samples (10-20 µl) and a molecular weight ladder (5 µl) were loaded into appropriate wells, and the gel was run at 200V, 400mA for 50 min.

**3.2.10 Coomassie Blue staining**
Staining SDS-PAGE gels with the Coomassie Blue dye allows visualization of proteins as blue bands on a clear background. When the dye is applied to the SDS-PAGE gel, it will bind to proteins through ionic interactions between sulfonic acid groups and positive protein amine groups through Van der Waal attractions.

**Reagent:**
- Coomassie Blue R-250 (0.3mM) (Sigma- Aldrich, St. Louis, Missouri)
- Methanol (Sigma- Aldrich, St. Louis, Missouri)
- Acetic acid
- dH2O

**Procedure**
Coomassie Blue was first made by solubilizing 0.25 g Coomassie Blue R-250 in 400 ml methanol, followed by addition of 70 ml acetic acid. dH2O was added up to 1 L. The Coomassie Blue staining solution was added to the SDS-gel, until it covered the entire gel and incubated at room temperature for one hour. After incubation, the Coomassie Blue staining solution was removed and washed with dH2O 2 times for 5 min.

To remove excess Coomassie Blue staining, a de-stain solution was added to the gel. This de-staining solution was made up by 200 mL methanol, 100 mL acetic acid, and 700 mL dH2O. The SDS-gel was incubated at room temperature with the de-staining solution for 30 min, and then washed with the de-staining solution until the redundant color from the Coomassie Blue solution was removed.

**3.2.11 Western Blotting**
Western blotting is a common technique used to probe for proteins with antibodies. After SDS-PAGE, the proteins separated in the gel are transferred to a membrane (typically nitrocellulose or PVDF), and the membrane is stained with antibodies towards proteins of interest. Primary antibodies are used to mark the target proteins, and secondary antibodies coupled to a reactive enzyme (horse radish peroxidase, HRP) are used to label the marked protein, and the complex can be measured upon incubation in an ECL substrate solution. The presence of probed proteins will be visual as bands after developing the membrane by autoradiography.

**Reagents:**
- PierceG2 Fast blotter (Thermo Scientific™, Waltham, Massachusetts)
- Western blotting filter paper, extra thick Filter (Bio-Rad, Hercules, California)
- PVDF membran (Merck Millipore, Billerica, Masachusetts)
- Blocking buffer (see appendix 3)
Anti CD63 antibody (TS63) (Life technologies, Waltham, Massachusetts)
Donkey anti mouse HRP-conjugated antibody (Jackson ImmunoLabs)
Methanol (Sigma-Aldrich, St. Louis, Missouri)
dH2O
1-step Transfer Buffer (Thermo Scientific™, Waltham, Massachusetts)
SuperSignal® West Pico Luminol/Enhancer solution (Thermo Scientific™, Waltham, Massachusetts)
SuperSignal® West Pico stable Peroxide Solution (Thermo Scientific™, Waltham, Massachusetts)
ChemiDoc™-MP Imaging System (Bio-Rad, Hercules, California)
Image Lab 4.1 (Software)
CURIX 60 (AGFA, Greenville, South California)

**Procedure**

The proteins separated by the SDS-PAGE gel was transferred to a PVDF membrane using a PierceG2 Fast blotter, this is called electrophoretic transfer. First, the PVDF membrane was activated in methanol for 20 sec. The membrane was then washed for 1 min in dH2O till it was hydrophobic, and then placed in 1-step Transfer Buffer for 5 min. Two extra thick blotting filters were also soaked in the transfer buffer. The following “sandwich” was composed from bottom to top: filter, PVDF membrane, gel, filter. The transfer was run at 23V, 2.1A for 8:30 min. After transfer, the membrane was immediately transferred to a blocking buffer (5% skimmed milk – see Appendix 3), and incubated for 45 min on a rocker at room temperature. The blocking buffer prevents the antibodies to bind to the membrane non-specifically. Next, the blocking buffer was removed and replaced with the CD63 primary antibody in 5 % skimmed milk at 1/500 dilution. The membrane was incubated overnight in a cold room.

The next day, the primary antibody was removed, and the membrane washed 3x 5 min with TBS-T, before adding the secondary antibody diluted 1/8500 in TBS-T for one hour on the rocker. The secondary antibody contain the enzyme HRP (horseradish peroxidase), which is detected by the signal it produces corresponding to the position of the target protein. After the secondary antibody, the membrane was washed again 3x for 5 min with TBS-T. The washing with the TBST-T removes unbound antibodies and minimizes the background. After the last wash, the membrane was covered with 2 mL ECL substrates 1:1, and incubated for 5 min. The ECL substrate contains SuperSignal® West Pico Luminol/Enhancer solution, and SuperSignal® West Pico Stable Peroxide Solution. The luminol is oxidized in the presence of H2O2 by the enzyme HRP. After the oxidation the luminol is in an excited state, which decays to the ground state by emitting light. Using this method, it is possible to detect membrane immobilized specific antigens. After incubation, the membrane was developed by Hyperfilm in dark room by CURIX 60 and/or ChemiDoc™-MP Imaging System.

**3.2.12 Protein concentration measurement**

According to previous reports, cancer cells secrete a higher amount of exosomes compared to healthy cells. If this also applies to acute leukemia, exosome samples from plasma obtained from acute leukemia patients would contain higher amount of proteins, than exosomes from healthy controls. This was tested by measuring the protein concentration.
3.2.12.1 Infrared spectrometer
Direct Detect® infrared spectrometer measures amide bonds in protein chains, and thereby accurately determines an intrinsic component of every protein without relying on amino acid composition, dye properties, or reduction-oxidation (red-ox) potential. This technique was used to measure protein concentration of samples exosomes isolated from the Total Exosome Isolation kit.

Reagents:
Direct Detect® infrared spectrometer (Merck Millipore, Billerica, Massachusetts)
Direct Detect® spectrometer card (Merck Millipore, Billerica, Massachusetts)
SDS-lysis buffer for exosomes
dH2O

Procedure:
5 µl dH2O was mixed with the 5 µl isolated esosomes from the Total isolation of exosomes kit. As blank the SDS – lysis buffer (see Appendix 3) was diluted 1:1 with dH2O. Three µl of the sample was applied to 3 spots on the Direct Detect® spectrometer card, and the blank on a fourth spot.

3.2.12.2 Pierce BCA Protein Assay Kit
Pierce BCA Protein Assay Kit is a colorimetric protein assay, which is based on bicinchoninic acid (BSA) for the colorimetric detection and quantification of total protein. This method combines the reduction of Cu2+ to Cu1+ by protein in an alkaline medium with the highly sensitive and selective colorimeter detection of the Cu1+ using a reagent containing BSA. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one Cu1+. This complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentration over a broad working range. This assay was used to measure the total protein concentration of the resulting fractions from exosome isolation by SEC.

Reagents:
BCA Reagent A (Thermo Scientific™, Waltham, Massachusetts)
BCA Reagent B (Thermo Scientific™, Waltham, Massachusetts)
Albumin Standard Ampules (BSA) (2 mg/mL) (Thermo Scientific™, Waltham, Massachusetts)
VICTOR3™ (PerkinElmer precisely, Waltham, Massachusetts)
Elution buffer (see Appendix 3)

Procedure
Preparation of Diluted Albumin (BSA) standards
The standards with Albumin Standard Ampules BSA were made from 4000 µg/mL and diluted two-fold 8 times in total with elution buffer (PBS w/ 0,32% trisodiumcitrate). The elution buffer was used for dilution since the exosomes was eluted with this buffer during isolation by SEC.

Preparation of the BCA Working Reagent (WR)
Working Reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part BCA Reagent B (50:1, reagent A:B).
Measurement of the samples and standard
25 µl of each of the 26 fractions or each standard were added to a flat-bottom 96-well plate in duplicates, followed by addition of 200 µl of the WR. The plate was placed on a plate shaker for 30 seconds to mix, and thereafter incubated at 37°C for 30 min. After incubation the plate was cooled to room temperature and the absorbance was measured at 562 nm by VICTOR³™ multilabel plate reader.

3.2.13 Mass spectrometry preparation
Mass spectrometry allows analysis of the total proteome of the samples. In this thesis it was used for plasma-derived exosomes from patients and healthy controls.

The mass spectrometry analysis, sample preparation (lysis and trypsinization) was done by a technician, Maria Stensland, at the Proteomics Core Facility at Oslo University Hospital. The exosome samples (fractions) were delivered right after their isolation without any addition of reagent. The procedure below was done by Stensland, and is included for an overview of the process.

Reagents:
- PBS with 1% SDS
- FASP 10kDa filter
- Urea 8M
- Tris/HCl 0.1M pH 8.5
- Iodoacetamide 0.05M
- Ammonium bicarbonate
- Trypsin
- NaCl 0.5M
- 1% TFA
- MeOH
- ACN
- 1% FA
- EASY Spray PepMap®RSLC, C18, 2 µl, 100Å, 75 µm x 50 cm

Procedure:
Exosome lysis
100 µl 1% SDS in PBS was added to 100 µl exosome samples, the samples were vortexed and heated at 98 °C for 10 min, before sonicated for 10 min. Thereafter the samples were centrifuged at 14000 g for 10 min.

Filtering of exosome samples, FASP
Since SDS is not a detergent ideal to use when analyzing samples with mass spectrometry, it had to be filtered to not disturb the instruments.

75 µl of the lysed exosome samples was added to 10kDa FASP filter and mixed with 200 µl of 8M Urea in 0.1M Tris/HCl pH 8.5. The samples were centrifuged for 40 min at 14000g, and the flow through discarded. Then 100 µl then 100 µl iodoacetamide (0.05M iodoacetamide in 8M Urea in 0.1M Tris/HCl pH8.5) was added and mixed by 600 rpm for 1 min, and incubated for 5 min (without mixing). Next the samples were centrifuged at 14000g for 30 min, and added 100ul of 8M Urea in Tris/HCl. After this, the same step was repeated 3
more times, but the centrifugation time was set for 40 min. After adding the 100 μl of 8M Urea in Tris/HCl the fourth time, the samples were centrifuged again at 14000g for 40 min, then for 7 min twice. After the last centrifugation the filter was transferred to clean collection tubes and added 200 μl ammonium bicarbonate with 1 μg Trypsin, and incubated overnight in a wet chamber at 37°C. The next day, the samples were centrifuged for 40 min at 14000g and added 50 μl NaCl and centrifuged at 14000g for 20 min.

**C18 purification**
The supernatant from the previous centrifugation was added 100 μl 1% TFA, and the samples were centrifuged at 14000g for 10 min. Next the samples were purified on C18 micro columns. 3 discs were assembled in 200 μl pipette tip and activated by 50 μl MeOH, followed by 50 μl ACN. This was equilibrated twice by 80 μl 0.1% TFA before samples were added. The samples were then washed twice by 80 μl 0.1% FA and eluted by 80 μl 80% ACN/0.1% FA. Next the samples were speed vacumned in order to remove CAN and volume was adjusted to 14 μl using 0.1% FA, and the samples were frozen at -20°C until analysis.

**LC-MS/MS analysis**
The samples were analyzed by an EASY-LC coupled to QExactive with the column EASY Spray PepMap®RSLC, and column temperature was 60°C. Analyzed by a 120 min grad in single injections, 3 μl injected.

**3.2.14 Electron microscopy (EM)**
An electron microscope uses an “electron beam” to produce the image of the object and magnification is obtained by electromagnetic fields. This differs from light or optical microscope, in which light waves are used to produce the image and magnification is obtained by a system of optical lenses.

The samples of isolated exosomes were delivered to a technician, Sverre-Henning Brorson, at The Electron Microscopy Core Facility at Oslo University Hospital for images of the isolated exosomes as a tool to verify the techniques for isolation. Briefly, SEC fractions or exosomes from the Total Exosome Kit (solubilized in PBS) were delivered to the core facility. The exosomes were fixed in 2 % paraformaldehyde, and aliquots transferred to Formvar-carbon coated electron microscopy grids.
4 Results

4.1 Test runs of potential reference genes for qPCR analysis

The fluorescence–based qPCR assay has the ability to detect and measure minute amounts of nucleic acids in a wide range of samples from numerous sources. Several candidates as reference gene for normalization of the data were picked. Fig. 11 shows two qPCR runs for each reference gene to determine how stably expressed they were in different samples. All the experimental conditions were the same for all of the samples. We chose TBP, a TATA box binding protein, for use in normalization of the qPCR done on patients, healthy controls, and cell lines. The reason for choosing TBP was because we did not expect ligands for NK cell receptors to be highly expressed, and therefore needed to match with a weakly expressed reference gene. As Figure 11 illustrates, TBP had low Ct values. TBP was also observed to vary minimally after running it twice by qPCR, and also with minimal variation between samples. Therefore this was an ideal reference gene for our purpose. The TBP reference gene was suggested in a study to be the most reliable reference gene for the study of lymphoid malignancies [73] (see Appendix 1), it was shown to be expressed at low abundance and exhibited low variability among different types of lymphoid cells.

![Figure 11: Potential reference genes for normalization of qPCR data for expression analysis of ligands.](image)

The reference genes TBP, 18S rRNA, PKRG1, and GAPDH were run twice by qPCR with two healthy adult controls, and four patients diagnosed with acute leukemia. The expressed Ct values in the different samples indicate how stably the expression of the reference genes is. The expression of all the reference genes was stable, and the highest expression was found for 18S rRNA, while the lowest expression was found for TBP.
4.2 qPCR analysis of NKG2D, DNAM-1, and NKp30 receptor ligands

As previous studies have shown that cancer cells may express ligands for NK cell activating receptors, this was investigated for acute pediatric leukemia in this thesis. NKG2D have been shown to play an important role in tumor immune surveillance, and expression of its ligands MICA, MICB, and ULBP1-5 were therefore of interest. In addition the ligands for DNAM-1 and NKp30 were also investigated. By using qPCR the expression levels of the ligands in pediatric patients diagnosed with acute leukemia was tested, both acute lymphoblastic and acute myeloid leukemia. The qPCR measured the quantity of the ligand expression in real time from cDNA. The cDNA was generated by reverse transcription of extracted RNA from peripheral blood mononuclear cells from the patients, healthy adult controls, and from cell lines by reverse transcription PCR. As positive controls, the leukemia cell lines K562 and Jurkat were used as positive controls. These cell lines are known to express several ligands for NK cell activating receptors. By semi-quantitative PCR, expression of ligands for NKG2D, DNAM-1, and NKp30 were tested in Jurkat and K562 in order to test primer specificity and to test the ligand expression (see Appendix 6).

4.2.1 Expression of ligands for NKG2D in pre-B ALL, T-ALL, and AML patients

The cell lines K562 and Jurkat expressed some of the NK cell activating ligands. A semi-quantitative PCR were done for the ligands used in this thesis (see Appendix 6). K562 was observed to express MICA, MICB, ULBP4, BAG6, and CD155, while Jurkat cells was observed to express MICA, MICB, ULBP1, ULBP2, ULBP4, ULBP5, BAG6, and CD155. ULBP3 was not expressed in any of the cell lines by semi-quantitative PCR.

**MICA and MICB**

There was a trend towards higher expression of MICA in AML patients compared to healthy controls (p=0.0562, fig.12A). MICA was also observed to be expressed at somewhat higher levels in both pre-B ALL and T – ALL, although the expression levels were not significantly different from healthy controls. Neither pre-B ALL, T-ALL, or AML patients were observed to express MICB significantly different from healthy controls (fig.12A). The expression of MICA in K562 and Jurkat was very low by qPCR, especially for K562. This agrees with our result of the semi-quantitative PCR. In contrast, both K562 and Jurkat had high expression of MICB as expected.

**ULBP1-5**

Pre-B ALL samples were observed to variably express ULBP ligands. Some patients had low expression, while others had high expression of several ULBP ligands. In particular, it appears that a majority of the patients may express ULBP5 (fig.12A), although it was not to significance in this study. We only had access to 3 AML samples, but these samples all showed up-regulation of all ULBP ligands significantly different from healthy controls (fig. 12A). The highest significant difference was observed for ULBP1. In the one T-ALL sample, higher expression of ULBP1 and ULBP5 was observed, while expression of ULBP2-4 was comparable to healthy controls.

The expression of ULBP1 was observed to be very low for both K562 and Jurkat, this partially agrees with the semi-quantitative PCR data. Further, both K562 and Jurkat
expressed ULBP2 by qPCR, while semi-quantitative PCR suggested no expression by K562 cells. Further, Jurkat cells appear to express ULBP3 (not observed by semi-quantitative PCR) and ULBP5, while K562 cells weakly express ULBP5 as assessed by qPCR and not expressed as assessed by semi-quantitative PCR.

4.2.3 Expression of ligands for DNAM-1 in pre-B ALL, T-ALL, and AML patients
The AML samples significantly (p=0.0045) expressed the DNAM-1 ligand CD155, while pre-B ALL expressed comparable levels as healthy controls (fig. 12B). The T- ALL sample was observed to express the DNAM-1 ligand CD155 (fig. 12B). The K562 cells were not observed to express CD155, while the Jurkat cells seemed to express CD155, but at very low levels. Both of the cell lines were observed to express CD155 when semi-quantitative PCR was done.

4.2.1 Expression of ligands for NKp30 in pre-B ALL, T-ALL, and AML patients
Both AML (p=0.0013) and T-ALL patients were observed to express BAG6 at much higher levels than healthy controls, and almost at the same level. The pre-B ALL samples appeared to express BAG6 higher than healthy controls, although the difference was not to significance. Also the difference was lower than for both AML and T-ALL samples (fig.12C). K562 did not express BAG6, but Jurkat was observed to express BAG6. Also this ligand was observed to be expressed in both of the cell lines by semi-quantitative PCR (Appendix 6).
Figure 12: qPCR analysis of ligand expression for NKG2D, DNAM-1, and NKp30 activating NK cell receptors. The analysis was performed on eight healthy adult controls, seven pre-B ALL, one T-ALL, and three AML patients, including K562 and Jurkat cell lines. qPCR measurement of the expression of (A) ligands of the NKG2D receptor (B), the DNAM-1 receptor, and (C) the NKp30 receptor. RNA from mononuclear cells of healthy donors and acute leukemia patients was extracted and reverse transcribed. Relative copy numbers of NKG2D, DNAM-1, and NKp30 ligands were determined by quantitative PCR using specific primer pairs for the ligands and normalized with TBP expression. Statistical significance was calculated with a non-parametrical t-test with Welch correction.
4.3 Establishing a protocol for isolation of exosomes from plasma
One of the aims of this thesis was to identify ligands for activating receptors or immunosuppressive proteins in exosomes from leukemia patients. This could represent a mechanism used by tumor cells for immune evasion. The hypothesis is that tumor cells secrete exosomes to counteract tumor immune surveillance. With that another aim of this thesis was to establish a protocol for exosome isolation with minimal contamination of plasma proteins.

4.3.1 Exosome isolation by differential ultracentrifugation
Isolating exosomes have recently got a lot of attention, and there exist several protocols for isolating exosomes from different body fluids. Currently, there exist no consensus for isolation protocols, therefore verification is extremely important. By using electron microscopy, it is possible to verify whether the isolation of exosomes is accomplished or not. Several studies done on exosomes have employed differential centrifugation for isolating exosomes. The first method used in this thesis to isolate exosomes was therefore differential ultracentrifugation of serum-free culture supernatants from K562 or Jurkat cells. The resulting pellet was processed for electron microscopy, but the images did not show any structures that resembled exosomes (data not shown).

4.3.2 Total Exosome Isolation Kit
Since the differential ultracentrifugation protocol failed to isolate exosomes from plasma, we next tested a commercial kit (The Total Exosome Isolation from plasma kit) claiming to be simple and reliable for exosome isolation from plasma.

4.3.2.1 Electron microscopy images of exosomes isolated with the total exosome isolation kit
As the images from the electron microscopy in fig.13 shows, the isolation of exosomes using the commercial kit was successful. As mentioned in the introduction, exosomes have a cup shaped morphology. This cup shaped morphology was found in our sample after isolating exosomes, the arrows in fig.13 shows the typically cup shape of exosomes. The vesicles seemed to be in the expected size range (30-100 nm).
4.3.2.2 Protein content in exosomes isolated with kit

The exosomes isolated with the kit were analyzed by mass spectrometry to characterize presence of ligands for activating NK cell receptors as well as possible immunosuppressive molecules. Precipitated exosomes were lysed in an SDS-lysis buffer and further processed for mass spectrometry. The data from the mass spectrometry detected a very high amount of plasma proteins in the exosome samples, with 14 % Ig-proteins and 26 % other plasma proteins, in total making up 40 % of the proteins characterized (fig.14). This high amount of contamination was not ideal for our proteomic analysis of the exosomes, as the high abundance plasma proteins potentially could mask detection of low-abundance proteins such as ligands for activating NK cell receptors.

![Pie chart showing protein distribution in exosomes isolated with kit](image)

**Figure 14: Electron microscopy images of exosomes isolated with the Total Exosomes Isolation (from plasma) kit.** 2 representative photos from one sample processed for electron microscopy. The arrows shows examples of the cup shaped morphology of exosomes. The scale bar is at 500 nm.

4.3.3 Exosome isolation by size exclusion chromatography (SEC)

The isolation by the total exosome kit had high contamination of plasma protein as shown in fig.13. As our aim was to isolate exosomes with minimal plasma protein contamination, we therefore tested a third method where exosomes were isolated by SEC. SEC separates proteins according to size, and has been shown to be useful for separation of exosomes from plasma. The SEC protocol resulted in 26 fractions of 0.5 mL each.

4.3.3.1 Protein distribution of the fractions

To get an overview of the protein content in each of the 26 fractions isolated from SEC, an SDS gel was run and stained with Coomassie Blue to get an overview of the total protein content in the fractions. Previous SEC studies have observed exosomes to be located within fractions 5-10. From fraction 1 – 4 there were very low amounts of proteins, but from fraction 5 the amount of the proteins seemed to increase. The assumption was made that fraction 11 till 18 contained mainly plasma proteins, because of the high amount shown in fig. 15, especially in fractions 14 - 17. The remaining fractions (19-26) contain again a low amount of proteins.
Based on the result in fig.15 and previous literature, fractions 5-6, fractions 7-8, and fractions 9-10 were pooled. These fractions were first assessed by electron microscopy to verify presence of exosomes, and then further analyzed by mass spectrometry for analysis of exosomal protein content.

**Figure 13: Analysis of isolated exosomes by mass spectrometry.** The analysis from mass spectrometry of exosomes from Total Exosome Isolation (from plasma) kit showed high content of plasma proteins. Annotations of protein hits into functional clusters were performed with the DAVID database with gene names as input. ECM, extracellular matrix.

Based on the result in fig.15 and previous literature, fractions 5-6, fractions 7-8, and fractions 9-10 were pooled. These fractions were first assessed by electron microscopy to verify presence of exosomes, and then further analyzed by mass spectrometry for analysis of exosomal protein content.

**Figure 15: Protein analysis of SEC fractions.** SDS-PAGE and Coomassie Blue analysis of the 26 fractions from SEC, to visualize the protein content in each of the 26 fractions. Fraction 5-10 were chosen for further analysis.
4.3.3.2 Electron microscopy images of isolated exosomes by SEC
The electron microscopy images taken of fraction 5-6, 7-8, and 9-10 proved a successful isolation of exosomes from plasma. It seemed like fractions 9-10 (fig.16C) contained vesicles of the most uniform size, around 40-50 nm, compared to fraction 5-6 (fig.16A) and 7-8 (fig.16B). Particularly fraction 5-6 contained a heterogeneous mixture of vesicles in size range from 60-70 nm upwards to 150 nm. However, cup shaped exosomal vesicles were identified in all three samples as shown with an arrow in fig.15. This verification of exosome isolation led us to continue analysis of the exosomal proteome by MS.

4.3.3.3 Proteomic analysis of exosomes
Mass spectrometry is a tool that provides the ability to analyze the proteome of biological samples, including exosomes. Pooled fractions 5-6, fractions 7-8, and fractions 9-10 were processed for mass spectrometry, and subjected to SDS lysis to release membrane proteins prior to trypsinization. A total of 312 proteins were identified, and some of these are presented in the fig.17, 18, 19, and 20. One of the aims of this thesis was to characterize possible immunosuppressive molecules from acute leukemia-derived exosomes. As fig. 17 illustrates, the fractions 5-10 contained minimal plasma proteins, while fractions 11-13 and 21-26 contains the majority of the plasma proteins. The first 10 fractions seem to contain the least plasma proteins. This agrees with what was observed in fig.15, where the protein content was higher from fraction 11 onwards. However, there are still plasma proteins in the fractions containing exosomes, but as fig.17 illustrates, the intensity is lower for the majority of the plasma proteins compared to fraction 11 and upwards. For example, the protein coded by SERPING1 and the complement protein coded by C1QC are detected with high signal intensity. The latter is only found in the exosome fractions. According to the mechanism of SEC, this is an expected result, since the small molecules are excluded first and the largest least. Some Ig proteins/antibodies are also found in the exosome fraction, but at a lower intensity, compared to the fractions 11-26.
Characterization of exosome markers

Having verified that exosomes may be recovered in fractions 5-10, we chose to continue further analysis with fractions 7-8. These fractions displayed more homogenous size-distribution of exosomes than fractions 5-6 as evaluated by electron microscopy, and less contaminating plasma proteins than fractions 9-10. Exosomal fractions 7-8 from 2 healthy children controls and 8 pre-B ALL patient samples were analysed by MS. Among the MS data, four previously suggested exosome markers were detected (fig.18). CD9 is one of the most widely used markers out of the four markers we detected. Despite being so commonly used as an exosome marker, we observed it only in four of the samples (fig.18B). Exosomes are found to contain heat shock proteins, which were also identified in our exosomes. Fig. 18A illustrates the observation of Hsp70 (HSPA8), but only in half of the samples. In contrast, the LGALS3BP and CD5L (fig.18E and F) proteins were observed in all the samples, except for one in case of CD5L. LGALS3BP and CD5L were recently suggested as the most reliable markers for plasma-derived exosomes [74]. Besides the four suggested exosome markers, our MS analysis indicated presence of the protein coded by PRG4 in all the samples (fig.18D).

Importantly, the selectivity of the detected exosome markers found in our mass spectrometry analysis is illustrated in fig.19. HSPA8 is observed to be detected also in fractions 1-4 that do not contain exosomes (fig.19A). However, LGALS3BP, CD5L, and PRG4 are detected predominantly in the exosome fractions, in particular fractions 7-8 (fig.19B and C). PRG4 was only found in fractions 7-10, and with highest intensity in fraction 7-8. PRG4 has not been suggested as an exosomal marker previously (fig.19D), but appear to be a new candidate.
A common issue when isolating exosomes from plasma, seem to be a high contamination of exosomes from platelets. Platelets are known to secrete a high amount of exosomes, which is characterized by the expression of the integrin GPIIb/IIIa [75]. In our samples, we observed the integrin GPIIb (ITGA2B) in only 2 of the samples (Fig. 18C).

**Figure 18: Exosome markers.** Signal intensity values of four suggested exosome markers in exosomal preparations from 2 controls and 8 patients. (A) HSPA8 (B) CD9 (C) ITAG2 (D) PRG4 (E) CD5L (F) LGALS3BP. The proteins are named in the figure by their gene names.
Cancer associated proteins

Interestingly, we detected TGF-β1 in 5 of the 8 patient samples, and in none of the 2 control samples (fig. 20A). This indicates that TGF-β1 is only found in the exosomes from some of the acute leukemia patients. TGF-β1 is known to have an inhibitory effect on immune cells. In addition, purvate kinase muscle isozyme (PKM), a protein with two isoforms PKM1 and PKM2, were detected in exosomes of 2 of the patients. Which of the isoforms that was detected in the mass spectrometry data is not known. PKM have been associated with the energy production in cancer cells [76], and as fig.20C illustrates, this protein is not found in the exosomes from healthy controls. Three of the patient exosomes are observed to contain CD99, a cell surface glycoprotein (fig.20B). This protein is associated with several human tumors [77], and is reportedly highly expressed by pre-B ALL blasts. Another protein detected only in the exosomes from patients, was CD13 that is also associated with tumor cells and with pre-B ALL blasts (fig.20D). None of the known ligands for NKG2D, DNAM-1, or NKp30 were detected in the exosomes by mass spectrometry.
4.4 Measurement of the protein concentration from isolated exosomes and healthy controls

As discussed in the introduction, previous studies have observed an increase in secretion of exosomes in cancer patients compared to healthy controls. We wanted to investigate this phenomenon by measuring the protein concentration in the isolated exosomes from patients and healthy controls.

4.4.1 Protein concentration of isolated exosomes from the Total exosome isolation kit

The original project goal was to enrich for leukemia-derived exosomes using beads to separate CD19⁺ (pre-B ALL), CD14⁺ (AML), and CD7⁺ (T-ALL) blast-derived exosomes from the other exosomes in the plasma samples. This was not accomplished due to technical reasons. As a substitute clinical analysis, the total protein concentration was measured of all the exosomes isolated from the acute leukemia patient samples, in comparison to healthy controls. All separations were done from 1 ml plasma to allow direct comparisons. Detect® infrared spectrometer is a fast and easy method to measure protein concentration, and was therefore used for this purpose. The total protein concentration was significantly (P<0.0125) elevated in pre-B ALL patients (fig.21), which nicely fits with data from other cancer patients.

Figure 20: Cancer associated proteins detected in patient exosomes. (A) TGF-β1, (B) CD99, (C) PKM, and (D) CD13 is only detected in some of the acute leukemia pediatric patient samples, and in none of the healthy controls. Here only pre-B ALL samples are included. The proteins are labelled in the figure by their gene names.
We were also interested in the concentration of the proteins in the different fractions resulting from the exosome isolation by SEC. The Pierce BCA Protein Assay Kit was used for this purpose, and fig.22 gives an overview of the first 15 fractions of the total 26. As fig.22 illustrates, the protein concentration increase with the fraction number. The graph agrees with what was observed in fig.15. The protein concentration was only measured till fraction 15, as we knew the exosomes were in fraction 5-10, therefore fractions after fraction 15 was not of interest.

**Figure 22: Total protein concentration of fractions 1-15.**
The Pierce BCA Protein Assay Kit was used to measure the total protein concentration of fractions 1-15 from Size Exclusion Chromatography (SEC). The remaining fractions 16-26 was not measured total protein concentration.
Fractions 5-10 were confirmed to contain exosomes by electron microscopy (see fig. 16). We observed a trend towards increased protein concentration in fraction 5 in the pre-B ALL patients (fig. 23A), and this increase was significant in fraction 7 (p=0.0356) and 8 (p=0.0130) between pre-B ALL and the healthy controls (fig. 23B). After fraction 10 (fig. 23C) the difference between pre-B ALL and healthy controls were not significant anymore, likely due to the increased dominance of plasma proteins in the samples.

Figure 23: Total protein concentration of pre-B ALL higher than the negative controls. The exosomal fractions from pre-B ALL patients contains a higher concentration of proteins, than the healthy controls. (A) Fraction 5 and 6. (B) Fraction 7 and 8. (C) Fraction 10 and 13.
5 Discussion

5.1. qPCR analysis of ligands for activating NK cell receptors

5.1.1 Healthy controls used in qPCR analysis
When analyzing the gene expression of ligands for NKG2D, DNAM-1, and NKp30 by qPCR, no controls from healthy children were included, only healthy adults. It would have been preferable to have had included age-matched children as the control group, since the leukemia samples are from pediatric patients. The reason for not including any children controls was because of shortage of cells from this group. The analysis of pediatric leukemia patients is part of another on-going project in the lab, and so far there has been no surplus of cells from healthy children for qPCR analysis. However, the lack of inclusion of healthy children controls may not affect the interpretation of the results in this context, since PBMC from neither healthy adults nor healthy children should express the ligands for activating NK cell receptors.

5.1.2 Patient samples used for qPCR analysis
Pediatric T-ALL is a very rare disease, and only 1-2 new cases are diagnosed each year in Norway. Due to this reason, we had only material from one T-ALL patient. For AML, we had three samples. AML is also relatively rare amongst children, with 3-4 new cases each year. In contrast, more than 20 new cases are diagnosed each year with pre-B ALL. Therefore the majority of the acute leukemia patients samples in our study were pre-B ALL. The qPCR results of T-ALL and AML should therefore be interpreted with care, and more samples are needed to reach a conclusion on their expression of ligands for activating receptors.

5.1.3 Melt curves for ligands and reference genes
When running qPCR it is important to look at the melt curves for the primers used. The reason is to be sure that only one amplicon is detected. A high specificity primer will result in only one amplicon, detected as one main peak in the melt curve. In this thesis, we detected one main peak for all the primers used, but a small peak before the main peak was often observed (see Appendix 5). The small peak does not necessarily mean that our primers were not specific, but perhaps not as specific as intended. Semi-quantitative PCR was also run for all the primers towards ligands. The references genes were designed in a previous study, or was a commonly used reference gene in the HLA typing unit at the Institute of Immunology at Oslo University Hospital. Therefore the reference genes were not tested by semi-quantitative PCR. Surprisingly, the melt curves indicated that the reference genes also had a small peak before the main peak. This was not expected due to the reference genes being well characterized for this purpose. MICA and MICB barely had a second peak, even though their semi-quantitative PCR results indicated more than one amplicon. This might have been avoided if the semi-quantitative PCR settings were optimized even more. CD155 and BAG have a small second peak, but the semi-quantitative PCR results shows only one amplicon, this is an example that the peaks do not necessary mean that the primer are unspecific. The ULBP1 and ULBP2 melt curves were observed to have a minor second peak, but their semi-quantitative PCR indicate only a single amplicon. The ULBP3 primer had a small peak as well, but no bands at all were shown in the semi-quantitative PCR results. A possibility for the latter is that the primer might be more optimal for qPCR analysis.
ULBP4 and ULBP5 primers were observed to contain mainly one amplicon from the semi-quantitative PCR result. Of note, the melt curves for each primer were almost identical between different samples.

5.1.4 Ligands for NK cell activating receptors
The NK cell activating receptors of interests were NKG2D, DNAM-1, and NKp30. These receptors were chosen as their ligands are well characterized. They have also all been implicated in tumor immune surveillance by NK cells. Several other receptors, such as NKp44 and NKp46 are also implicated in tumor recognition, but their ligands are unknown. For this reason, these receptors were not included in this thesis. The known ligands for DNAM-1 are CD155 and CD112, initially both of them were set to be included, but the primer for CD112 turned out to be non-specific, and was therefore excluded. As for NKp30 ligands, the initial plan was to test for both BAG6 and B7-H6, but B7-H6 was mistaken for the T cell ligand B7-H3 (CD276). This mistake was detected very late, and therefore B7-H6 was not included. The CD16 receptor, which is the most powerful activating NK cell receptor was not included. The reason is that the CD16 receptor target antibodies. Another central activating receptor, NKG2C, was also not included as this receptor recognizes HLA-E in complex with viral peptide and the ligand thus not easily defined.

5.2 Methodological consideration
5.2.1 Reference genes for normalization of the qPCR measurement
In this thesis we decided to use TBP as a reference gene to normalize qPCR expression data. It was considered to use 18S rRNA as an additional reference genes, but due to its high expression level, the 18S rRNA reference genes was not included in normalization of the measured expression of the ligands for NK cell activating receptors, which are expressed at much lower levels. Using more than one reference genes for normalization provides a more accurate result. The reference gene PRKG1 was considered to be used as the second reference gene since this was the second lowest expressed reference gene after TBP, but lack of time to perform more experiments, limited us to only use TBP for normalization. As shown in the results (see section 4.1), TBP expression was very stable, and it is also a recommended reference gene for lymphoid malignancies [73], but another reference gene should had been included for normalization.

5.2.2 The use of SYBR green instead of TaqMan for qPCR analysis
When qPCR analysis was done for the ligand expression of NK cell activating receptors, SYBR green was used. There are many advantages to use SYBR Green; it is sensitive, simple to use, and inexpensive. TaqMan is known to be more specific, since the SYBR Green dye can bind to any dsDNA, including primer dimers, therefore it can give false positive signals. By analysing melt curves, one can assess the specificity of the qPCR. This is done in the thesis, as discussed earlier. The TaqMan probe (containg probe/reporter and quencher) is more specific because it uses a probe, which SYBR Green lack. For that reason TaqMan can be used to run multiplex qPCR assays to simultaneously amplify and detect different target sequences in the same tube. Since our melt curves turned out to have relatively high specificity, we decided to use SYBR green for our experiments.
5.2.3 Exosome isolation

Exosomes have caught several researchers’ attention during the last few years, but a consensus for a standard method for isolation of exosomes from plasma has not yet been reached. It was therefore necessary to establishing a reliable protocol for isolating exosomes from plasma with high purity.

There are both advantages and disadvantages with the three methods used in this thesis to isolate exosomes from plasma. The first method tried out was differential centrifugation of plasma to isolate exosomes based on low-speed centrifugation to remove cells and large vesicles, followed by high-speed ultracentrifugation to pellet exosomes. Differential centrifugation is the most common method used for this purpose, and seems to be the “golden standard” for isolating exosomes. Several researchers claim the ultracentrifugation to be an efficient method for exosome isolation. As mentioned in the results, this was not the case in this thesis. The images taken of the isolated samples by the electron microscope showed no signs of anything resembling the morphology of exosome vesicles. Besides being a method requiring a lot of time, the main disadvantage with this method was that it was less efficient to isolate exosomes from plasma. The method might be more efficient for biological fluids other than plasma and serum, because of their high viscosity. A study done on this observed that viscosity affects sedimentation of microvesicles (MVs) using ultracentrifugation. They hypothesized that increasing the duration of the ultracentrifugation might be a solution for plasma and serum [78]. Another disadvantage, which previous reports have observed, is high contamination of plasma proteins. Since the plasma-derived exosomes were to be used for protein analysis, it was an important factor that the isolation contained as little plasma proteins as possible.

The isolation kit from Thermo Scientific isolates specifically exosomes from plasma. The isolation kit showed to be very successful and gave a high yield of isolated exosomes. This was a less time consuming technique, the only issue was the high contamination with plasma proteins. The aim was to get as pure isolation of exosomes as possible from plasma. With that, this technique was not a preferable one. fig.14 illustrates the high plasma contamination. The isolation kit contained Proteinase K in addition to the Exosome Precipitation Reagent. Proteinase K was recommended in the protocol provided by Thermo Scientific, to remove plasma proteins, but it could partially degrade proteins expressed on the surface of the exosomes. This would have affected our results, since one of the aims was to test for presence of ligands for activating NK cell receptors, that would be found in the exosomal membrane. For this reason it was chosen to not use Proteinase K.

This further led to try SEC, after discovering a paper published in the end of 2015 [79], comparing ultracentrifugation with SEC. As the paper claimed, this was a very efficient technique to isolate exosomes from plasma with minimal plasma protein contamination, by separating several fractions. This was an easy one-step technique to isolate exosomes from plasma, but it was a very time consuming process because of the long run time through the SEC column during separation of exosomes. In addition it required much time to wash the column between every sample and before storage in the fridge, which limited simultaneous isolation of multiple plasma samples. However, despite these disadvantages, SEC was the only method that fulfilled the purpose of isolating exosomes with the least plasma protein contamination.
This process of establishing a reproducible and viable protocol for isolating plasma-derived exosomes consumed much time, which left less time for different analysis of the exosomes than the ones included in the thesis.

5.2.4 Isolation of leukemia-derived exosomes
Plasma contains exosomes from all the cells found in blood. In the case of a leukemia patient, the plasma also contains exosomes from leukemia cells. Therefore the purpose was to isolate the leukemia-derived exosomes, and analyze them by mass spectrometry. That way a comparison of exosomes secreted from leukemia, and other healthy blood cells could be done. This isolation turned out to not be successful, and due to lack of time further experimental optimizations of their separation was not possible. It is a challenge to work with exosomes, well known and established protocols may not work as well on exosomes, and further optimizing or modification is necessary. Both the MACS beads and Dynabeads are often used for isolation of a cellular target by means of antibodies coupled to these beads. In the case of this thesis, Dynabeads with CD14 and CD3 antibodies were used after isolation of the exosomes by differential ultracentrifugation. However, no exosomes were isolated. We also tested separation of leukemia-specific exosomes on the exosome population isolated by the Total Exosome Isolation Kit using MACS beads, but also with no success. Likely, more optimization of this protocol is needed.

5.2.5 Methods to measure protein concentration of isolated exosomes
In this thesis both Direct Detect®infrared spectrometer and Pierce BCA Protein Assay Kit was used to measure the protein concentration of exosomes after isolation. After using the Total Exosome Isolation (from plasma) kit for isolation, Direct Detect®infrared was used to measure the protein concentration. When exosomes were isolated by using SEC, it resulted in 26 fractions for each plasma sample. With so many plasma samples from both patients diagnosed with acute leukemia and healthy controls, it was a very expensive technique to use for so many samples. Therefore the Pierce BCA Protein Assay Kit was used to measure the protein concentration from the fractions from SEC.

5.2.6 Exosome markers
A widely used method to verify exosome isolation is Western blot analysis with a putative exosome marker. This can be done in addition to electron microscopy, but alone western blot is not reliable to verify the isolation of exosomes as there are still no consensus for standard markers for exosomes. In this thesis, several western blots were done with CD63 with exosomes isolated by both the Total Exosome Kit and by SEC. CD63 is a widely used marker, but all the blots were negative. We used both ChemiDoc™MP Imaging System and the CURIX 60 film developer to exclude sensitivity issues by either developer, but with no success. It seems like the CD63 protein, which has been used by several researchers as an exosome marker, did not work for us. Although it could be that the samples loaded to the SDS gel contained too few exosomes, we detected high protein levels in exosomes from the Total Exosome Isolation kit so we consider this unlikely. Moreover, we did not detect CD63 in any of the samples processed for MS. Other markers for exosome are also being used, as CD81, CD82, and CD9 (see section.1.4.2), and future experiments should include tests of these. Also, the new marker detected in our findings should also be tested to confirm it as a novel exosome marker, in addition to the two already suggested markers that were detected in almost all samples (see section 5.3.2)
5.3 Findings

5.3.1 Ligand expression for NKG2D, DNAM-1, and NKp30 in acute leukemia cells

**NKG2D**
In previous studies of the ligand expression on cancer cells for NK cell activating receptors, relative quantification assay have been a common method used for these ligands ([80], [81], and [82]). The NKG2D ligands MICA, MICB, and ULBP1-5 have been reported to be expressed in several tumors as extrahepatic cholangiocarcinoma (EHCC), colorectal cancer, and breast cancer ([83],[84], and [85]). In this thesis the aim was to examine the expression of the NKG2D ligands in acute leukemia in children. The pre-B ALL patient samples expressed all the NKG2D ligands, while T-ALL, and AML patient samples expressed all the ligands except MICB. Also, T-ALL did not express ULBP2. Previous studies have also shown that MICB is expressed at low levels in leukemia patients [25]. The AMLs have earlier been shown to express low levels of ULBP1, 2 and 3 in a study containing several AML samples, but only few expressed the 3 ULBPs [86]. Based on the pre-B ALL expression of the NKG2D ligands, the pre-B ALL is observed to have a heterogeneous expression of NKG2D ligands. This has been reported in previous studies as well for MICA, ULBP1, 2 and 3 [25]. In the T-ALL sub-group, only one patient sample was included, therefore a conclusion was not made by this sample alone. As fig. 12 illustrates, even though many pre-B ALL patient samples were included, the expression of the ligands vary considerably. Only three patient samples of AML were included, but a minimal variation is seen in them, this reinforces the result seen, but more samples should be included before reaching a firm conclusion. Also in the pre-BALL patient group, more samples should be included, due to the high variation from patient to patient.

**DNAM-1**
All the acute leukemia samples were observed to express CD155, and the expression is high in T-ALL compared to the two other acute leukemia groups, but again this is based on only one sample. The expression of CD155 is not to elevated in pre-B ALL compared to the healthy control, but the AML expression is significant. This has been observed in an earlier study as well. CD155 were observed to be expressed on almost all AMLs, independently on the FAB subtype, but less frequently expressed in lymphoblastic leukemias [86].

**NKp30**
The last activating receptors ligand we analyzed is NKp30 and its ligand BAG6. It was observed to be expressed in all the acute leukemia samples. The pre-B ALL samples are again seen to vary in expression level for BAG6. The NKp30 ligand BAG6 has been detected previous in plasma of CLL patients [87]. Even though we did not analyze the expression of B7-H6, it is a ligand found mainly in certain tumors such as gastric cancer and non-small lung cancers ([88] and [89]).

**K562 and Jurkat**
The acute leukemia cell lines K562 and Jurkat were used as positive controls. The ligand expression by these cell lines seem to vary between qPCR and semiquantitative PCR. In Table 4, an overview of the cell lines’ expression of ligands is shown for both of the methods.
Overall, we conclude that K562 and Jurkat may be useful as positive controls for most ligands, except for ULBP1.

**Table 4. Overview of K562 and Jurkat ligand expression.** The table compares the expression the cell lines expressed in qPCR with the expression by semiquantitative PCR.

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### 5.3.2 Detection of immunosuppressive molecules by mass spectrometry analyses

The mass spectrometry analysis of isolated exosomes from acute leukemia patients, did not result in any detection of the ligands for NKG2D, DNAM-1, or NKp30. This indicates that acute leukemia cells might not use exosomes as an immune evasion mechanism in context of expression of activating ligands, as hypothesized. It may also be that these ligands are below threshold for detection by MS, and that more sensitive techniques such as flow cytometry should be used to investigate this expression. Expression of immunosuppressive molecules in exosomes was also of interest. In our analysis, the cytokine TGF-β1 was detected in exosomes from several pre-B ALL patients. This cytokine was not detected in healthy children controls. TGF-β1 is known to be involved in the immune evasion of tumors. Studies done on tumor-derived exosomes from mesothelioma cell lines have reported observation of TGF-β1 in the exosomes, which seemed to downregulate NKG2D expression ([90] and [91]). Other proteins characterized as potential cancer-associated proteins in our study were CD13, CD99, and PKM. The PKM protein is typically expressed in cancer cells where it has an oncogenic function [92]. CD13 is expressed by tumor-associated blood vessels and is also shown to be highly expressed by pre-B ALL blasts. Likewise CD99 is also associated with several tumors, and again is highly expressed by pre-B ALL blasts [93]. These findings indicate that tumor cells might use exosomes as a survival mechanism as well as tumor evasion, and that exosomes may contain a signature of the proteins expressed by the tumor cells. Without further analysis it is not possible to be sure of the origin of the immunosuppressive proteins discovered. Here, isolation of blast-specific exosomes would have been useful.

**Exosome markers**

The CD63 tetraspanin was not found in the mass spectrometry analysis, which would explain why the western blot membrane did not show any signals for this protein. But CD9 was found in the exosomes from some patients. This is one of the most used markers for exosomes, still it was only found in four of the exosome samples. A study done on identifying novel markers for plasma-derived exosomes, suggested CD5L and LGALS3BP as the most reliable markers [93]. These two proteins were also detected in our mass spectrometry analysis, and expressed in almost all of the exosome samples. CD5L was
expressed in all of the samples except from one, while LGALS3BP was expressed in all of our samples. This verifies their study of using those two proteins as exosome markers, and it also verifies our exosome isolation to be successful with SEC. In addition we detected another possible novel exosome marker, PRG4. This was seen in all of the exosome samples analyzed by mass spectrometry. It was the only exosome marker, which was only detected in the exosome fractions 7-10, CD5L and LGALS3BP were also observed in other fractions than 7-10 as well. The B-cell marker CD19 was not observed in any of our pre-B ALL samples, this could explain why the isolation of exosomes with MACS CD19 beads did not work.

In addition, the protein coded by ITGA2, associated with platelet exosomes, is considered as a major contamination when isolating exosome from plasma. The platelets are known to produce a high amount of exosomes, therefore many platelet exosomes are included in the resulting exosome isolation sample. In our case, the protein was only detected in 2 of 10 the samples, which indicates that our sample preparation before SEC isolation have been very efficient in excluding the platelets, by filtration.

5.3.3 Fractions containing exosomes from SEC
Exosomes isolated by SEC was detected in fractions 5-10, but after analysis of these 6 fractions by electron microscopy and mass spectroscopy, we conclude that the fractions 7-8 are the best fractions for exosome analysis. Fraction 7 -8 seems to be the fractions that contains most homogenously sized vesicles as judged by electron microscopy, and with the least contamination of plasma proteins as assessed by mass spectrometry. In addition, fig.19 shows that the exosome markers are found with highest intensity in the fraction 7-8.

5.4 Conclusion
One of the aims of this thesis was to analyze pediatric patients diagnosed with acute leukemia for expression of ligands for NKG2D, DNAM-1, and NKP30. Our data suggest pre-B ALL to variably express all the NKG2D ligands analyzed. The ligand for DNAM-1, CD155, was not expressed in pre-B ALL, but BAG6 for NKp30 was expressed. We did not detect ligands for activating receptors in exosomes from patients. However, we did detect immunosuppressive molecules, such as PKM, TGF-β1, CD13, and CD99. Therefore, exosomes might have a role in the immune evasion as suppressors of immune activation. In addition we confirmed two of the earlier suggested exosomes markers, CD5L and LGALS3BP to be good exosome markers, and surprisingly we found another protein, PRG4, which have the potential to become a novel exosome marker. The identification of three exosome markers defined by MS in combination with electron microscopy shows that the exosome isolation by SEC is a very efficient method to isolate exosomes.
6 Future perspectives

In this thesis we have established a protocol for exosome isolation from plasma using size-exclusion chromatography. With a combination of mass spectrometric analysis and electron microscopy, we were able to define fractions 7-8 as containing exosomes with least plasma protein contamination. These fractions also contained relative homogenously sized vesicles within the size-range of exosomes, and selective expression of two acknowledged exosomal markers. In this thesis we did not detect the exosomal marker CD63 by western blotting, an option might be to use LGALS3BP and CD5L instead. Also, the size of the isolated exosomes should be quantified by the use of the nanosight technology.

Further studies should include MS analysis of more control samples, as only exosomes from 2 healthy controls were included in this study. Also, exosomes from AML and T-ALL patients should be investigated by MS and compared to the results from pre-B ALL patients. Moreover, another attempt at isolating blast-specific exosomes should be made, as we never attempted their separation from the SEC fractions. MS analysis of blast-enriched exosomes could yield detection of low abundance proteins, e.g. ligands for activating receptors, which could be below threshold for detection in a heterogeneous population as investigated here.

Also a protocol to perform immunophenotyping of the exosomes by flow cytometry could be developed. There exists protocols to capture exosomes on latex beads, which allows staining exosomes with antibodies for flow cytometric analysis. The advantage with this approach is the high sensitivity of antibodies that could lead to identification of low abundance proteins not detected by MS.

It would be interesting to test whether exosomes derived from patients have suppressive effects on NK cells. This could be achieved by culturing PBMC with exosomes, and analyze functional activity and expression of activating NK cell receptors by flow cytometry. We isolated exosomes for future miRNA/RNA analysis, and a future directive could be to investigate whether the exosomes contain RNA for activating ligands or miRNA species that may influence gene expression of activating receptors in NK cells.

In terms of analysis of expression of activating ligands on leukemic cells, more samples should be included. In a follow-up study, also B7-H6, the other ligand for NKp30, and CD112 for DNAM-1 should be included. Also, it could be desirable to include flow cytometric analysis of the ligands, as mRNA expression levels not necessarily predict protein expression.
References


## APPENDIX 1: PRIMERS

### PRIMER SEQUENCES

**Appendix Table 1:** Primer sequence, size, and accession numbers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Search numbers</th>
<th>Reverse and forward primers (5’ → 3’)</th>
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<td>MICB</td>
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<td>F: GTCGCCTTCCCTTTTGCAC R: TCCGGGGATAGAAGCTGGAA</td>
<td>647 bp</td>
</tr>
<tr>
<td>ULBP1</td>
<td>NM_025218.2</td>
<td>F: TCAAGGCGCTGTTGATGAAA R: GCCAGAGAGGTGTTTTTGTT</td>
<td>465 bp</td>
</tr>
<tr>
<td>ULBP2</td>
<td>NM_02517.2</td>
<td>F: CCGCTACCAAGATTCTTTTCTGT R: CCAGGATGAAACGCTGGCA</td>
<td>458 bp</td>
</tr>
<tr>
<td>ULBP3</td>
<td>NM_024518.1</td>
<td>F: TCGCGATTTCTCGTACTCTG R: GGAAGTCCCTAAGCCAGCTC</td>
<td>538 bp</td>
</tr>
<tr>
<td>ULBP4</td>
<td>AY25119.1</td>
<td>F: TGTGAAGGCGAGGTTCTTTTCTGT R: CTGCCACTCCACACTTTTGCC</td>
<td>595 bp</td>
</tr>
<tr>
<td>ULBP5</td>
<td>NM_00101788</td>
<td>F: GCTGCTGTATCAATGGGCG R: AGCTCTCCTGAACCTCTGGT</td>
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</tr>
<tr>
<td>CD155</td>
<td>NM_006505.4</td>
<td>F: TGTGTCACTGCTGACTTGGG R: GCTGGGATGCTCTGACTGTT</td>
<td>968 bp</td>
</tr>
<tr>
<td>CD112α</td>
<td>NM_00285.2</td>
<td>F: GTACCTCGGCGGTACTGATG R: CACACACAAATGCGCCTG</td>
<td>605 bp</td>
</tr>
<tr>
<td>CD112β</td>
<td>NM_001042724.1</td>
<td>F: GTTTCTCGGCGGTACTGATG R: CACACACAAATGCGCCTG</td>
<td>636 bp</td>
</tr>
<tr>
<td>BAG6</td>
<td>NM_004639</td>
<td>F: GAATGCCTGGCCTAATCCCG R: CTTAGAGGAGCAGGCTGGG</td>
<td>553 bp</td>
</tr>
<tr>
<td>18S sRNA**</td>
<td></td>
<td>F: CGGCTACACATCAGGCGAA R: GCTGGAAATCCACCGCT</td>
<td>*</td>
</tr>
<tr>
<td>TBP</td>
<td>**</td>
<td>F: GCACAGGGGAGCAAAGGTGA R: TCAAGGATCCACCATGTT</td>
<td>*</td>
</tr>
<tr>
<td>PRGK1</td>
<td>**</td>
<td>F: GGGAAAAAGATCTTCTGGAAG R: TTGAAAAATGGAAGCTCGGAA</td>
<td>*</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Provided by the HLA typing unit at Institute of Immunology, OUS</td>
<td>F: CCACCCATGCGCAATTCCATGGCAA R: TCTAGACGGGACGTCAACCGACC</td>
<td>*</td>
</tr>
</tbody>
</table>

*Primers only used for qPCR. ** Primer sequences from [73]
### Primes optimized annealing temperature and PCR cycles for semi-quantitative PCR

*Appendix Table 2:* Optimized primer annealing temperatures and total cycles.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Annealing temp.</th>
<th>Total cycles</th>
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<tbody>
<tr>
<td>MICA</td>
<td>54°C</td>
<td>40 cycles</td>
</tr>
<tr>
<td>MICB</td>
<td>54°C</td>
<td>40 cycles</td>
</tr>
<tr>
<td>ULBP1</td>
<td>59°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>ULBP2</td>
<td>59°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>ULBP3</td>
<td>63°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>ULBP4</td>
<td>54°C</td>
<td>40 cycles</td>
</tr>
<tr>
<td>ULBP5</td>
<td>54°C</td>
<td>40 cycles</td>
</tr>
<tr>
<td>CD155</td>
<td>63°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>CD112α</td>
<td>63°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>CD112β</td>
<td>63°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>BAG6</td>
<td>54°C</td>
<td>40 cycles</td>
</tr>
<tr>
<td>18S sRNA</td>
<td>54°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>TBP</td>
<td>54°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>PRGK1</td>
<td>52°C</td>
<td>30 cycles</td>
</tr>
<tr>
<td>GAPDH</td>
<td>59°C</td>
<td>30 cycles</td>
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</table>
APPENDIX 2: Patient and healthy control information

Patients used in this study

*Appendix Table 3:* Patients diagnosed with acute leukemia; pre-B ALL, T-ALL, and AML.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Birth year</th>
<th>Diagnosis</th>
<th>Risk group</th>
<th>Sample date</th>
<th>WBC at diag x10^9/L</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>P01</td>
<td>2000</td>
<td>AML M4</td>
<td>M4/M5 SR</td>
<td>03.01.14</td>
<td>99,2</td>
<td>qPCR</td>
</tr>
<tr>
<td>P02</td>
<td>2007</td>
<td>T- ALL</td>
<td>IR</td>
<td>03.01.14</td>
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<tr>
<td>P05</td>
<td>2002</td>
<td>pre B-ALL</td>
<td>SR</td>
<td>03.03.14</td>
<td>7,9</td>
<td>qPCR</td>
</tr>
<tr>
<td>P11</td>
<td>2011</td>
<td>pre B-ALL</td>
<td>IR</td>
<td>02.09.14</td>
<td>110</td>
<td>qPCR</td>
</tr>
<tr>
<td>P20</td>
<td>2004</td>
<td>pre B-ALL</td>
<td>IR (T21)</td>
<td>07.01.15</td>
<td>44,5</td>
<td>qPCR</td>
</tr>
<tr>
<td>P21</td>
<td>2004</td>
<td>pre B-ALL</td>
<td>IR</td>
<td>12.01.15</td>
<td>3,9</td>
<td>MS</td>
</tr>
<tr>
<td>P25</td>
<td>2008</td>
<td>pre B-ALL</td>
<td>HR</td>
<td>30.01.15</td>
<td>19,6</td>
<td>qPCR and MS</td>
</tr>
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<td>AML</td>
<td>M4/M5</td>
<td>15.04.15</td>
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<td>2001</td>
<td>pre B-ALL</td>
<td>SR</td>
<td>14.04.15</td>
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<td>2012</td>
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<td>IR (T21)</td>
<td>15.04.15</td>
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<td>qPCR and MS</td>
</tr>
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<td>SR</td>
<td>04.05.15</td>
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<tr>
<td>P44</td>
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<td>pre B-ALL</td>
<td>IR</td>
<td>01.12.15</td>
<td>9,50</td>
<td>MS</td>
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</table>
Healthy controls

*Appendix Table 4:* HAD = Healthy adult control, HCD = Healthy child donor. All the healthy controls were from humans.

<table>
<thead>
<tr>
<th>Controls</th>
<th>Adult/child</th>
<th>Sex</th>
<th>Age (years)</th>
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</thead>
<tbody>
<tr>
<td>HAD1</td>
<td>Adult</td>
<td>Female</td>
<td>42</td>
</tr>
<tr>
<td>HAD2</td>
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<td>Female</td>
<td>41</td>
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<tr>
<td>HAD3</td>
<td>Adult</td>
<td>Female</td>
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<tr>
<td>HAD4</td>
<td>Adult</td>
<td>Male</td>
<td>29</td>
</tr>
<tr>
<td>HAD5</td>
<td>Adult</td>
<td>Male</td>
<td>52</td>
</tr>
<tr>
<td>HAD6</td>
<td>Adult</td>
<td>Female</td>
<td>30</td>
</tr>
<tr>
<td>HAD7</td>
<td>Adult</td>
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<td>HAD8</td>
<td>Adult</td>
<td>NN</td>
<td>Unknown</td>
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<tr>
<td>HCD1</td>
<td>Child</td>
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<td>10</td>
</tr>
<tr>
<td>HCD2</td>
<td>Child</td>
<td>Male</td>
<td>16</td>
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<td>HCD5</td>
<td>Child</td>
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<tr>
<td>HCD6</td>
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</table>
APPENDIX 3: Buffers

Buffer recipes

1.5% Agarose gel solution (200mL)
Agarose 3g
1xTAE 200mL

Blocking buffer (skim milk)
Skim milk 2.5 g
1X TBS 50 mL

Buffer A
KCl 15 mM
Hepes 30 mM
EDTA 1 mM
MgCl$_2$ 1 mM

Elution buffer (PBS w/ 0.32% trisodiumcitrate)
PBS 1 mL
Trisodiumcitrate 32 mg
pH 7.4

SDS lysis buffer
Buffer A 900 μl
3% SDS 100 μl
PMSF (100mM) 10 μl
Protease inhibitor (100x) (Sigma) 10 μl
TCEP (100mM) 10 μl
NaVO$_3$ 10 μl

1x NP-40 Lysis buffer
NaCl, 300mM/Tris-HCl 50 mM 500 μl
NP-40 100 μl
dH$_2$O 370 μl
NaVO$_3$ 10 μl
NaF 10 μl
PMSF 10 μl

Lysis buffer for red blood cells
NH$_4$Cl, 155 mM
KHCO$_3$, 10 mM
EDTA, 0.1 mM
10x PBS (1L)
NaCl (1.37 M) 80 g
KCl (27 mM) 2 g
Na$_2$HPO$_4$ · 2 H$_2$O (43 mM) (anhydrous: 6.1 g Na$_2$HPO$_4$) 7.7 g
KH$_2$PO$_4$ (14 mM) 2 g
H$_2$O 1 L

1xPBS (1L)
10xPBS 100mL
ddH$_2$O 900 mL
pH 7.4

PBS+2%FBS
1xPBS 800 mL
FBS 16 mL

1xSDS sample buffer
2-ME, 0.1%
Bronephenol-blue 0.1% (Sigma Aldrich)
Glycerol, 10% (Sigma Aldrich)
SDS, 2% (Sigma Aldrich)
Tris-Hcl, 63mM pH 6.8

SDS Running Buffer (1 L)
10x TBS 50 mL
dH$_2$O 950 mL

10x TBS (tris-buffered salin) buffer (1L)
Tris-Cl (1M, pH 7.5) 24g
NaCl (1.5 M) 88g
Tris base 56g
dH$_2$O 900mL

TBS-T (1L)
10xTBS 100mL
dH$_2$O 900mL
Tween20 (Sigma Aldrich) 500µl

TAE (Tris/acetate/EDTA) buffer 50x stock solution
Tris base 242 g
Na$_2$EDTA-2H$_2$O 37.2 g
dH$_2$O 800 mL
Acetic acid 57.1 mL
dH$_2$O till 1L
Appendix 4: Ladders

Agarose gel electrophoresis – DNA ladder

**Appendix Figure 1**: FastRuler – Low Range, DNA Ladder (Thermo Scientific™) (www.thermofisher.com)

**SDS-PAGE - Ladder**

**Appendix figure 2**: Precision Plus Protein™ Dual Color Standards (Bio-Rad) (www.bio-rad.com)
Appendix 5: Melt curves of genes used for qPCR analysis

Appendix figure 4:
Appendix 6: Images of polymerase chain reaction (PCR) products

cDNA analysis by PCR

Appendix figure 4: Semi-quantitative PCR done for NK cell activating receptors, on the cell lines K562 and Jurkat.

RNA quality control by PCR

Appendix figure 5: Quality control of the isolated RNA, which is used to generate cDNA.
Appendix 7: Abbreviations

\( \mu l \) microliter
\( \mu g \) microgram
AML Acute myeloid leukemia
ALL Acute lymphoid leukemia
BSA bovine serum albumin
bp base pairs
CLL Chronic lymphoid leukemia
CML Chronic myeloid leukemia
cRPMI complete Roswell Park Memorial Institute
cDNA complementary DNA
DEPC diethyl pyrocarbonate
d\text{H}2\text{O} distilled water
DNA deoxyribonucleic acid
dNTP deoxynucleotide
dsDNA double stranded DNA
DTT dithiothreitol
FAB French-American-British
FBS fetal bovine serum
HAD Healthy adult donor
HCD Healthy child donor
HRP Horseradish peroxidase
MDSCs myeloid-derived suppressor cells
miRNA microRNA
M-MLV Moloney Murine Leukemia Virus
mRNA messenger RNA
MS Mass spectrotomery
MVBs multivesicular bodies
NCBI National Center of Biotechnology Information
NCRs natural cytotoxicity receptors
NK cells natural killer cells
\text{mL} milliliter
\text{mM} millimolar
\text{nm} nanometer
PCR polymerase chain reaction
PBMC peripheral blood mononuclear cell
PBS phosphate buffered saline
PMSF phenylmethane sulfonyl fluoride
pmol picomol
PVDF polyvinylidene difluoride
qPCR quantitative polymerase chain reaction
RNA ribonucleic acid
RNasin ribonuclease inhibitor
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC Size Exclusion Chromatography
TAE Tris acetate EDTA
TBS Tris buffered saline
TBS-T Tris buffered saline -Tween
TCEP Tris(2-carboxyethyl)