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In vitro cultivation of natural killer (NK) cells for autologous and allogenic cellular immunotherapy

Master's Thesis

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Terms and definitions

ADCC	**a**ntibody **d**ependent **c**ellular **c**ytotoxicity
ALL	**a**cute **l**ymphocytic **l**eukemia
AML	**a**cute **m**yelogenous **l**eukemia
BCR	**B c**ell **r**eceptor
BM	**b**one **m**arrow
BMt	**b**one **m**arrow **t**ransplantation
CCR	**C-C** chemokine **r**eceptor
CML	**c**hronic **m**yelogenous **l**eukemia
CTL	**c**ytotoxic **T l**ymphocytes
GvHD	**G**raft-**v**ersus-**H**ost **D**isease
GvL	**G**raft-**v**ersus-**L**eukemia
HLA	**h**uman **l**eukocyte **a**ntigen
HSC	**h**ematopoietic **s**tem **c**ell
HSCt	**h**ematopoietic **s**tem **c**ell **t**ransplantation
KIR	**k**iller cell **I**mmunoglobulin-like **r**eceptor
NK cell	**N**atural **K**iller **c**ell
NKR	**NK** cell **r**eceptor
PBMC	**p**eripheral **b**lood **m**ononuclear **c**ells
PBMCt	**p**eripheral **b**lood **m**ononuclear **c**ell **t**ransplantation
TCR	**T c**ell receptor
TLR	**T**oll-**l**ike **r**eceptor

Introduction

Natural Killer (NK) cells are lymphocytes of innate immune system that are critical in host defense and immune regulation. They have a fascinating capability to distinguish normal healthy cells from abnormal ones such as virus-infected cells or transformed tumor cells. Unlike cytotoxic T lymphocytes (CTL), NK cells do not require somatic recombination to detect target cells.

As recent data from Ruggeri *et al.* 2005 indicate, NK cells may be used after Bone Marrow transplantation (BMt) as method to prevent Graft-versus-host Disease (GvHD) and express Graft-versus-Leukemia activity (GvL), with survival ratio up to 100%. There are only 10-15% of NK cells circulating in peripheral blood, so the main limiting factor for such therapy is the amount of peripheral NK cells.

Current work is based on the results of some pilot experiments in the "NK cells usage in immune therapy" project supported by Competence Center for Cancer Research in collaboration with Tartu University Department of Hematology and Oncology. The main goal of this project is to establish a protocol for NK cells' expansion *in vitro*, including medium, supplements, growth conditions and development of suitable vessels.

First autologous bone marrow transplantation in Estonia was performed in 1993 by professor Hele Everaus. During the period 1993-2006 there has been 149 autologous and 27 allogenic PBMCts performed. Development of new immunotherapy methods would lead estonian transplantology to better outcomes and prolonged survival of patients.

The main goals of the current work are characterization of donor and patient NK cells proliferation dynamics *in vitro* and NK cells' cytotoxicity differences.

1. Literature overview

1.1. The biology of human NK cells' subsets and their functions

In the 1970s, several groups described a spontaneous cytotoxic antitumor activity in the spleens of unmanipulated mice and rats and the peripheral blood of normal human subjects (Rosenberg, McCoy *et al.* 1974; Kiessling, Klein *et al.* 1975). Although initially received by scientific community with a healthy dose of skepticism, the initial observations were rapidly confirmed, and the term natural killer cell durably entered the immunological lexicon (Di Santo 2006).

NK cells are granular lymphocytes that develop from bone-marrow precursors. Although they resemble T and B cells in many respects, NK cells do not express antigen receptors encoded by genes that undergo recombination-activating gene-dependent recombination. So NK cells are considered to be cells of the innate immune system (Herberman 1974; Herberman, Nunn *et al.* 1975; Kiessling, Klein *et al.* 1975; Raulet and Vance 2006).

There are about 5-15% NK cells among peripheral blood lymphocytes (Cooper, Fehniger *et al.* 2001). NK cells have different functions: they have the ability to attack cells that lack expression of MHC class I molecules or have decreased expression of MHC class I molecules (*see principles of "Missing self" hypothesis*) or virus-infected cells, to produce numerous cytokines and chemokines (Raulet and Vance 2006) and also to serve as a bridge between innate and adaptive immunity by expressing some co-stimulatory ligands for T or B cells (Blanca, Bere *et al.* 2001; Zingoni, Sornasse *et al.* 2004; Orange and Ballas 2006).

NK cells can kill certain virally infected cells and tumor target cells regardless of their MHC expression. NK cells possess relatively large numbers of cytolytic granules, which are secretory lysosomes containing perforin and various granzymes. Upon contact between an NK cell and its target cell, an immunological synapse is formed and the contents are extruded to effect lysis.

Morphologically, most NK cells are large granular lymphocytes. They are bigger than normal lymphocytes and have more cytoplasm (Orange and Ballas 2006). NK cells lack expression of the antigen receptors that are expressed by B cells and T cells, the B

cell receptor (BCR) and T cell receptor (TCR) respectively (Shi and Van Kaer 2006). Phenotypically, NK have several unique markers on their surface, but are most traditionally characterized by being $CD56^{+}CD3^{-}$. The CD56 antigen is an isoform of the human neural-cell adhesion molecule on NK cells. Its function in hematopoietic system remained unknown, until recently. Nowadays there is increasing body of evidence that one of the CD56 functions is maintenance of hematopoietic stem cells (HSCs) (Zhao, Wang *et al.* 2006; Wang, Hisha *et al.* 2007).

Human NK cells are a heterogeneous population. Early studies have revealed that NK cells can be divided into two main subsets, based on their cell-surface density of CD56 – $CD56^{bright}$ and $CD56^{dim}$ each with distinct phenotypic properties and distinct roles in human immune response (Cooper, Fehniger *et al.* 2001; Farag and Caligiuri 2006). These NK cells' subsets show important differences in their cytotoxic potential, capacity for cytokine production, and responses to cytokine activation. More than 90% of peripheral blood NK cells belong to the $CD56^{dim}$ subset, which possess cytotoxic activity against target cells. $CD56^{bright}$ NK cells' subset has reduced cytotoxic activity and comprises only 10% of peripheral blood NK cells. However, it is dominant in lymph nodes (about 90%) and tissues. This population is responsible for cytokine and chemokine production (Penack, Gentilini *et al.* 2005; Farag and Caligiuri 2006).

Almost 90-95% of peripheral NK cells are $CD56^{dim}/CD16^{bright}$. CD16 is the low-affinity FcγRIII on the surface of NK cells, which binds to antibody-coated targets and signals through associated subunits containing an immunoreceptor tyrosine-based activation motive (ITAM) to direct antibody-dependent cellular cytotoxicity (ADCC). This NK cells' subset shows lymphokine activated killing activity and natural cytotoxicity (Cooper, Fehniger *et al.* 2001)[a]. $CD56^{dim}$ cells are rich in lytic granules such as perforin and granzyme serine proteases, thus morphologically these cells seem more granular (Cooper, Fehniger *et al.* 2001; Farag and Caligiuri 2006; Moretta, Bottino *et al.* 2006). Target cell killing by NK cells mainly relies on degranulation, which leads to release of perforin- and granzyme-containing lysosomes upon formation of immunological synapse between NK cell and target cell. Granzymes, predominantly granzyme A and B, then initiate caspase-dependent and caspase-independent apoptotic pathways, which rapidly lead to target cell death (Voskoboinik and Trapani 2006). The $CD56^{dim}$ NK cells' subset has high-level expression of killer immunoglobuline-like receptors (KIRs) and C-C

chemokine receptors (CCR) and low-levels of C-type lectin receptors like CD94/NKG2A (Cooper, Fehniger *et al.* 2001; Farag and Caligiuri 2006).

The other 5-10% of NK cells are $CD56^{bright}$ NK cells are – primary population of NK cells that produces immunoregulatory cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)- α, TNF-β, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-10, and IL-13 following monokine stimulation. These cells are poor in CD16, so they are less effective mediators of ADCC and natural cytotoxicity (Cooper, Fehniger *et al.* 2001; Hokland and Kuppen 2005; Penack, Gentilini *et al.* 2005; Farag and Caligiuri 2006). $CD56^{bright}$ NK cells have high-level expression of the inhibitory CD94/NKG2A C-type lectin NK receptor but have low-level expression KIRs. This NK cells' subset expresses a number of cytokine and chemokine receptors constitutively, including the high-affinity interleukin-2 receptor (IL-2Rαβγ) and chemokine receptor 7 (CCR7), is involved in trafficking to secondary lymph nodes, is also found in $CD56^{bright}$ NK cells (Farag and Caligiuri 2006). Summary for receptors and functions of both NK cells subset is in Figure 1.

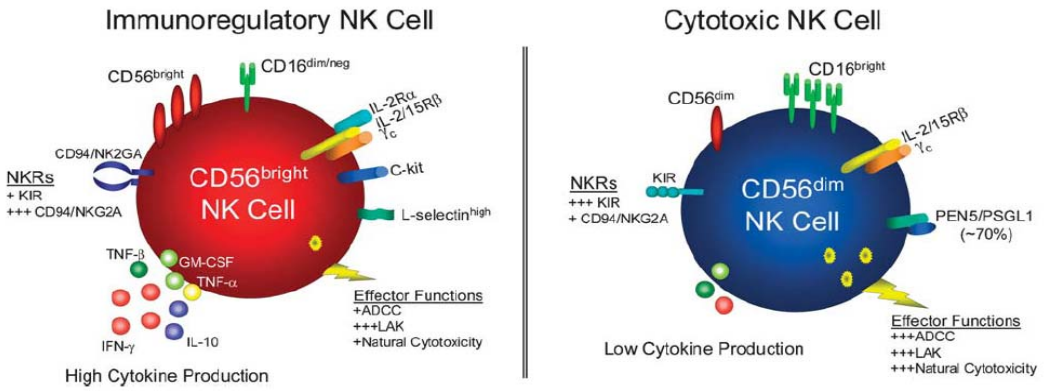


Figure 1. Human NK cells' subsets. **a)** Immunoregulatory NK cell. CD56^{bright} NK cells produce high levels of cytokines following stimulation with monokines. This subset has low-density expression of CD16 and exhibits low natural cytotoxicity and antibody dependent cellular cytotoxicity (ADCC), but potent lymphokine-activated killer activity. CD56^{bright} NK cells have high-level expression of the inhibitory CD94/NKG2A C-type lectin NK receptor (NKR) but have low-level expression of killer Ig-like receptors (KIRs). This NK cells' subset expresses a number of cytokine and chemokine receptors constitutively, including the high-affinity interleukin-2 receptor (IL-2Rαβγ) and C-C chemokine receptor 7 (CCR7), is involved in trafficking to secondary lymph nodes, is also found in CD56^{bright} NK cells. **b)** Cytotoxic NK cell. By contrast, CD56^{dim} NK cells produce low levels of NK-derived cytokines but are potent mediators of ADCC, LAK activity and natural cytotoxicity, and have a more granular morphology than CD56^{bright} NK cells. The CD56^{dim} NK cells' subset has high-level expression of KIRs and chemokine receptors. CD56^{dim} NK cells lack L-selectin but highly express PEN5-P selectin glycoprotein ligand-1 (PSGL-1), another adhesion molecule. Abbreviations: γc, common chain; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon γ; TNF, tumor necrosis factor. Figure adapted from Farag and Caligiuri 2006, text by Cooper, Fehninger *et al.* 2001; Farag and Caligiuri 2006.

1.2. Principles of "Missing self" hypothesis

The "missing self" hypothesis has been a guiding principle for understanding target cell recognition by NK cells for more than 15 years. According to this hypothesis NK cell receptor engagement by MHC class I inhibits NK cell-mediated lysis of target cell expressing MHC class I (self), thereby directing the cytolytic activity of NK cells against virally infected or tumor cells that lost MHC class I expression (non-self) (Ljunggren and Karre 1990).

Although NK cells are prepared to kill abnormal cells and rapidly release cytokines, they are normally restrained by inhibitory receptors that recognize target-cell-expressed MHC class I molecules and allow NK cells to survey tissues for normal MHC class I expression (Karre 2002). When MHC class I molecules are downregulated or absent, NK cells are released from the inhibitory influence of these receptors and kill target cells more efficiently(Karre 2002; Orange and Ballas 2006; Yokoyama and Kim

2006). It has been proposed that NK cells all express at least one inhibitory receptor that recognizes self MHC to provide NK cell tolerance and to prevent inappropriate NK cell responses directed at self (Karre 2002; Yokoyama and Kim 2006). However, release from inhibitory receptor effects does not automatically lead to NK cell activation against cellular targets (Figure 2). NK cells also express different combinations of various activation receptors, allowing them to respond to ligands on potential target cells (Ljunggren and Karre 1990; French and Yokoyama 2004; Kumar and McNerney 2005; Raulet and Vance 2006). Thus, "missing self" recognition model represents a delicate balance between activatory and inhibitory signals that are delivered by cell-surface receptors belonging to many families. These signals either cooperate with or antagonize each other (Long 1999; Vivier, Nunes *et al.* 2004; Anfossi, Andre *et al.* 2006; Bryceson, March *et al.* 2006).

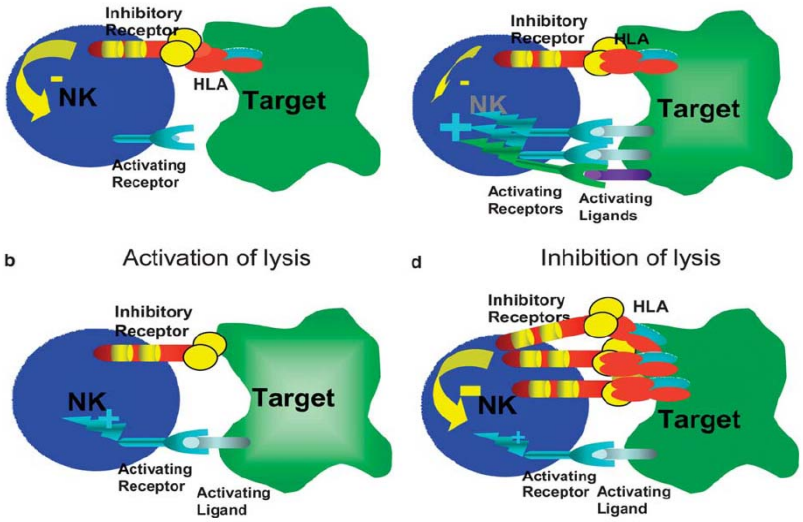


Figure 2. Regulation of NK cell response by activatory and inhibitory receptors. The response of NK cells is regulated by a balance of signals from activatory and inhibitory receptors. a) In the absence of an activatory receptor/ligand interaction, lysis is inhibited when inhibitory receptors engage cognate HLA class I molecules on the surface of the target cell. **b)** Lysis occurs when activatory receptors engage their ligands on target cells in the absence of inhibitory receptor/ligand interactions. **c)** The activatory receptor/ligand interactions predominate over weaker inhibitory receptor/ligand signals with the net result of NK cell activation and target cell lysis. This may occur when activation receptors and/or ligands are up-regulated thereby amplifying the net activation signal to exceed the inhibitory signal. **d)** A predominance of inhibitory receptor/ligand interactions result in a net negative signal that prevents NK cell lysis. Figure and text adapted from Farag and Cligiuri 2006.

1.3. Receptors of NK cells and their functions

NK cells are programmed to kill and require inhibitory signals from normal autologous cells to prevent unwanted cell death. Activatory receptors of NK cells can initiate adhesion, cytotoxicity, and cytokine release. The key regulators, however, are inhibitory receptors (Stewart, Vivier *et al.* 2006).

Functional types of NK cell receptors are: **a)** inhibitory: KIRs and LIRs (leukocyte immunoglobulin-like receptor molecules) that discriminate different allelic groups of MHC class I molecules (in human HLA – Human Leukocyte Antigen class I molecules), CD94-NKG2A -heterodimer, that belongs to the lectin family of proteins and recognize HLA-E (Moretta *et al.,* 2004) and **b)** activatory: some KIRs, natural cytotoxicity receptors, toll-like receptors, NKG2D and CD16 (causes antibody dependent cellular cytotoxicity).

1.3.1. Inhibitory KIR molecules

Encoded on chromosome 19q13.4 in the leukocyte receptor complex, KIRs comprise a family of 15 receptors that include both inhibitory and activatory members (Rajalingan 2002; Anfossi, Andre *et al.* 2006; Gasser and Raulet 2006; Raulet 2006). On human NK cells, members of the KIR family (also referred to as CD158), mediate recognition of various HLA-A, -B, -C alleles (KIR3D for HLA-A and B and KIR2D for HLA-C) (Moretta, Bottino *et al.* 2006). These receptors are type I membrane proteins that contain either two or three extracellular Ig-like domains, and hence are designated KIR2D or KIR3D respectively (Figure 3). The cytoplasmic domains of the KIRs can be either long – depicted as L or short depicted as S, corresponding to their function as inhibitory or activatory receptors, respectively (Boyington, Motyka *et al.* 2000; Natarajan, Dimasi *et al.* 2002; Rajalingan 2002; Santourlidis, Trompeter *et al.* 2002).

Ligation of inhibitory KIRs by their HLA ligands on healthy cells results in the inhibition of NK cell activation and protection of HLA^+ cell from NK cell lysis (Boyington, Motyka *et al.* 2000). As inhibitory KIRs contain one or two immunoreceptor tyrosine-based inhibitory motif (ITIM**)** sequences in their cytoplasmic domains that, when tyrosine phosphorylated, it recruits and activates SHP-1 phosphatase, leading to inhibition of signaling (Natarajan, Dimasi *et al.* 2002).

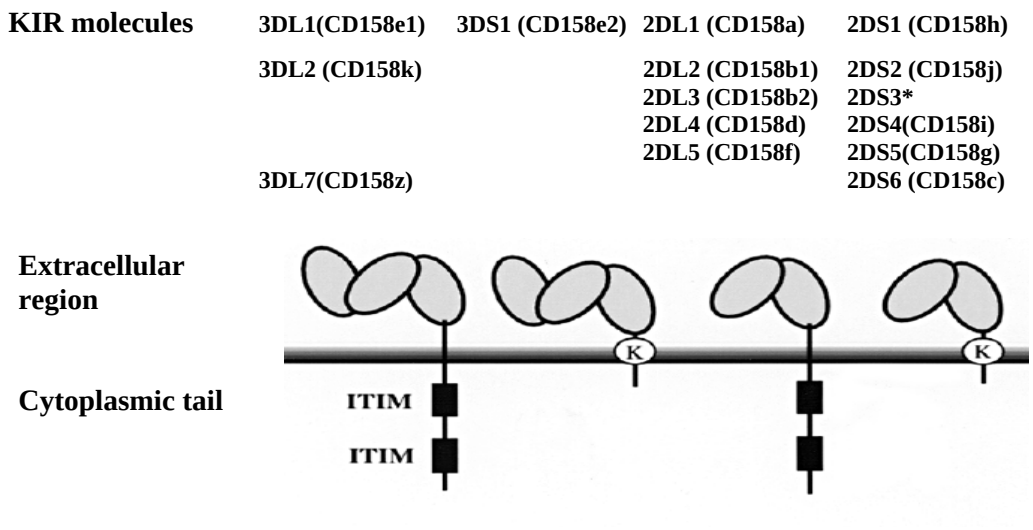


Figure 3. Activatory and inhibitory KIR and KIR nomenclature. KIR molecules contain either two or three Ig-like domains, and hence are designated KIR2D or KIR3D, respectively. The cytoplasmic domains of the KIRs can be either long (L) or short (S), corresponding to their function as inhibitory or activatory receptors, respectively. Inhibitory KIRs contain one or two ITIM sequences in their cytoplasmic domains. Activatory receptors, on the other hand, do not signal directly signal. KIR nomenclature is also depicted.

Figure adapted from (Rajalingan 2002). Text adapted from Natarajan, Dimasi *et al.* 2002. *- no CD assigned (Santourlidis, Trompeter *et al.* 2002).

1.3.2. Activatory receptors

The term "activatory receptor" generally refers to those receptors that trigger release of cytolytic granules and typically induce cytokine production (Lanier, 2005; Kirwan & Burshtyn, 2007). Established activatory receptors on NK cells include toll-like receptors, activatory KIRs, ADCC mediator – CD16 and also natural cytotoxicity receptors - e.g. NKp30, NKp44 and NKp46, NKG2D, and NKRP1 (Diefenbach, Hsia *et al.* 2003; Raulet 2003; Hayakawa and Smyth 2006). Ligands for several but not all of these receptors are known (Di Santo 2006; O'Connor, Hart *et al.* 2006). The activatory receptors are in many cases expressed on all NK cells (Santourlidis, Trompeter *et al.* 2002; Johansson and Hoglund 2006).

Generally, activatory receptors form complexes with adaptor proteins that have immunoreceptor tyrosine-based activatory motifs and by recruiting Src and Syk family kinases instigate calcium fluxes production, cytoskeletal remodeling that induce process of degranulation (Bryceson, March *et al.* 2006; Kirwan and Burshtyn 2007).

1.4. Cytotoxicity of NK cells

Cytotoxicity mechanism of NK cells, known as perforin-dependent cytotoxicity, is the major mechanism of NK cell-mediated lysis (Hokland and Kuppen 2005; Orange and Ballas 2006). When a target cell is identified, the content of NK cells' lytic granules is released by exocytosis into the immunological synapse formed between killer and target cell (Trapani and Smyth 2002; Davis and Dustin 2004). The ammunition stored in these granules includes the serine proteases granzyme A and B, the membrane-disrupting protein perforin, and the antimicrobial lytic molecule granulysin. Inside the granules, perforin and granzymes are complexed with proteoglycan serglycin (Metkar, Wang *et al.* 2002; Lieberman 2003).

Recent results suggest that, perforin enters target cell and enables release of lytic complexes from endocytic vesicles of the target cell, where the granzymes would otherwise get trapped. Upon cytosolic delivery of granzymes, cell death is then induced through triggering of different, caspase-independent (granzyme A and B) as well as caspase-dependent (granzyme B) apoptotic pathways (Metkar, Wang *et al.* 2002; Lieberman 2003; Uhrberg 2005).

1.5. Alloreactivity of NK cells

NK cells may exert alloreactivity either in the graft versus host or the host versus graft direction. NK cells' alloreactivity in host versus graft direction was first described – albeit not understood – as the phenomenon of "hybrid resistance" in a mouse transplantation model in the 1960s. Parental bone marrow grafts were rejected by a subset of host F1 NK cells that was not equipped with the correct inhibitory receptor to recognize donor MHC class I alleles and was, therefore, activated to kill (Metcalf 1963). Moreover, as the hybrid recipient mouse tolerated skin and organ allografts, the model indicated that NK cells' alloreactivity is restricted to lymphohematopoietic targets (Figure 4) (Cudkowicz, Rossi *et al.* 1972; Yu, George *et al.* 1996; Ruggeri, Mancusi *et al.* 2005).

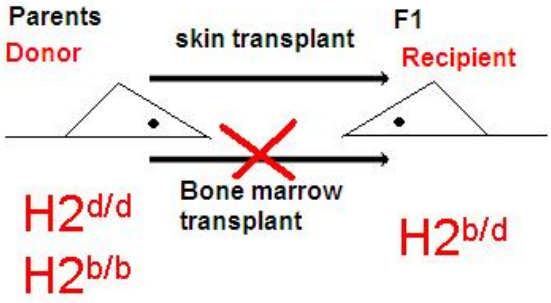


Figure 4. Hybrid resistance transplant model. The phenomenon of hybrid resistance occurs when irradiated F1 hybrid mice reject bone marrow cells donated by either parent, but not skin transplant (Cudkowicz, Rossi *et al.* 1972; Yu, George *et al.* 1996).

In humans, alloreactions can occur because KIRs discriminate between groups of HLA class I molecules. KIR2DL1 is the receptor for HLA-C group 2 alleles; KIR2DL2/3 receptors are specific for HLA-C group 1 alleles (Table 1). NK cells' alloreactions are generated between individuals who are mismatched for HLA-C allele groups and/or the HLA-Bw4 group (Ruggeri, Mancusi *et al.* 2005).

Table 1. HLA-class I allele specificity of the main inhibitory KIR.

KIR	HLA-class I specificity
KIR2DL1 (CD158a)	Group 2 HLA-C alleles expressing Lys80 (such as, HLA-Cw2, -Cw4, -Cw5, -Cw6)
KIR2DL2/3(CD158b)	Group 1 HLA-C alleles expressing Asn80 (such as HLA-Cw1, -Cw3, -Cw7, -Cw8
KIR3DL1	HLA-Bw4 alleles (e.g. HLA-B27)
KIR3DL2	HLA-A3 and HLA-A11
KIR2DL4	HLA-G

Alloreactions can occur because KIRs discriminate between individuals who are mismatched for HLA-C allele groups and/or the HLA-Bw4 group (Ponte, Cantoni *et al.* 1999; Rajalingan 2002; Ruggeri, Mancusi *et al.* 2005).

Similarly to "hybrid resistance" model, donor versus recipient NK cells' alloreactivity derives from mismatch between donor NK clones (carrying specific inhibitory receptors for self-MHC class I molecules) and MHC class I ligands on recipient cells. When faced with mismatched allogeneic targets, these donor NK clones sense the

missing expression of self-HLA class I alleles and mediate alloreactions without causing GvHD.

1.6. Clinical studies

1.6.1. Cancer immunotherapies

Cancer immunotherapy attempts to harness the exquisite power and specificity of the immune system for the treatment of malignancy. Although cancer cells are less immunogenic than pathogens, the immune system is clearly capable of recognizing and eliminating tumor cells (Blattman and Greenberg 2004). Cancer immunotherapy recruits organism immune system to reject cancer by stimulation the patient's immune system to attack the malignant tumor cells. This can occur either through immunization of the patient by infusion of *in vitro* cultivated either T, or NK cells or through the administration of therapeutic antibodies as drugs, in which case the patient's immune system is recruited to destroy tumor cells by the therapeutic antibodies (like therapy with rituximab) (Arai and Klingemann 2003; Morse, Lyerly *et al.* 2004).

Rituximab is a chimeric monoclonal antibody (MoAb) directed against CD20, an antigen found most B-cell malignancies, including non-Hodgkin follicular lymphoma and CLL. As a single agent, rituximab induces objective responses in more than 50% of CLL patients with minimal toxicity because of its B-cell selectivity (Dalle and Dumontet 2007). These favorable results have led to considerable interest in combining rituximab with other agents in CLL (Farag, Flinn *et al.* 2004). It is known that rituximab is able to induce ADCC as well as complement dependent cytotoxicity and apoptosis. ADCC caused by rituximab can be explained by its strong association with CD16 (Dalle and Dumontet 2007).

Cellular immune responses can produce an anticancer effect. Two types of cellular immune responses appear to be clinically relevant: antigen specific immunity mediated by T cells that recognize tumor-associated peptide antigen expressed on surface HLA class I or class II molecules, and antigen non-specific immune responses mediated by NK cells that are activated by the failure to recognize “self” HLA class I molecules (Arai and Klingemann 2003). The Graft-versus-Leukemia (GvL) effect of allogeneic transplants using HLA-matched donors is mediated by antigen-specific T cells, while allogeneic transplantation using HLA-mismatched donors is mediated by NK cells (Morse, Lyerly *et al.* 2004; Waller 2004). In the case of hematologic malignancies, the activity of donor T

cells against tumor cells is regulated by dendritic cells, which can augment or inhibit cellular immune responses. Future successes in enhancing the patients' own cellular immune responses to cancer will likely be based on combination of both branches of immunotherapies: monoclonal antibody treatment and cellular immunotherapy (activation of T cells by the appropriate dendritic cells' subset, in the case of tumors that have down-regulated HLA class I expression by activated NK cells) (Waller 2004).

1.6.2. Leukemic diseases

Leukemia is a broad term covering a spectrum of diseases. Clinically and pathologically leukemia is split into its acute and chronic forms. Furthermore, the diseases are classified according to the type of abnormal cell found in the blood: leukemia affecting lymphoid cells is called lymphocytic leukemia. Leukemia affecting myeloid cells is called myelogenous leukemia. Combining these classifications provides a total of four main leukemia categories: acute lymphocytic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myelogenous leukemia (CML). The most common forms in adults are AML and CLL (Holland 2006). In present thesis two different patient subsets, which are diagnosed either AML or CLL, are described and researched.

Among the adult population of the Western world chronic lymphocytic leukemia (CLL) is the most common form of leukemia and – because of its relatively longer survival – the one with the highest prevalence among all leukemias, accounting more than 22% of leukemias diagnosed (Caligaris-Cappio 2000; Holland 2006). B cell chronic lymphocytic leukemia is an accumulative disease of slowly proliferating, functionally incompetent $CD5^+$ B lymphocytes (Chiorazzi and Ferrarini 2003). The international workshop for CLL recommends a blood lymphocyte threshold of 10^9/L (Holland 2006). The clinical course of chronic lymphocytic leukemia shows a marked heterogeneity, with a median survival ranging from 2 to 20 years at different disease stages (Hallek, Kuhn-Hallek *et al.* 1997).

Acute myelogenous leukemia (AML) is a clonal expansion of myelogenous blasts in bone marrow, blood or other tissues (Kileen 2001). According to the widely used World Health Organization classification system, the diagnosis of AML requires that myeloblasts constitute 20% or more of bone marrow cells or circulating white blood cells (Holland 2006). The acute myelogenous leukemias are a relatively heterogeneous group

of diseases (Kileen 2001). AML is the most common variant of acute leukemia occurring in adults, comprising approximately 80% of cases of acute leukemia diagnosed in individuals greater than 20 years of age. As AML affects adults of all ages it is especially common in older adults. The median age of *de novo* AML is approximately 55 years, and the median age at diagnosis is probably 65 to 70 years (Holland 2006). Induction therapy of AML with standard-dose chemotherapy results in 52% to 72% of patients achieving a complete remission (CR) on bone marrow morphology, however, with very high risk of relaps and mortal outcome (Bishop 1997).

1.6.3. NK cells immunotherapy

Adoptive immunotherapy using natural killer cells may prove useful, especially in situations where infusion of T cells is impractical such as in recipients of haploidentical stem cell transplantation (HSCt) from haploidentical donors (Passweg, Stern *et al.* 2005; Suck 2006). Potential benefits and harms of NK cells' alloreactivity are depicted in Table 2.

Table 2. Potential benefit and harm of adoptive immunotherapy using NK cells in HSCt. Adapted from Passweg, Stern *et al.* 2005.

Potential benefits of NK cells' alloreactivity	***Comment***
Targeting host T lymphocytes	Decrease rejection
Targeting Host dendritic cells	Decrease antigen presentation by host dendritic cells and hence GvHD
Targeting leukemic cells	Decrease relapse
Improved immune reconstitution	Decrease infection risk

The effectiveness of NK cells' alloreactivity as revealed in the haploidentical transplant setting, has led to the establishment of specific criteria for donor selection which have enhanced survival rates of leukemia patients (Igarashi, Wynberg *et al.* 2004; Ruggeri, Mancusi *et al.* 2005). This results will encourage extending the use of mismatched transplants to more leukemia patients without a matched donor (Ruggeri, Mancusi *et al.* 2005; Suck 2006) or to use patient own cells with reconstituted cytotoxic activity (Lister, Rybka *et al.* 1995; deMagalhaes-Silverman, Donnenberg *et al.* 2000).

They might also be infused post-transplant to help prevent or control leukemia relapse (Kalinski, Giermasz *et al.* 2005; Miller, Soignier *et al.* 2005; Passweg, Stern *et al.* 2005).

There are number of allogenic as well as autologous NK cells' infusions performed after BMt so far. Approaches were quite different, however, all of them were based on previous treatment of NK cells with IL-2 that positively influences NK cells cytotoxic ability (Kay and Zarling 1987; Uharek, Zeis *et al.* 1996; Salcedo, Andersson *et al.* 1998; Raulet, Vance *et al.* 2001). For example Slavin *et al.* 2004 used IL-2 activated NK cells following transplantation from haploidentical sibling, or unrelated donor. No GvHD was observed. One patient achieved complete remission. Four patients are alive; one with disease; three with no evidence of disease at 9-22 month post-HSCt (Slavin, Morecki *et al.* 2004). Study of Koehl *et al.* showed that 4 patients with KIR mismatches in GvHD direction reached complete remission 4 weeks post-HSCt, which was accompanied by complete donor chimerism (Koehl, Sorensen *et al.* 2004).

Very encouraging results were obtained after autologous NK cells' infusions by Lang *et al.* (Lang, Pfeiffer *et al.* 2002), Passweg *et al.* (Passweg, Tichelli *et al.* 2004), Rosenberg *et al.* (Rosenberg and Dudley 2004). Autologous NK cells immunotherapy may serve also as a therapy for AIDS (Rosenberg and Dudley 2004) and gastric cancer (Jiang, Xu *et al.* 2006)

There are a lot of questions concerning NK cells immunotherapy to be answered. Open issues include NK cells' doses, timing, and appropriate selection of donor and recipients. The use of NK cells for adoptive immunotherapy will help to better define the clinical impact of NK cells' alloreactivity including the importance of KIR mismatching. Whether these cells should be used preemptively or as a salvage treatment is unknown (Passweg, Stern *et al.* 2005). There is also a gap in the data, how NK cells act in complex therapy with monoclonal antibodies like rituximab.

Aims of study

The main goals of the current work are:

1. Description of NK cells' expansion kinetics and quantities in donor and CLL and AML patient samples.
2. Comparison of the specific cytotoxicity ratio of NK cells of healthy donors, CLL and AML patients.
3. Investigate influence of rituximab MoAb on NK cells ADCC *in vitro* of two different patient samples subsets (CLL and AML).
4. Study of *in vitro* NK cells' cytotoxicity of CLL and AML patient samples against day 1 autologous cells.
5. Investigate the influence of NK cells' preservation at -150°C.

2. Materials and Methods

2.1. Samples and cell lines

Patients and Donor blood samples

Donor samples (D) were obtained from Tartu University Hospital blood bank from healthy blood donors. We do not have access to their private data. HLA typing was performed in United Laboratories of Tartu University Hospital Department of Immunoanalyses by using *AllSet+* SSP Assay kits (Dynal Biotech, Oslo, Norway) for low resolution and Pel-Freez SSP UniTray kits (Invitrogen Ltd, Paisley, Scotland) for high resolution HLA typing.

Patient samples (P) were provided by Tartu University Hospital before the chemotherapy. Patients were selected according to diagnosis CLL or AML respectively, in order to compare two principally different diseases for ability to expand NK cells and cytotoxicity rates.

All procedures were approved by Ethics Review Committee on Human Research of the University of Tartu, protocol nr: 146/4, 27.02.2006. Donor and patient information is presented in Table 3.

Lymphopreparation

To obtain sample lymphocytes, modified Ficoll-Paque PLUS^{TM} separation method was used. Whole blood or buffy coat samples (25 ml) were mixed with D-PBS (25 ml) (phosphate buffered saline without $CaCl_2$ and $MgCl_2$, Invitrogen, Gibco, Grand Island, NY, USA) in 50 ml tubes. Diluted blood cells were gently added in volume of 25 ml to tubes with 15 ml of Ficoll-Paque, in order to obtain two layers of diluted cells and Ficoll-Paque (Note: layers should not be mixed). Tubes were centrifuged 400×g, 30 minutes, 18°C. After centrifugation, lymphocytes were gently collected through the formed layer of plasma, trying not to mix the layers. If needed, cells were used for further analyses or cryopreserved.

Cryopreservation and thawing of cells

Peripheral blood mononuclear cells (PBMCs) obtained by Ficoll-Paque™ PLUS separation were cryopreserved as further described. Cells were washed twice with D-PBS, resuspended in 0.5 mL 20% human serum albumin (HSA, Octapharma, Wien, Austria), then left on ice for 20 minutes. These cells were gently mixed with 0.5 mL 20% human serum albumin containing 20% dimethylsulfoxide (DMSO, AppliChem, Darmstadt, Germany) (to reach the final DMSO concentration of 10%) and stored at -150ºC for future use. When needed, cells were thawn at room temperature, then gently mixed with D-PBS of room temperature (RT), and spun down in 400×g, 5 minutes, 20ºC. Supernatant was aspirated. Cells were then cultured according to cell culture treatment protocol.

Table 3. Donor and patient samples.

Donor/ Patient	A1	A2	B1	B2	C1	C2	HLA group	Diagnosis	Gender
D1	03	-	0702	-	0702	-	A+B0Cw3	Healthy	
D2	02	-	13	18	0602	0701/ 0702	A0B+Cw34	Healthy	
	02	03	27	44	0704	-	A+B+Cw3	Healthy	
D4	03	26	3503	4102	0401	1703	A+B0Cw4	Healthy	
D5	01	02	08	13	0602	0701	A0B+Cw34	Healthy	
D10	02	23	2705	4435	0102	0401	A0B+Cw34	Healthy	**M**
D13	02	03	3501	3519	0401	0602	A+B0+Cw4	Healthy	**M**
P15*								AML	**F**
P18*								AML	**F**
P19*								CLL	**F**
P25*								CLL	**M**
P29*								AML	**F**
P35*								AML	**M**
P39*								AML	**F**
P40*								CLL	**M**
P41*								CLL	**M**

The donors (**D**) and patients (**P**) were genotyped serologically (HLA-A and B) and by high-resolution PCR (HLA-B and C). The HLA group is derived from the alleles' belonging to NK reactivity groups. **A0**, there is no ligands for KIR3DL2; **A+**, KIR3DL2 reactivity; **B0**, no ligand for of KIR3DL1; **B+**, KIR3DL1 reactive; **Cw3**, no ligand for KIR2DL2 (CD158b); **Cw4**, no ligand for KIR2DL1 (CD158a) (Rajalingan 2002; Ruggeri, Mancusi *et al.* 2005).

* – HLA data absent.

Human serum (HS)

We used AB+ whole blood samples (without anticoagulants) from healthy male donors, provided by Tartu University Hospital blood bank. Whole blood was incubated for 4 hours at 37ºC to achieve blood clotting. After incubation, serum was aspirated and centrifuged at 4000×g, 30 min, 4ºC. For future use, serum was stored in -20ºC in propylene tubes (BD labware Europe, Maylan Cedex, France). When needed, samples were thawed and held at +4ºC.

Tumor cell lines

The K562 human HLA class I-deficient erythroleukemia cell line was chosen as direct target cell line for NK cells.

Namalwa – Burkitt lymphoma cell line (HLA class I positive) was chosen in order to investigate the influence of specific monoclonal antibody rituximab, which is used as treatment of several lymphomas, on specific cytotoxicity of NK cells. Namalwa HLA class I was genotyped in Huddinge Immunology laboratory (Stockholm, Sweden) (Table 4).

Table 4. Namalwa HLA genotyping result.

Namalwa HLA class I type		HLA group	Reactive KIR
HLA-A	0301 ,6802	A+	KIR3DL2
HLA-B	0702/0735, 4901	B+	KIR3DL1
HLA-Cw	0701, 0702	Cw3	KIR2DL1

2.2. Cell culture

Patient and Donor cell culture

After thawing, cells were counted and cultured in tissue culture 6-well plates (Falcon by BD, Le Point De Claix, France) at concentration of 10^6 cells/ml. CellGro SCGM serum-free medium (CellGenix, Freiburg, Germany) (Lot number: 0492K, 0693K, 0818H) with the addition of 5% HS was supplemented with 500 U/ml IL-2, anti-CD3 antibody (Orthoclone OKT-3, Ortho Biotech Inc., Raritan, NJ, USA) at final

concentration of 10 ng/ml, doxocycline and itraconazol at final concentration 3,3µg/ml and 1,1µg/ml respectively. Cultures were replenished with fresh medium and four supplements every 2-3 days through the culture period of 21 day ((Carlens, Gilljam *et al.* 2001), modified protocol).

All cell cultures were incubated at 37ºC, under 5% CO_2 in atmosphere. Cells were counted in a Bürker hematocytometer (Brand GMBH, Wertheim, Germany).

Tumor line cell culture

Namalwa and K562 cell lines were cultivated *in vitro* in RPMI-1640 (Invitrogen, Gibco, Grand Island, NY, USA) medium supplemented with 10% fetal bovine serum (**FBS**) (Invitrogen, Gibco, Grand Island, NY, USA). The cultures were replenished every 3-4 days through the culture period.

2.3. Flow cytometry based methods

Flow Cytometry and Antibodies

The cell phenotype was analyzed by flow cytometry on days 0, 5-6, 10-11, 15-16 and 20-21 (and also on days, when cytotoxicity tests were performed). Three-color fluorescence was analyzed according to standard procedures. Briefly cells were collected into 1.5 mL tubes (Axygen Scientific Inc., Union City, CA, USA) at volume of 0.1 – 0.5 mL.

Cells were spun down by centrifugation at 500×g, 5 minutes, 20ºC. Mix of primary antibodies (CD3-PerCP-Cy5.5, CD56-PE, and CD158a (KIR2DL1 and KIR2DS1)-FITC) (all antibodies by BD Biosciences Pharmingen, San Jose, CA, USA) at final volume of 20 µL in D-PBS supplemented with 0,1% HSA was added to cell pellet, resuspended and incubated for 15-30 minutes at RT. After incubation cells were spun down at analogous conditions (500×g, 5 minutes, 20ºC), mix of MoAbs was aspirated and cells were resuspended in 500 µL of DPBS supplemented with 0,1% HSA.

For the CLL patient vs. CLL patient cytotoxicity assay further primary antibody mix was used: CD3-APC-Cy7, CD56-PE, CD158a-FITC, CD19-PE-Cy7 (all antibodies by BD Biosciences Pharmingen, San Jose, CA, USA) at final volume of 20 µL in D-PBS

supplemented with 0,1% HSA. This antibody mix allows differentiation of B-cells and NK-cells in cytotoxicity assay.

For AML patient vs. AML patient cytotoxicity assay further primary antibody mix was used: CD3-APC-Cy7, CD56-PE, CD158a-FITC, CD33-PE-Cy7 (all antibodies by BD Biosciences Pharmingen, San Jose, CA, USA) at final volume of 20 μL in D-PBS supplemented with 0,1% HSA. This antibody mix allows differentiation of myeloid lineage cells of most acute myelogenous leukemias and NK cells in cytotoxicity assay.

For data acquisition and analysis, FACSsort (BD) flow cytometer was used with CellQuest (BD) software. For 6-colour fluorescence analysis (cytotoxicity test patient vs. patient directions) LSR II (BD) flow cytometer was used with FACSDiva (BD) software. In each sample, a minimum of 10 000 cells was acquired in the analysis of viable cells, using log-amplified fluorescence and linearly amplified side- and forward-scatter signals.

Cytotoxicity assay

In vitro expanded NK cells cytotoxicity against K562 and Namalwa cell lines was performed on different days of cell culture, as at the beginning of the cell culture as well as at the end, and evaluated in 4-hour FACS-based cytotoxicity assay, using commercial CellTrace™ CFSE Cell proliferation kit (Molecular probes, Leiden, Netherlands).

Cells to be stained with CFSE (target tumor cells or part of donor target cells, approximately $3\times10^4 – 10^5$ cells), were collected into 1,5 mL tubes in equal volumes and centrifuged at 500×g, 5 min, 23° C, resuspended in essential volume of fresh RPMI 1640. According to the manufacturer's protocol, prepared target cancer cells were labelled with CFSE (1 ng/mL in RPMI 1640) for 15 minutes at 37˚C, patient cells were labelled with 0,25 ng/mL of CFSE in RPMI 1640.

Unlabeled donor cells were also collected in equal volumes, washed in RPMI1640 and gently mixed with labeled donor or tumor cells in V-bottomed 96-well microtiter plate (Deltalab S.L., Barcelona, Spain) in effector (E): target (T) ratio 10:1, doubled and incubated for 4 hours at 37˚C. In case for tests, where rituximab MoAb influence should be measured, 10× dilution of 10mg/mL MoAb stock was made in RPMI 1640 medium and was added test sample to achieve final concentration of 10 μg/mL.

After incubation, 7-amino-actinomycin D (7-AAD) at final concentration of 10 µg/mL was added to stain dead cells. Plates were directly centrifuged at 20°C, 500×g, 5 min. Supernatant was aspirated, and cells were resuspended in 150 µL of D-PBS. For cytotoxicity reactions for patient vs. patient reactions, cells were resuspended in 20 µL of primary antibody mix in D-PBS.

Cells were collected into FACS tubes (Falcon by BD, Le Point De Claix, France) and resuspended in D-PBS to obtain a final volume of 0.5 mL.

The specific cytolysis (L) is calculated according to the Equation 1.1,

$$L = \left(\frac{Dead\ Target\ Cells}{All\ Target\ Cells} \times 100\% \right) - spontaneous\ lysis\ of\ Target\ cells \quad (1.1)$$

where $Dead\ Target\ Cells$ is number of dead target (K562 or Namalwa) cells; $All\ Target\ Cells$ is number of all target (K562 or Namalwa) cells; $Spontaneous\ lysis\ of\ Target\ cells$ is background value for K562 or Namalwa, was measured in the absence of effector cells, which was normally around 5±2%; $Spontaneous\ lysis\ of\ Target\ cells$ is background value for patient sample results, was measured in absence of effector cells as dead target cells' number of particular donor divided by all target cells' number of the same donor.

Specific lysis of $CD19^+$ or $CD33^+$ cells was calculated by subtraction of spontaneous lysis of either $CD19^+$ or $CD33^+$ cells, respectively. Spontaneous lysis of the $CD19^+$ or $CD33^+$ cells was measured in the absence of effector cells.

Total cell number calculation equations

In order to calculate the potential total cell number (D_n) and total NK cell number (D_{NK}) in replenished sample at indicated day, recursive Equation 1.4 and Equation 1.5, respectively were proposed by our work group. However these equations have exclusions for day 0 and day 1, which are explained in Equation 1.2 and Equation 1.3, respectively. Definitions for the equations are explained in Table 5.

Statistic analyses were performed with Microsoft® Excel® software.

Table 5. Definitions for equations 1.2 - 1.5

		Pre-previous day* $d(n-2)$ **or** $d(0)$	**Previous day†** $d(n-1)$ **or** $d(1)$	**Day of interest** $d(n)$
Cell number ×10⁴ cell/mL	A	A_{n-2} or A_0	A_{n-1} or A_1	A_n
Volume left mL	B	B_{n-2} or B_0	B_{n-1} or B_1	
Volume added mL	C	C_{n-2} or C_0	C_{n-1} or C_1	
Total Volume mL	V	V_0		
Total cell number	D	D_0	D_{n-1} or D_1	D_n
Total NK cell number	D_{NK}	$d(0)D_{NK}$	$d(1)D_{NK}$	$d(n)D_{NK}$
NK cells' share	NK	NK_0	NK_1	NK_n

Recursive Equation 1.4 and Equation 1.5 allow to calculate total cell number and total NK cell number in replenished sample by day 21 starting from day 2 (equation 1.4 is invalid for days 0 and 1). Cell concentration at day 0 is $10^6 cells/mL$. Exclusions for day 0 and day 1 are brought by Equations 1.2 and 1.3, respectively.

*- pre-previous supplement replenishing or day 0.
†- previous supplement replenishing or day 1.

(1.2) $$D_0 = 10^6 \times V_0$$

(1.3) $$D_1 = \frac{A_1}{A_0} \times \left(\frac{B_0 + C_0}{B_0} \right) \times D_0$$

(1.4) $$D_n = \left(\frac{A_n \times (B_{n-1} + C_{n-1})}{A_{n-1} \times (B_{n-2} + C_{n-2})} \right) \times \left(\frac{B_{n-1} + C_{n-1}}{B_{n-1}} \right) \times D_{n-1}, \quad n \in \{2, ..., 25\}$$

(1.5) $$d(n)D_{NK} = D_n \times NK_n, \quad n \in \{0, ..., 25\}$$

2.4. Scheme of experiments

In the current work a series of tests were performed:

1. Medium testing for donors and patients depicted in "*Patients and Donor blood samples",* Table 3. Samples were cultivated during 21 days of culture and analyzed by flow cytometer on different subsets of lymphocytes presence. *This test gives answers to questions: how do different donor and patient lymphocytes expand in vitro, how NK cells total number differs within the samples, how homogeneous these samples are and how successful are the chosen growth conditions.*
2. *In vitro* specific cytotoxicity measurement against K562 tumor cell line, using samples from donors and patients noted in section 1. During cell culture period of 21 days, cytotoxicity tests were performed on different days of cell culture. *This test gives us overview how cells from healthy donor samples react against target cells and how these results differ from patient sample results.*
3. *In vitro* measurement of rituximab interference with specific cytotoxicity of NK cells against Namalwa cell line, using patient samples noted in section 1. *This test represents results for interference of these two immunotherapies.*
4. Specific cytotoxicity measurement of patient day 15 and day 21 NK cells against appropriate patient day 1 cells. *This test gives answer to questions if we are able to use patient cells for autologous NK cells' immunotherapy.*
5. Comparison of day 21 NK cells' share and cytotoxicity against K562 cell line in cryopreserved samples. Analyzed samples of 5 donors.
   This test gives answer to question, how do NK cells from healthy donors preserve at -150ºC and how it influences the ability to kill target cells.

3. Results

3.1. Cells' expansion rates

Since our medium protocol differs from protocol described by Carlens *et al.* 2001, we tested modified cultivation protocol on 7 healthy donor samples and 9 patients (depicted in Table 3) with two different diagnoses CLL and AML, respectively. PBMCs originating from 7 human donors expanded from the starting number to average of 153-fold after 21 days of culture in CellGro medium with presence of OKT3 (10 ng/ml), IL-2 (500 U/ml) and 5% HS. PBMCs of 4 CLL and 5 AML patients were able to expand 185-fold and 120-fold, respectively. Average results of 3 tests with standard deviations are presented (Figure 5).

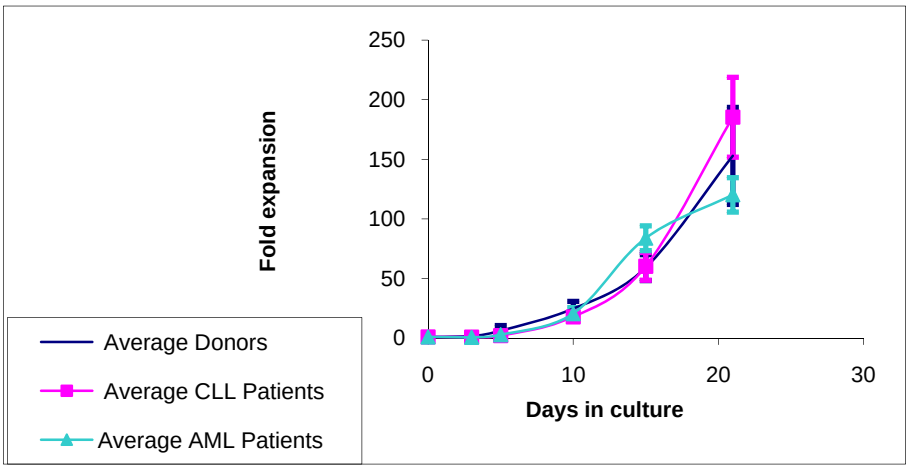


Figure 5. Total cell number for healthy donors, CLL and AML patients. Cell samples of 7 healthy donors, 4 CLL and 5 AML patients were analyzed for the ability to expand in proposed growth conditions: CellGro medium, supplemented with OKT3 (10 ng/ml), IL-2 (500/ml) and 5% HS. Average results of 3 tests with standard deviations are presented.

3.1.1. Expansion of NK cells

We measured total NK cells' expansion and NK cells' share dynamics in cell culture. Cells of donor and patient samples were collected and counted in a Bürker hematocytometer. Samples were analyzed by flow cytometer for expression of CD3, CD56 and CD158a. On the Figure 6, results of 7 healthy donors and 9 patients (4 CLL and 5 AML) are depicted. We also present results for culture during period of 25 days. NK cells' share differed between the donors and patients. The average of three results for

NK ($CD56^+CD3^-$) cells' share in healthy donors at day 21 equalled 66% and for CLL patents 49%. AML patients showed the maximum of NK cells' share at day 15 of cell culture, which equalled 34%. Tests were performed on samples, which had been preserved at -150ºC no longer than 3 months.

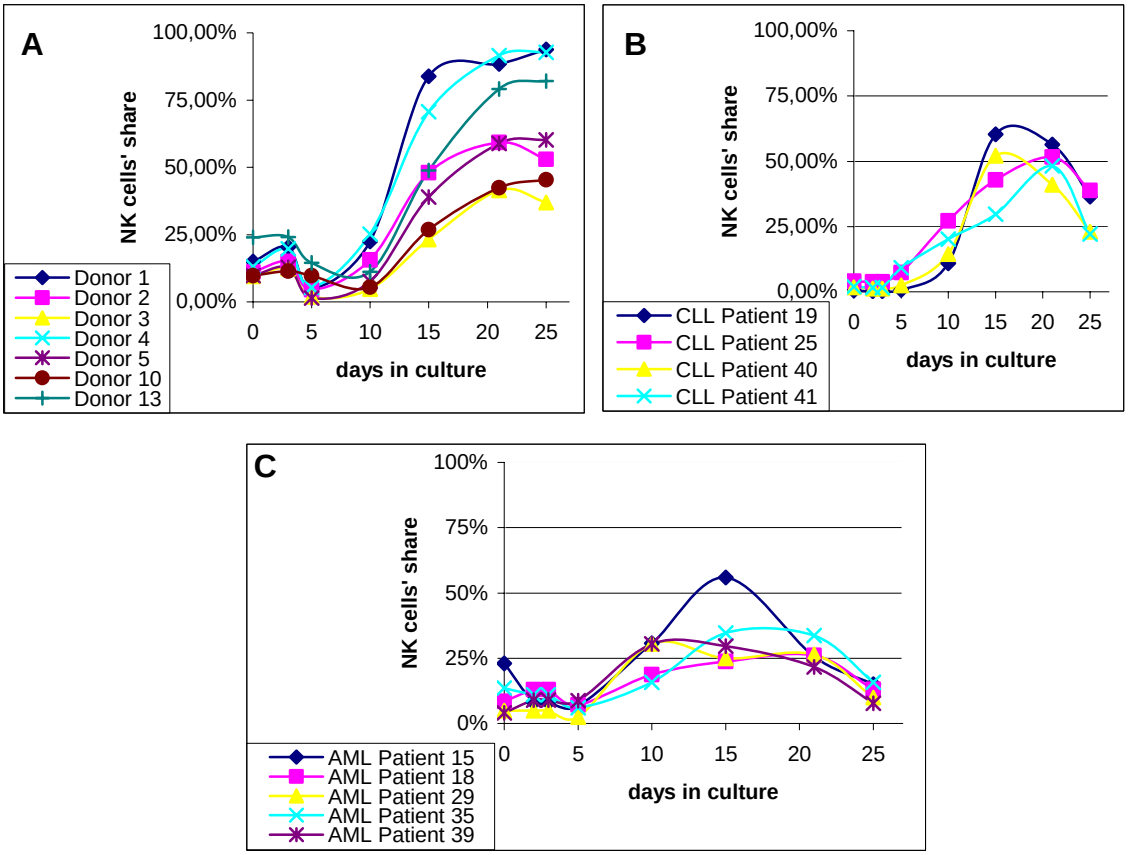


Figure 6. NK cells' share dynamics in cell culture period of 25 days. Cell samples of 7 healthy donors and 4 CLL and 5 AML patients were analyzed by flow cytometer for expression of CD3, CD56 and CD158a. $CD56^+CD3^-$ cells' share is depicted. Average results of three tests are presented. Analyses were performed with cells that were kept at -150ºC no longer than 3 months.

Figure 7 represents total NK cells number variation between 7 healthy donors and 9 patients (4 CLL and 5 AML patients) in cell culture at the day of maximum NK ($CD56^+CD3^-$) cells' share (either day 21 or 15). In order to calculate total NK cells number, NK cells' share (data from Figure 6) is multiplied with total cell number, acquired by counting cells at indicated day in Bürker hematocytometer, and applying the Equation 1.5 (for total NK cells number count).

According to calculations, our cultivation protocol allows to obtain by day 21 on average $2{,}5\times10^8$ NK cells for healthy donor samples (starting from 10^5 – $1{,}2\times10^5$ NK cells), $1{,}79\times10^8$ NK cells for CLL patients (starting from $0{,}09\times10^5$ – $0{,}8\times10^5$ NK cells).

Results for AML patients are given to the 15th day of cell culture. It comprised on average $1{,}07\times10^8$ NK cells for AML patients (starting from $0{,}8\times10^5 - 2{,}72\times10^5$ NK cells). Test was performed three times on samples that had been preserved at -150°C no longer than 3 months. On the plot the average results of three tests with standard deviations (on the average 20%) are depicted.

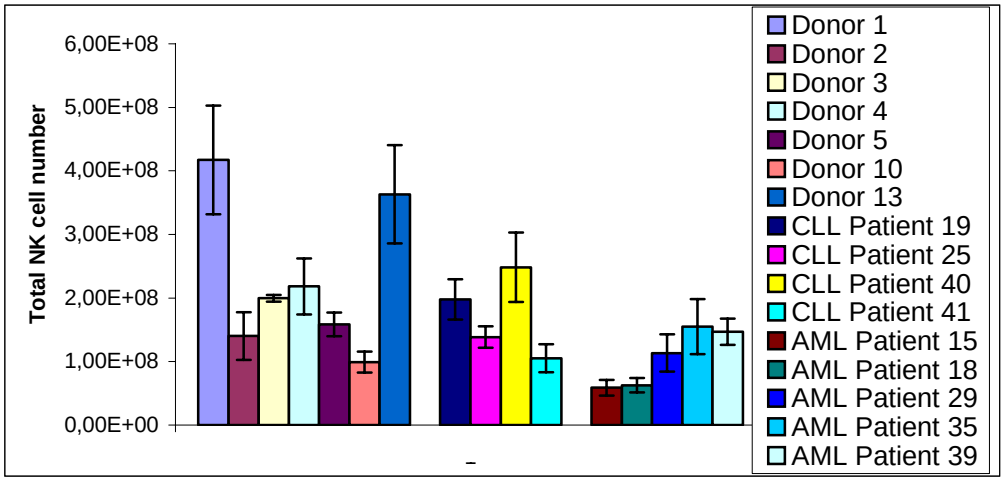


Figure 7. Maximum NK cells' number for donor and patient samples. Cell samples of 7 healthy donors and 4 CLL and 5 AML patients were controlled for the ability to expand NK cells ($CD56^+CD3^-$ cells) in cell culture by day 21 (donors and CLL patients) or day 15 (AML patients). Average results of 3 tests are presented with variability of 11-27%, within particular donor or patient sample. Samples which are tested were preserved at -150°C no longer than 3 months.

3.2. Cytotoxicity

3.2.1. Specific cytotoxicity of donor, CLL and AML patient samples during cell culture period

We measured the specific cytotoxicity of donor, CLL and AML patients NK cells against HLA class I deficient K562 cells at 10:1 ratio. NK cells' share ($CD56^+CD3^-$cells' share) in samples of 7 healthy donors (depicted in bright blue), 4 CLL patients (depicted in dark blue) and 5 AML patients (depicted in pink) is plotted against L- specific cytolysis (in percent) is calculated according to the Equation 1.1 (for specific cytotoxicity calculation) from data results of 4-hour FACS-based cytotoxicity assay. Linear trendlines are drawn through the data. Figure 8 depicts specific cytolysis variation during cell culture period at day 1, 3, 11 and at the end of culture (day 21). Tests were performed 3 times, representing results of the first cultivation. There seemed to be direct relation among NK cells' share and specific cytolysis ratio.

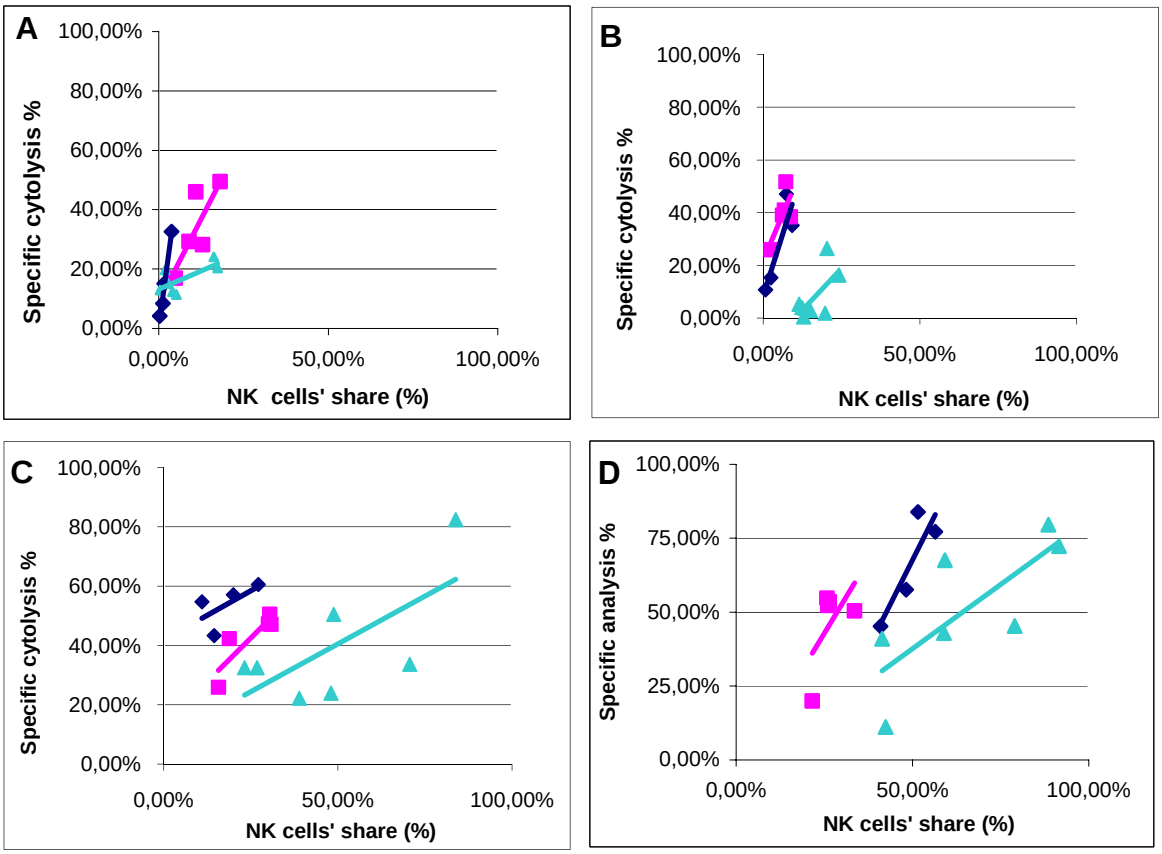


Figure 8. Differences in Specific cytolysis against K562 cells between healthy donors, CLL and AML patients. Plot represents results for specific cytotoxicity of samples of 7 healthy donors (bright blue), 4 CLL (dark blue) and 5 AML (pink) patient samples NK cells against K562 target cells, acquired in 4-hour FACS-based cytotoxicity assay during culture period of 21 day. Tests were performed at day 1 (A), day 3 (B), day 11(C) and day 21(D). Average results of 3 tests are depicted.

3.2.2. Rituximab influence *in vitro* on specific cytotoxicity of NK cells

CD20-specific MoAb – rituximab influence on NK cell-mediated ADCC was measured. Results of 7 healthy donors and 9 patient results are presented on Figure 9. On the plot NK cells ($CD56^{+}CD3^{-}$ cells) specific cytotoxicity results on day 1 (Figure 9A), day 3 (Figure 9B), day 11 (Figure 9C) and day 21 (Figure 9D) against Namalwa cell line as negative control and Namalwa with rituximab MoAb as control sample are presented. NK cells' share in sample at appropriate day was measured by flow cytometry for CD3, CD56 and CD158a expression and are plotted against L - specific cytolysis, is calculated with Equation 1.1, acquired from test results of 4-hour FACS-based cytotoxicity assay. Linear trendlines are drawn through the data. CLL patient results with and without

rituximab are depicted in green and dark blue trendlines, respectively as AML patient results with and without rituximab are depicted in bright blue and pink trendlines. Results of healthy donors with and without rituximab are depicted in purple and red, respectively. Rituximab was able to influence NK cell-mediated ADCC during only first two days of the culture period. The tests were performed three times; average results are presented.

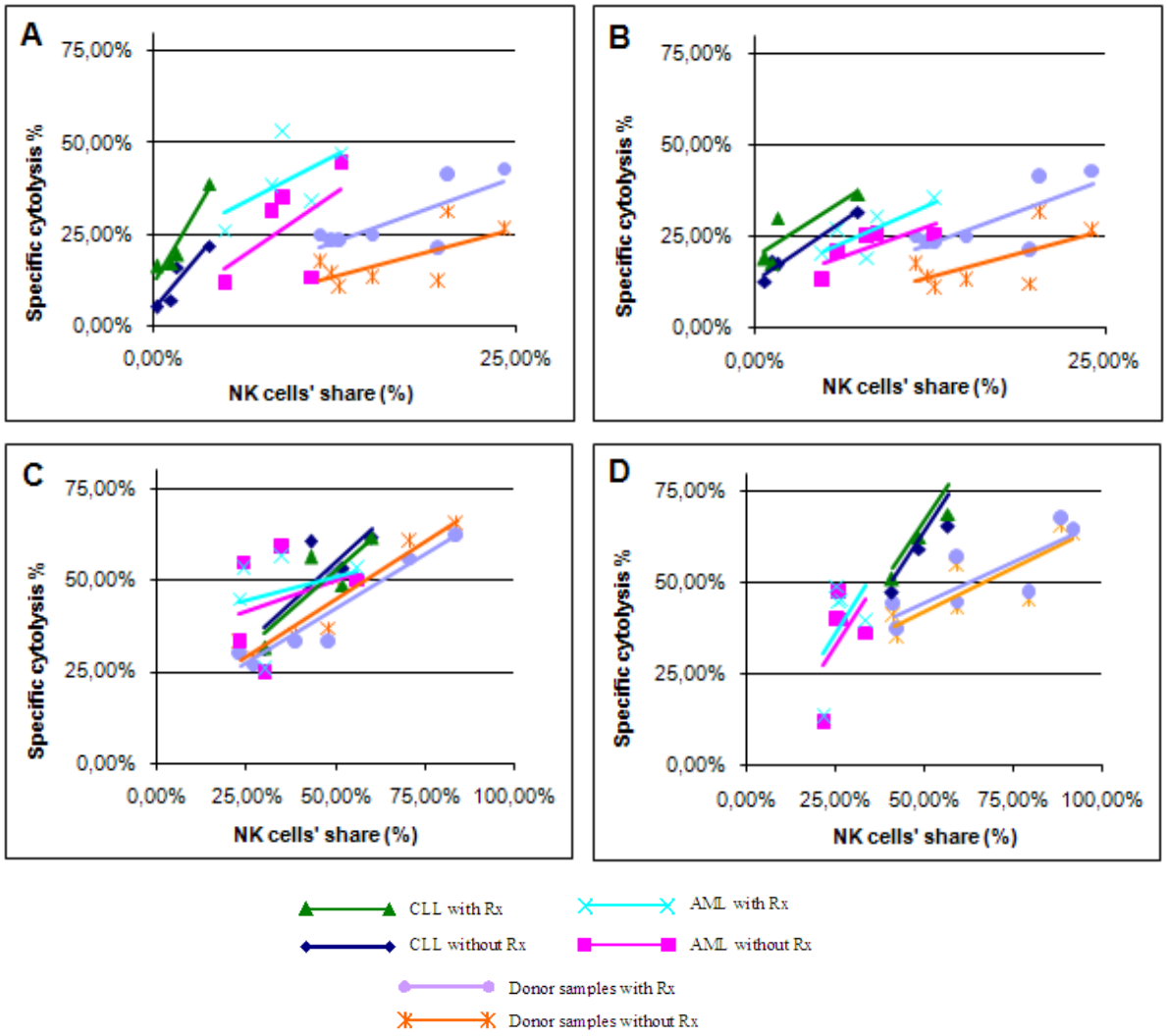


Figure 9. Rituximab influence on NK cell-mediated ADCC. 7 healthy donors, 4 CLL and 5 AML patient samples with and without rituximab (Rx) were analyzed for NK-cell mediated ADCC. On the plot we present results for day 1 (A), day 3 (B), day 11(C) and day 21(D) tests. Average results of three tests are presented.

3.2.3. Specific cytotoxicity *in vitro* against day 1 autologous cells

Figure 10 gives overview of cytotoxicity tests performed with autologous *in vitro* cultivated NK cells of two different patient subsets against day 1 appropriate patient PBMCs. Specific cytotoxicity percent was evaluated in 4-hour FACS-based cytotoxicity assay and is calculated according to Equation 1.1. Linear trendlines are drawn through the data. CLL patient results are depicted in dark blue, as AML patient results – in pink. NK ($CD56^{+}CD3^{-}$) cells' share was measured by flow cytometry for CD3, CD56 and CD158a expression. Tests were performed on day 15 (Figure 10A) and day 21 (Figure 10B) of the cell culture. Highest NK cell cytotoxicity ratio for CLL patients occurred at day 21, as for AML patients at day 15. Specific cytotoxicity for CLL was 26% in samples, where NK cells' share equalled 57%. Specific cytotoxicity for AML was 30% in samples, where NK cells' share equalled 40%. Average results of 3 tests are presented.

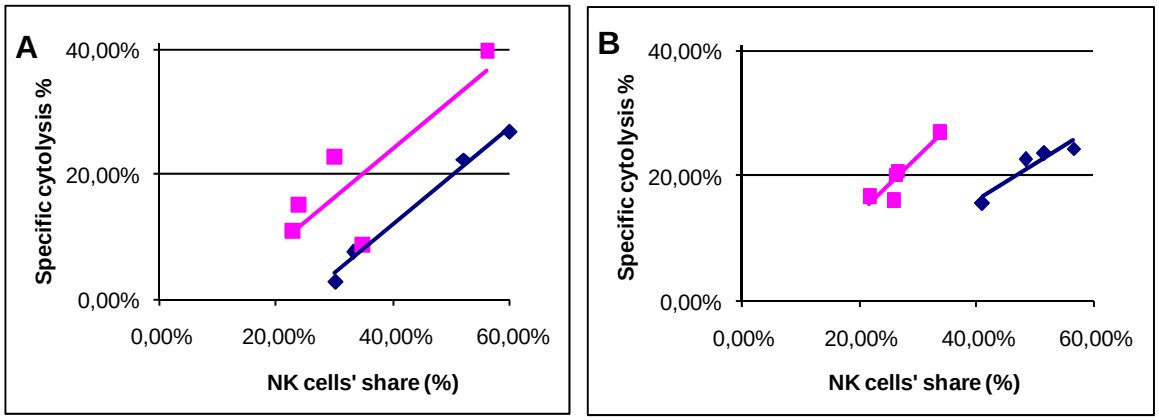
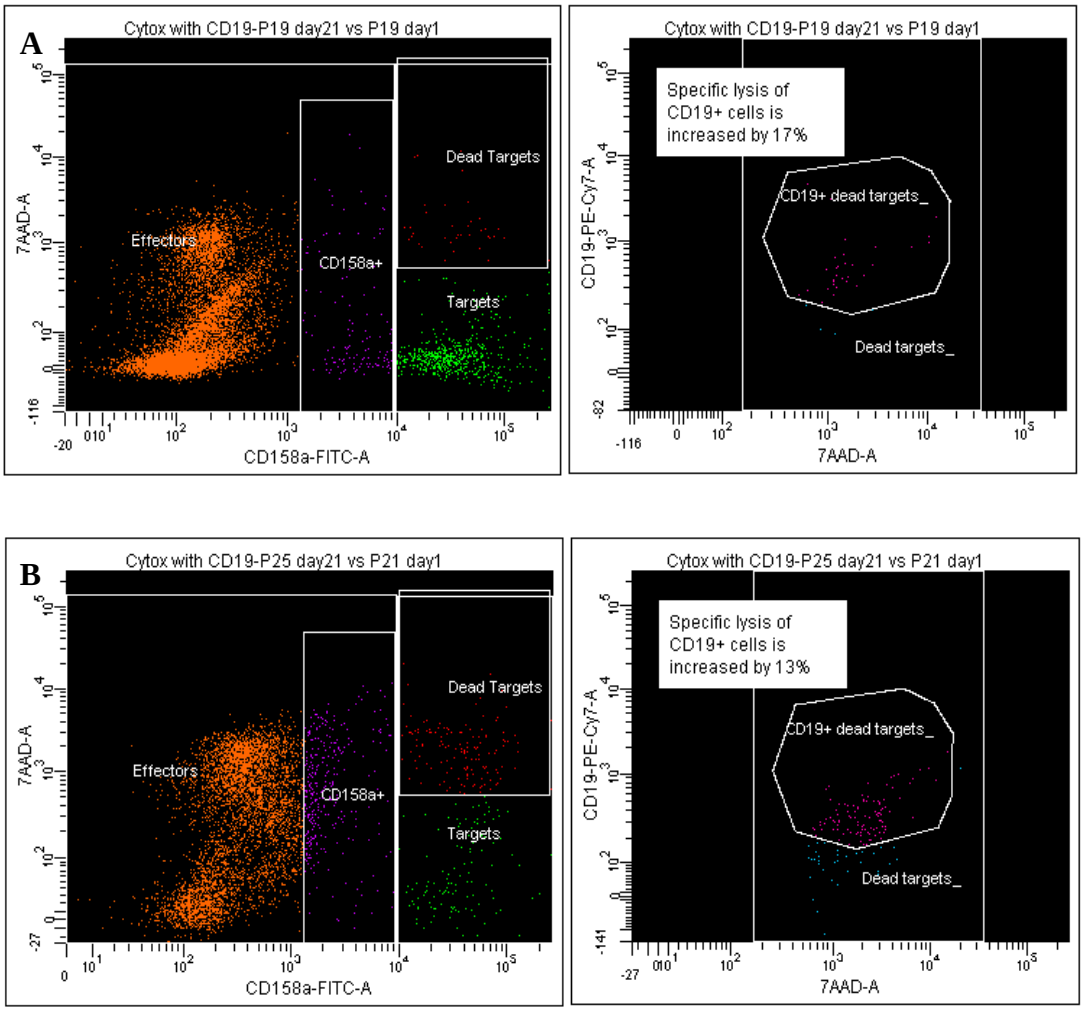


Figure 10. Specific cytotoxicity of *in vitro* patient NK cells against autologous day 1 PBMCs. Plot represents differences of NK cells of CLL and AML patients cytotoxic ability against autologous leukemic cells at different time points of cell culture. Results for CLL patients are depicted in dark blue, as AML patient results in pink. Tests were performed on day 15 (A), day 21 (B) of cell culture. Average results of three tests are represented.

We also measured $CD19^+$ and $CD33^+$ cells' share among dead target cells, killed during cytotoxicity test performed against autologous day 1 cells, described previously. Figure 11, shows dot plots for $CD19^+$ (for day 21 CLL) patients and Figure 12, shows dot plots for $CD33^+$ (for day 15 AML patients) cells' share in cytotoxicity tests against autologous day 1 cells. Specific cells' share was calculated by subtraction of spontaneous lysis of the $CD19^+$ or $CD33^+$ cells. Representative results for each patient are depicted.



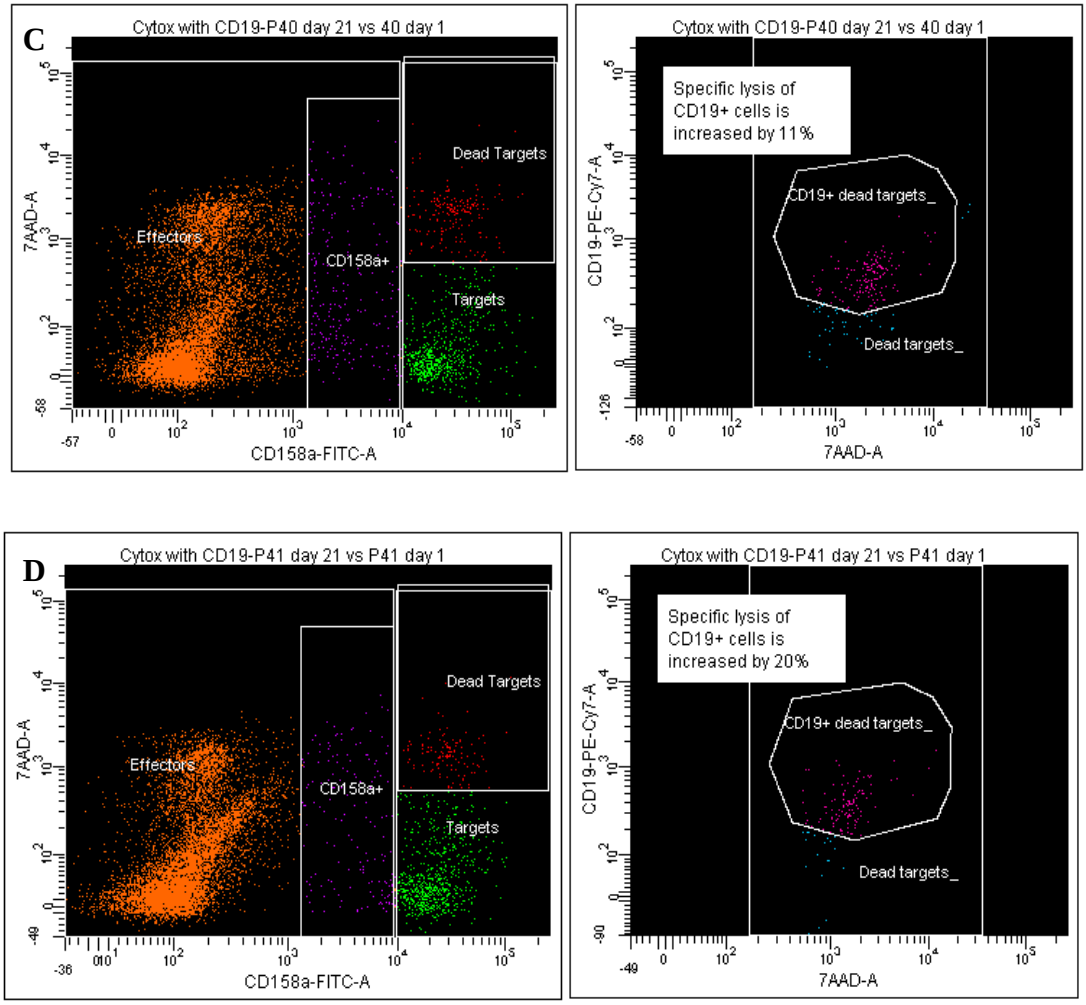
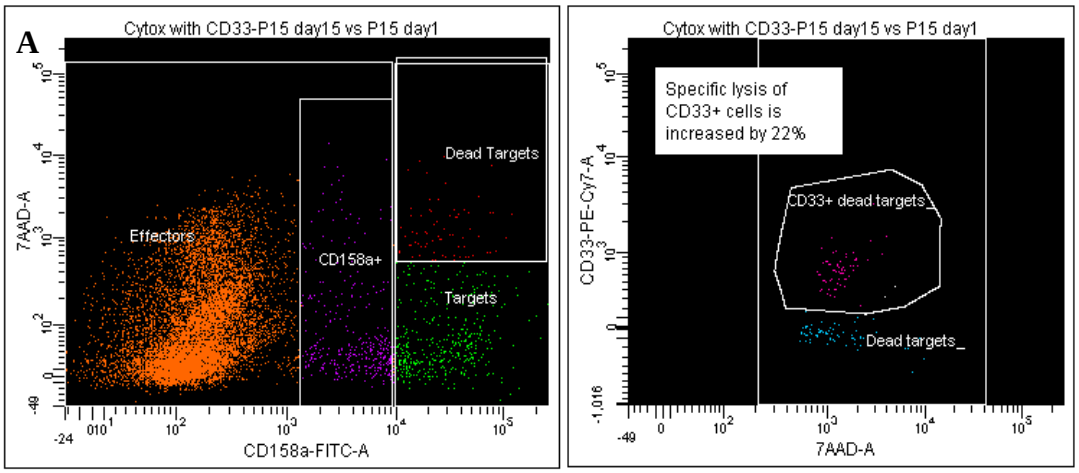
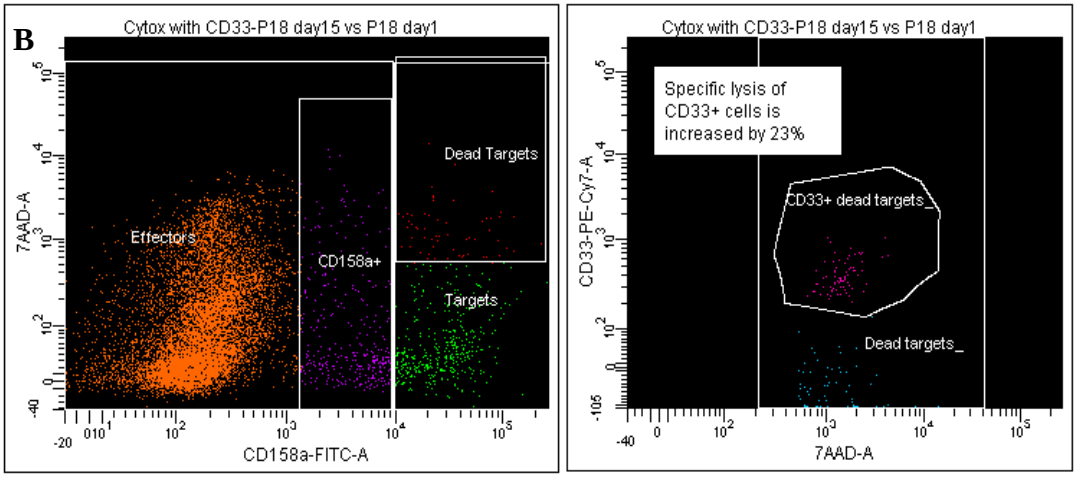
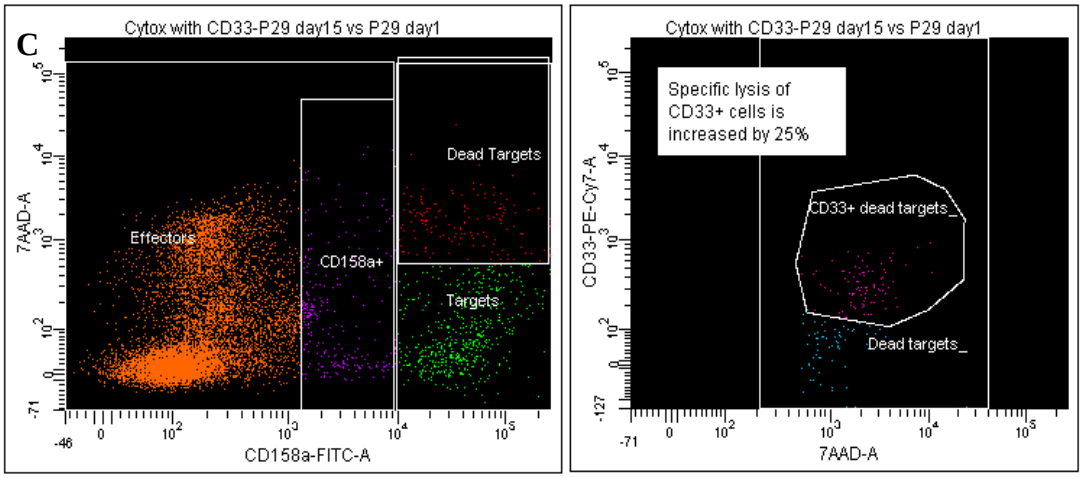


Figure 11. Specific lysis of CD19$^+$ cells in cytotoxicity test of CLL patients against autologous day 1 cells. Plot shows cytotoxicity test representative results for CLL patients (on the left), and CD19$^+$ cells' share of day 1 autologous dead cells. Specific lysis is also presented. A) CLL patient 19; B) CLL patient 25; C) CLL patient 40; D) CLL patient 41.


A
Cytox with CD33-P15 day15 vs P15 day1
Effectors
CD158a+
Dead Targets
Targets
7AAD-A
CD158a-FITC-A
Cytox with CD33-P15 day15 vs P15 day1
Specific lysis of CD33+ cells is increased by 22%
CD33+ dead targets_
Dead targets_
CD33-PE-Cy7-A
7AAD-A


B
Cytox with CD33-P18 day15 vs P18 day1
Effectors
CD158a+
Dead Targets
Targets
7AAD-A
CD158a-FITC-A
Cytox with CD33-P18 day15 vs P18 day1
Specific lysis of CD33+ cells is increased by 23%
CD33+ dead targets_
Dead targets_
CD33-PE-Cy7-A
7AAD-A


C
Cytox with CD33-P29 day15 vs P29 day1
Effectors
CD158a+
Dead Targets
Targets
7AAD-A
CD158a-FITC-A
Cytox with CD33-P29 day15 vs P29 day1
Specific lysis of CD33+ cells is increased by 25%
CD33+ dead targets_
Dead targets_
CD33-PE-Cy7-A
7AAD-A

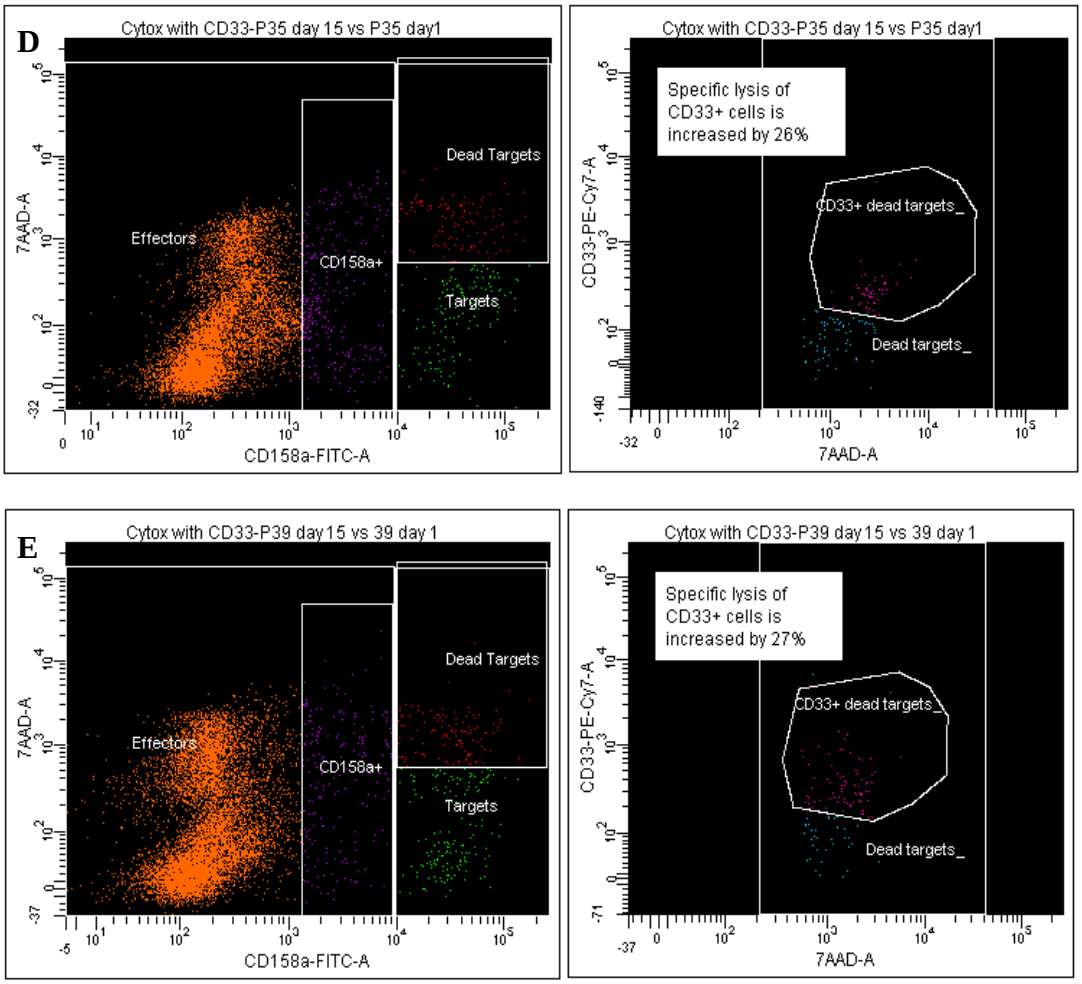


Figure 12. Specific lysis of CD33$^+$ cells in cytotoxicity test of AML patients against autologous day 1 cells. Plot shows cytotoxicity test representative results for AML patients (on the left), and CD33$^+$ cells' share of day 1 autologous dead cells. Specific lysis is also presented. A) AML patient 15; B) AML patient 18; C) AML patient 29; D) AML patient 35; E) AML patient 39.

3.3. NK cells' preservation at -150ºC

We compared NK (CD56$^+$CD3$^-$) cells' share at day 21 after different periods of sample preservation at -150ºC: 1 month, 3 months and 6 months. Results for 7 healthy donors are presented. Results indicate that sample preservation at -150ºC for period of more than 3 months influences NK cells' ability to expand by day 21 (Figure 13). Average results of one test, performed in duplicate, are presented. Standard deviations are not depicted, as they did not exceed 10%. Figure 14 shows how sample preservation influences on NK cells' cytolytic ability. On this plot NK cells share from Figure 13 is plotted against L-specific cytolysis (in percent), calculated according to Equation 1.1. Cytotoxicity results were evaluated in 4 hour FACS-based cytotoxicity assay at the end of

culture. Linear trendlines are drawn through the data. Data for donor 1 and 13 at 6th month are absent.

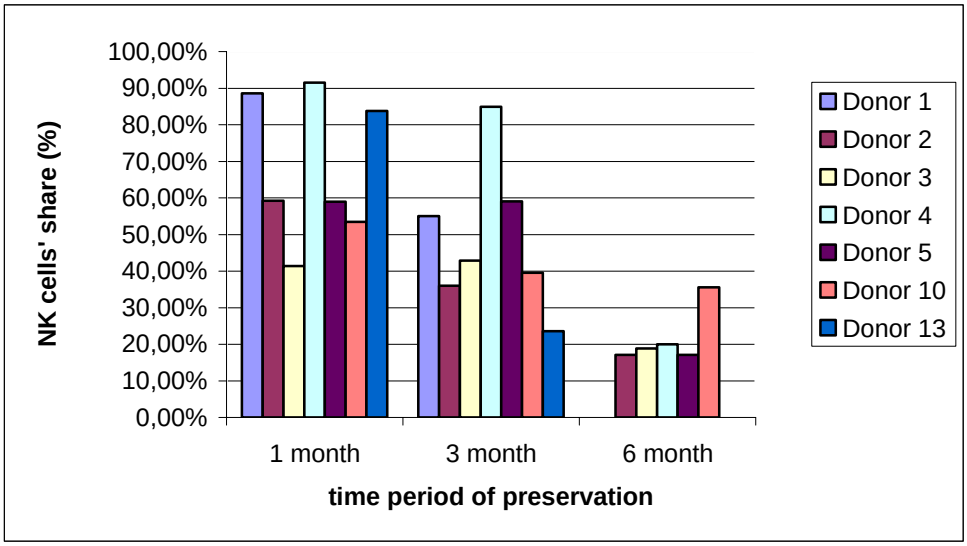


Figure 13. NK cells' preservation at -150°C. Plot represents results of NK ($CD56^+CD3^-$) cells' share expansion of 7 healthy donors at day 21 after different periods of preservation at -150°C. Results for one test are presented. Standard deviations (up to 10%) are not presented.

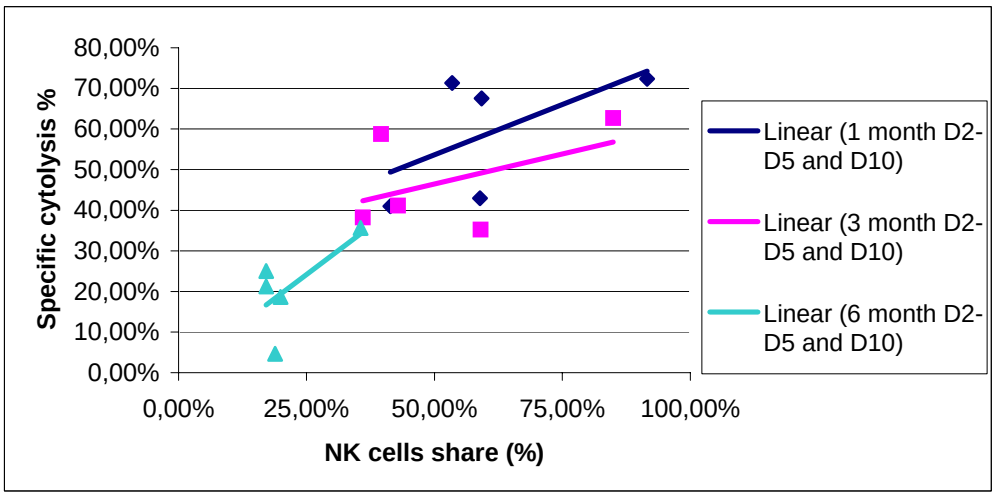


Figure 14. Cytolytic ability of cryopreserved NK cells during a time period of 6 months. Plot represents changes in cytotoxicity in 5 healthy donors (D2-D5 and D10) after 3 and 6 months of cryopreservation. Result for one test is presented.

Discussion

To answer the questions, proposed in the aims of study we have chosen 2 different groups of patients and healthy donors, which we tested during a 1.5 year period. It should be mentioned, that by healthy we mean individuals that were approved as healthy by Tartu University Hospital blood bank. All patients described were firstly diagnosed CLL or AML and all tests were performed on samples of patients before they have received chemotherapy. As CLL is the most common leukemia type among elder individuals, we wanted to test our protocol for NK cells' expansion, and also the reactivity of these cultivated cells against different target cells. As a second patients' subset, we have chosen AML, as another frequent disease. So in this work we have compared healthy donor s' results to patients' results, as well as CLL patients' results to AML patients' results. Altogether samples of 7 healthy donors, 4 CLL patients and 5 AML patients were tested.

The results for lymphocytes ability to expand in cell culture by day 21 are shown in Figure 5. This plot shows lymphocytes average expansion for 7 healthy donors, 4 CLL and 5 AML patients. We can conclude that our modified protocol allows cultivation of donor as well as patient samples.

There were two aspects that interested us in NK cell culture singularity: the NK ($CD56^+CD3^-$) cells' share and the total NK cells' number at the end of the culture. Results for the NK cells' share in the cell culture are presented in Figure 6. Figure 6A shows that at the end of the culture average NK cells' share achieved 66%. Donor 1 and 13 (both homozygous for HLA-B0 group of alleles), could expand NK cells up to 90% in the cell culture. We cultured cells until day 25, but after day 21 the cells began to die. Figure 6B and Figure 6C represent results for NK cells' share dynamics for CLL and AML patients, respectively. The NK cells' share started decreasing in both patient groups, after reaching maximum of 49% at day 21 and 34% at day 15, respectively.

Figure 7 represents total NK cells' number variation between healthy donors and patients. According to our calculations, our modified cultivation protocol allows to expand NK cells of healthy donors between 2×10^3 – $2{,}5\times10^3$-fold. Fold expansion for CLL patients was between $2{,}23\times10^3$- 2×10^4 and for AML patients we were able to obtain maximum NK cells at day 15, with expansion between $0{,}39\times10^3$ – $1{,}33\times10^3$-fold.

7 healthy donor samples, as well as 4 CLL and 5 AML patients were tested for cytotoxicity against target cell line that lack HLA class I molecules – K562

(erythroleukemia cell line). Receptors that may potentially be involved in generating cytotoxic reactions against this cell line include activatory receptors such as NKG2D and natural cytotoxicity receptors, also, a great role is played by KIR molecules, which activate the NK cells in the absence of HLA class-I-mediated inhibition. Results are presented in Figure 8. Interpreting the data, we can conclude that cytotoxicity of one NK cell at day 21 is higher for AML and lower for healthy donors. However, due to aspect that NK cells' share was on average higher for healthy donor samples, maximum cytotoxicity displayed NK cells of healthy donors. Nevertheless, all samples displayed considerable cytotoxicity against target cells, showing that NK cells remain reactive during *in vitro* cultivation.

We measured rituximab-mediated ADCC of NK cells on two patient subsets in comparison to normal samples (Figure 9). As K562 cell line is also deficient for CD20, which is a ligand for rituximab, we tested the potential reactivity on another target cell line Namalwa. Despite that we don't know patient genotypes, and thereby the potential KIR reactivity, we can conclude that higher cytotoxicity against Namalwa cell line showed samples with larger NK cells' share. From the Figure 9 we can also conclude that CD16 receptor, mediator for ADCC is absent from NK cells' surface on day 21, and disappears approximately at day 3-4. On the 1^{st} cell culture day, rituximab-mediated ADCC is increasing specific cytotoxicity of NK cells up to 15%, at day 3, it is influencing only up to 10%. In conclusion NK cells that had been cultivated *in vitro* during 15-21 days of cell culture do not participate in rituximab immunotherapy.

One of the most important tasks of this current thesis was to control two distinct subsets of patients NK cells' ability to kill autologous day 1 leukemic cells (Figure 10). Interpreting the data results, we may conclude that both patient subsets, CLL or AML, are able to kill autologous cells with almost equal efficiency despite difference in NK cells' share. And as for results against K562 cell line, cytotoxicity per one NK cell was higher in AML patient samples. We have also shown in Figure 11 and in Figure 12 that cells that were killed during cytotoxicity assay were mostly $CD19^+$ (for CLL patients) and $CD33^+$ (for AML patients) cells.

We have analysed the influence of cryopreservation on NK cells' function. In conclusion, Figure 13 and Figure 14 show that sample cryopreservation for periods longer than 3 months affects NK cells' share ability to expand and through this their cytotoxic potency.

Results of the current thesis indicate, that the *in vitro* cultivated NK cells maintain their cytotoxicity by day 21 of cell culture. The specific cytotoxicity is higher for AML patients, and NK cells' expansion rate is higher for healthy patients, homozygous for HLA-B0 group of alleles. Biological mechanisms for described differences need to be investigated further.

Summary

Complications, associated with bone marrow transplantation, are numerous: graft rejection and failure, virus diseases and regimen-related toxicity, drug reactions, acute and chronic Graft-versus-Host Disease and finally relapse. HLA-identical donor finding is in many respects question of luck. However, for how long person would remain in remission or achieve complete remission is the other side of the coin. Lots of immunotherapies are in use today in order to prolong graft survival and increase the probability of complete remission achievement.

NK cells' immunotherapy provides new approach to cellular immunotherapies, due to NK cells' fascinating capability of distinguishing organism "self" from "non-self" cells, reactivity against virus-infected cells or transformed cancer cells, ability to mobilize organism acquired immune system via interaction with dendritic cells and cytokine production. As NK cells' cytotoxic activity is restricted only to cells of hematopoietic origin, they could kill cancer cells of the leukemia patient and not rejecting the skin allograft at the same time.

This is a pre-clinical study supported by Competence Centre for Cancer Research in collaboration with Tartu University Department of Hematology and Oncology, that represents results for *in vitro* cultivation of NK cells for autologous and allogenic immunotherapy for leukemic diseases. We have shown that:

- Modified protocol allows cultivation of NK cells of healthy donors as well as of leukemic patients for allogenic or autologous immunotherapy.
- There is direct relation between NK cells' share in sample and cytotoxicity ratio, as NK cells remain cytotoxic in cell culture.
- Rituximab influences NK cell-mediated ADCC, only during first days of cell culture.
- We have also shown that cultivated NK cells are able to kill autologous leukemic cells *in vitro* with efficiency up to 30%. Cells that were killed were mostly either $CD19^+$ (for CLL patients) or $CD33^+$ (for AML patients).
- Higher NK cells' share and NK cells' number can be obtained from samples that preserved at -150°C for no longer than 3 months.

We can conclude that problems stated in the current thesis are solved. We would definitely continue our research work on NK cells cultivation in larger volumes.

Kokkuvõtte

Loomulike tapjarakkude (NK rakkude) *in vitro* kultiveerimine autoloogseks ja allogeenseks immunoteraapiaks

Leukeemiaravis kasutatava luuüditransplantatsiooniga võivad kaasneda mitmed komplikatsioonid: organite või kudede äratõuked, viirus- ja bakteriaalsed haigused, akuutne või krooniline transplantaat peremehe vastu haigus ning lõpuks relaps. HLA-identse doonori leidmine on keeruline ja aeganõudev ning see ei taga veel täielikku remissiooni saavutamist. Tänapäeval kasutatakse erinevaid teraapiaid parandamaks siirdatava materjali säilimist ja tõstmaks täieliku remissiooni tõenäosust. Viimasel ajal on seetõttu tähelepanu pööratud ka NK rakkude kasutusvõimalustele rakulises immuunteraapias.

Mitmed NK rakkude omadused soodustavad nende kasutamist immuunteraapias, sest NK rakkud on võimelised ära tundma viirusega nakatunud või transformeerunud rakke, eristama organismi enda rakke "mitte omadest", mobiliseerima organismi teisi immuunsüsteemi rakke ning produtseerima tsütokiine.

Antud töös kajastatakse prekliiniliste katsete tulemusi, mis saadi NK rakkude *in vitro* kultiveerimisel kaugema eesmärgiga kasutada neid autoloogseks ja allogeenseks immuunteraapiaks. Käesoleva töö tulemused ja järeldused olid järgmised:

- Täiendatud NK rakkude kasvatamise protokoll võimaldab kasvatada NK rakke tervetelt doonoritelt allogeenseks ja patsientidelt autoloogseks immuunteraapiaks.
- NK rakkude protsent koekultuuris ja tsütotoksilisuse määr on omavahel otseses sõltuvuses.
- Rituximab mõjutab NK rakkude tsütotoksilisust ainult kultiveerimise esimestel päevadel, hilisemat mõju tsütotoksilisusele ei täheldatud.
- Kultiveeritud NK rakkud on *in vitro* tingimustes võimelised hävitama autoloogseid leukeemiarakke effektiivsusega kuni 30%.
- Kõrgem NK rakkude protsent ja koguarvukus saadi proovidest, mida säilitati temepratuuril -150°C mitte rohkem kui 3 kuud.

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