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Due to the many medical advances in recent years, treatment of acute lymphoblastic leukemia (ALL) has improved tremendously. It has been estimated that about 80% of children that are diagnosed with this disease will likely go into remission. However, there is still a need for a more specific, less invasive treatment that lessens any toxic side effects of current cancer treatments and at the same time, lowers the risk of relapse.

Natural Killer (NK) cells, which are components of the lymphocyte population, can recognize and act on target cells under the control of their cell surface receptors. Binding of these receptors to specific ligands on the target cell results in signaling which either activates or inhibits NK cell effector functions. We have previously identified cell surface receptors 2B4 (CD244), CS1 (CRACC) and LIT1 playing a major role in NK cell activation. Along with receptors NKp30 and NKp46, previous studies have shown that these receptors play a role in leukemia and other cancers, however their significance and role in childhood ALL have not been evaluated. Based on this knowledge, this thesis tested the hypothesis that altered expression of these immune receptors may play a role in acute lymphoblastic leukemia in children. The results presented in this thesis demonstrate that there is indeed an alteration in the expression of receptors 2B4, CS1, LIT1, NKp30 and NKp46 in both the mRNA and surface protein level. This data can contribute to further understanding the functional role of these receptors that in turn can help develop a better mode of treatment for patients with childhood ALL.

ROLE OF NK-CELL RECEPTORS IN ACUTE LYMPHOBLASTIC LEUKEMIA IN
CHILDREN

THESIS

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Chapter I

Introduction

Leukemia is the most common type of cancer in children and adolescents. It is characterized by an abnormal increase of immature white blood cells that are devoid of regular cellular functions. Leukemia cells, or blast cells, can crowd out normal blood cells, which can lead to bleeding, anemia, infections and other serious problems. If left untreated, it can lead to severe complications that would ultimately lead to the patient's death. Despite the steady decline in death rate due to advancements in treatment, leukemia still causes more deaths among children and young adults under the age of 20 than any cancer combined ¹.

Although natural killer (NK) cells have long been known to mediate antigen independent tumor killing, the therapeutic potential of NK cell-based immunotherapy has yet to be realized. NK cells are large, granular lymphocytes that comprise 5-10% of the recirculating lymphocyte population that play an important role against cancer and various infections. They are an essential part of the innate immune system and play an important role in the adaptive immunity as well. They express several surface molecules that regulate NK cell function both positively and negatively, and that it is the overall sum of these signals that ultimately determines cell

function and activation. Also, they express Fc receptor molecule that allows them to target cells against which an antibody response has been mobilized and lyse the cells through antibody dependent cell-mediated cytotoxicity (ADCC). The molecular basis of NK cell target recognition is not fully understood. Towards this goal, our lab has identified three NK receptors 2B4 (CD244), CS1 (CRACC, CD319), and LLT1 that can activate NK cells upon the interaction with their ligands²⁰. Other significant receptors involved in NK cell cytotoxicity are the Natural cytotoxicity receptors (NCRs), NKp30, NKp44 and NKp46. Recent observations suggest that the ability of leukemia cells to escape immunosurveillance involving NK cells contributes to the poor outcome of the disease².

This research project explored the role of NK cell receptors in acute lymphoblastic leukemia (ALL) in children. Specifically, we investigated the expression and function of NK receptors 2B4, CS1, LLT1, NKp30 and NKp46 in peripheral blood mononuclear cells (PBMC) of childhood ALL subjects compared to healthy subjects. This will enable us to further understand the role of these receptors in immune dysregulation in childhood ALL.

1.1 Problem and Hypothesis

An issue with current cancer treatments is the lack of specificity when targeting leukemic cells. Chemotherapy, the preferred method in treating ALL, is efficient in killing leukemic cells but affects the peripheral (normal) cells as well. This leaves the patient susceptible to infections and other complications. Also, about 10-20% of children with ALL will suffer a relapse³. This usually is accompanied by poorer prognosis, and the salvage therapy that occurs as a result of the relapse comes with much more severe side effects³. A push for a more specific, less toxic cancer therapy has become a hot topic of research in recent years.

Immune receptors that are expressed on NK cells are known to play a role in NK cell killing of tumor cells. These receptors that are also expressed on B cells, T cells and monocytes, can either be inhibitory or activating depending on the context of their interactions. Previous studies by us and other laboratories showed that 2B4, CS1 and LLT1 receptors can differentially regulate the function of NK cells. However, their significance and role in ALL have not been evaluated. We believe that in leukemia, altered expression of these receptors could affect its function

We hypothesize that altered expression of NK cell receptors play a role in childhood Acute Lymphoblastic Leukemia.

1.2 Significance

Cancer treatment has undeniably progressed through the years. What was once considered a death sentence can now be managed by taking a proactive approach in caring for one's health, proper dosing of chemotherapy and, in some cases, a bone marrow transplant. These, however, do not diminish the fact that cancer treatments are costly, traumatic for everyone involved, not 100% effective and still plagued with toxic side effects that are damaging and can affect the patients far after they go on remission from cancer. The latter is an important aspect to consider especially in Acute Lymphoblastic Leukemia where approximately 60% of patients diagnosed are under the age of 20¹.

Previous studies have shown that receptors, including the ones that are part of this study, play a role in NK cell cytotoxicity of leukemic cells as well as in immune evasion mechanisms. Our attempt in this study is to analyze the expression of these receptors in patients with ALL and compare with healthy subjects, and then evaluate their function (inhibitory or activating) in

childhood ALL. Furthermore, by understanding the mechanism of action of these immune receptors, we may be able to develop a treatment by enhancing our own immune system's ability to target leukemic cells. Not only will this treatment be more specific, but, hopefully will also be less detrimental to the patient's health.

1.3 Background

Leukemia

Leukemia is a cancer of the early blood forming cells, most often, of the white blood cells. It starts in the bone marrow and in most cases, invades the blood fairly quickly. From there, it can go to other parts of the body such as the lymph nodes, spleen, liver, central nervous system and other organs

Any of the cells from the bone marrow can turn into leukemia cell. This is thought to occur when blood cells acquire mutations in their DNA⁵. Once this change takes place, leukemia cells fail to go through the normal process of maturing. They reproduce quickly and do not undergo apoptosis when they should. They build up in the bone marrow and eventually spill into the bloodstream and spread to other organs where they can keep other cells from functioning properly. The exact cause of leukemia is unknown although it has been thought to develop from a combination of genetic and environmental factors.

One way to classify leukemia is based on their speed of progression (acute or chronic). In acute leukemia, blood cells affected are normally immature blood cells called blasts. They lack normal cell function and multiply rapidly which requires an aggressive and timely treatment⁵. In chronic leukemia, blood cells affected tend to be more mature and retains some of its normal cell

function. Leukemia of the chronic kind can remain undetected for some time and more often harder to treat.

Another way leukemia is classified is based on which lineage of cell is affected. Leukemia can either be lymphocytic which affects the lymphoid cells that forms the lymphatic tissue, or myelogenous, which affects the myeloid cells that gives rise to red blood cells (RBCs), some white blood cells (WBCs) and platelet producing cells⁵. Based on these classifications, four major types of leukemia have been characterized. These are chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML) and the focus of this research, acute lymphoblastic leukemia (ALL)

Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia (ALL), a fast growing cancer of the lymphocyte forming cells called lymphoblasts, is due to a malignant disorder of lymphoid progenitor cells^{4,5}. ALL is most common in childhood, with peak prevalence between the ages of 2 and 5 years of age^{4,5}. Since, ALL is most often diagnosed early in childhood, it is difficult to apply preventative measures and there is no known reliable screening at this time. Physicians can determine the best course of treatment based on different factors, such as age of the patient and initial white blood cell (WBC) count at diagnosis.

Standard treatment for ALL includes chemotherapy, radiation therapy and stem cell transplant. Though these treatments have proven their efficacy, they are not without damaging side effects that in some cases can impact the patient's health even after years of remission. In chemotherapy, for example, drugs used tend to be relatively non-selective, often attacking healthy cells as much as the lymphoblasts⁶ thus leaving the patients susceptible to infections.

They are also at a higher risk of developing other cancers later in life ⁵. Patients who were treated with chemotherapy or radiation as children also have a higher risk for heart disease, stroke and osteonecrosis ⁵. In some cases, sexual development and the ability to have children can also be affected ⁵. Recent observations suggest that the ability of leukemia cells to escape immunosurveillance involving NK cells contributes to the poor outcome of the disease ².

Natural Killer Cells

NK cells are implicated in hematological malignancies and are part of the first line of defense against pathogens and cancer cells ^{7,8,9}. They are large, granular lymphocytes that comprise 5-10% of the recirculating lymphocyte population. The majority of NK cells is localized in peripheral blood, lymph nodes, spleen and bone marrow but can be induced to migrate toward inflammation site by different chemoattractants ¹⁰. Aside from their role against tumor and various infections, recent studies suggest that NK cells have memory. It has been observed that although NK cells do not rearrange the genes encoding their activating receptors, NK cells experience a selective education process during development, undergo a clonal like expansion during virus infection, generate long-lived progeny (i.e. memory cells) and mediate more efficacious secondary responses against previously encountered pathogens ¹¹.

The ability of NK cells to destroy leukemic cells as demonstrated by allogeneic transplantation of hematopoietic stem cells indicates that this cell type is implicated in the control and clearance of leukemia ¹². The functions of NK cells are regulated by a delicate balance of signal received through activating and inhibitory receptors ⁷. While activating receptors are stimulated by a wide variety of ligands ¹³, inhibitory receptors interact with self MHC class I antigens and protect normal cells from NK cell attack ¹⁴. Thus, NK cells attack cells

that fail to express sufficient levels of MHC class I molecules of the host ¹⁵ also known as the “Missing self-hypothesis”. A problem with this is that many malignant cells express MHC class I antigens which makes them resistant to NK cell killing ¹⁴. It has been shown, however, that engagement of activating NK cell receptors by ligands expressed on tumor cells can overcome inhibitory signals and activate NK cell even in the presence of MHC class I ¹⁴ (Figure 1). Indeed, progress in the field of NK cell receptors has revolutionized our concept of how NK cells selectively recognize and lyse tumor and virally infected cells while sparing normal cells ¹³. Identification of these receptors and the expression of their ligands on both normal and transformed cells (lymphoblasts) ¹³ are keys to understanding their function and may help us develop a way to manipulate these interactions which will gear NK cells towards specifically targeting lymphoblasts in ALL.

NK cell receptors

As previously mentioned, NK cell receptors are known to play a role in NK cell’s killing against tumor cells. These receptors, also expressed in B cells, T cells and monocytes, can either be inhibitory or activating depending on the ligand they interact with. Previous studies have shown that certain receptors can act specifically against different types of tumor/cancer cells. Identification of these receptors and the expression of their ligands on both normal and transformed cells are important to understand their function.

NK cell receptors that would be the focus of this study are 2B4, CS1, LLT1, NKp30 and NKp46.

The rationale for selecting these receptors in the study were based on our preliminary data and several studies that show these receptors to play a key role in the immune system by enhancing the NK-mediated cytolytic activity.

2B4 (CD244)

One of the receptors cloned in our lab, 2B4, is expressed on all NK cells, monocytes and CD8+ T cells, basophils and eosinophils ¹⁶. Cross linking with its specific antibody increases the cytolytic activity and cytokine secretion in NK cells. By generating 2B4 gene knockout mice, we have shown that 2B4 plays a role in the rejection of cancer cells and also play a central role in the immune system ^{17,18}. A previous study showed that 2B4 receptor has a strong co-stimulatory effect in NK cells targeting leukemic cells ¹⁴. In addition, human NK cells express two isoforms of 2B4, h2B4-A and h2B4-B that differ in a small portion of the extracellular domain ³³. Previous study demonstrated that these two isoforms differ in their binding affinity for CD48, which results in differential cytotoxic activity as well as intracellular calcium release by NK cells upon target cell recognition ³³.

CS1 (CD319, CRACC)

CS1, another receptor cloned in our lab, is expressed on NK cells, B cells, activated T-cells and mature dendritic cells (DC) ^{17,19,20}. It is known to be essential for triggering NK cell cytotoxic function in mice and humans and it induces B cell proliferation and autocrine cytokine secretion in humans ²¹. Previous study showed that two isoforms of CS1, CS1-L and CS1-S, are expressed in human NK cells that differentially regulate NK cell function ²². It has been found

that human B lymphocytes express only the CS1-L isoform, and its expression is up-regulated upon B cell activation with various stimulators²². Additionally, previous study on multiple myeloma showed that humanized antibody which specifically targets CSI has the potential to eliminate multiple myeloma cells via ADCC mediated by NK cells²³.

LLT1 (CLEC2D)

Lectin-like transcript 1 (LLT1), also cloned in our lab, is a receptor expressed on NK cells, T cells, B cells and all monocytes. It activates NK cells to produce IFN-gamma but does not induce killing of target cells. The natural ligand of LLT1 has been identified as CD161, an NK cell inhibitor receptor, known to play an important role in immune regulation²⁴. In a previous study, it was shown that glioblastoma cells escape NK cell killing by overexpressing LLT1, presumably by inhibiting NK cell killing via ligation of the inhibitory CD161 receptor²⁸.

NKp30 and NKp46

NKp30 and NKp46 belong to the family of natural cytotoxicity receptors (NCRs). NCRs are believed to be capable of mediating direct killing of tumor and virus infected cells and are specific for non-MHC ligands²⁵. NKp30 have different isoforms which can either be immunostimulatory or immunosuppressive²⁶. In patients with gastrointestinal sarcoma for example, it was shown that they have an over expression of the immunosuppressive isoform of NKp30²⁶. NKp46, on the other hand, has been shown to play a role in controlling tumor metastasis²⁷.

Although some of these receptors have been shown to play a role in leukemia, their function in childhood ALL has not been evaluated. Considering the high significance of these receptors in different cancers, it is imperative that its function and therapeutic potential in childhood ALL be investigated.

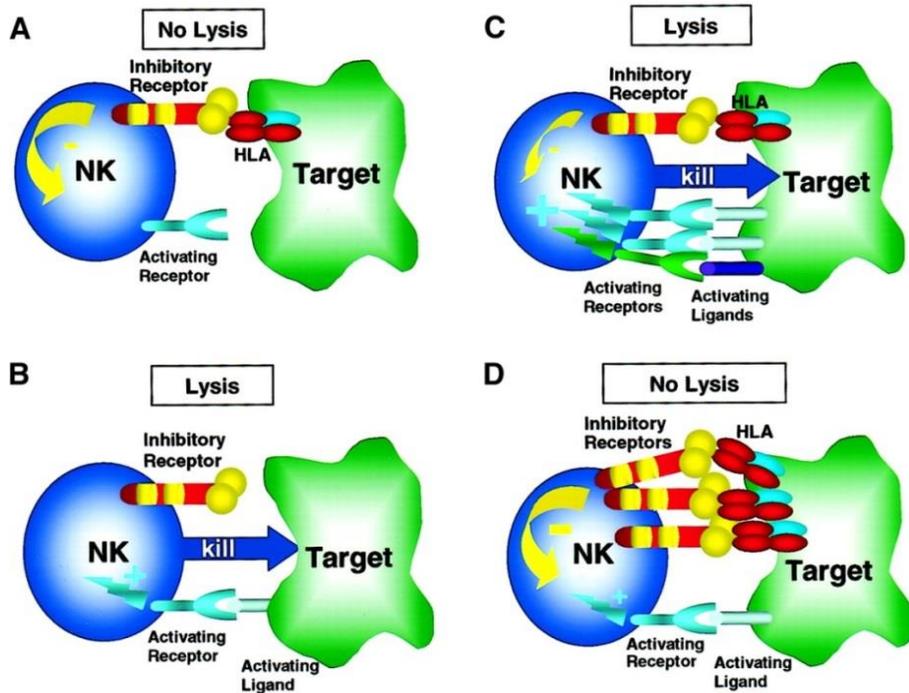


Figure 1 NK cells recognize target cells through multiple receptor-ligand interaction.

Chapter 2

Materials and Methods

2.1 Study Population and Subject Enrollment

Newly diagnosed ALL subjects between the ages of 2-21 years are enrolled in the study at the Hematology and Oncology clinic at Cook Children's Medical Center (CCMC) Fort Worth, TX by Dr. Paul Bowman. Healthy subjects for control that are matched to cases by age (+/- 3 years), gender, and ethnicity are enrolled in the study at the pediatric department of UNT Health Science Center (UNTHSC) Fort Worth, TX by Dr. John Fling.

Informed consent or parental consent/assent was obtained from all participants prior to enrollment. IRB approval from both CCMC and UNTHSC has been obtained.

2.2 Blood Collection

8 milliliters (mls) of blood were collected from ALL subjects at the time of diagnosis and after chemotherapy treatment (29 days after diagnosis). 8 mls of blood were also collected from healthy subjects

2.3 Sample Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood that was collected by layering the sample in the leukosep tube with histopaque (3 mls – Sigma Aldrich, St. Louis, MO) and lysing the RBCs with 5 mls of ACK (Ammonium-Chloride-Potassium) buffer for 5 minutes at room temperature. Lysing reaction was stopped by adding 20 mls of PBS+EDTA to the sample.

The PBMCs collected was separated into two portions. One portion, approximately 5 million cells, was used for flow cytometric analysis of surface expression of immune receptors and the other portion, another 5 million cells, was used to isolate mRNA through RT-PCR.

2.4 RT-PCR

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was used to determine the gene expression of the different immune receptors in the PBMC isolated from both ALL and healthy subjects. Total RNA was isolated with RNA stat 60 reagent according to the manufacturer's protocol (Teltest Inc, Friendswood, TX) and cDNA was synthesized using

Omniscript RT kit (Quiagen) reverse transcriptase and random primers in a volume of 20 uL²²
³³. Primers used for cDNA amplification of the different receptors are as follows: 2B4: (FP) 5'
GCT CTT TGC CTT CCA ATA CTT CC 3' (RP) 5' GGC CAA AAT CTG AAT TCC TGA
TGG GT 3'. CS1: (FP) 5' GTC TCT TTG TAC TGG GGC TAT TTC 3' (RP) 5' TTT CCA TCT
TTT TCG GTA TTT C 3'. LLT1: (FP) 5' TTC CTG TTG AGA TAT AAA GGC 3' (RP) 5'
CAG GAT AGG AAA CTG TCT TG 3'. NKp30: (FP) 5' TCT TGA TCA TGG TCC ATC CA
3' (RP) TGA ACT CTG GGG TTC CAT TC 3'. NKp46: (FP) 5' ACT TGG GGC ACC TAC
CTT TT 3' (RP) 5' CTT CCC AAG TGG AAG CTC TG 3'. GAPDH: (FP) 5' ATG ACA TCA
AGA AGG TGG TG 3' (RP) 5' CAT ACC AGG AAA TGA GCT TG 3'. Amplification and
annealing temperatures were optimized and reactions were resolved using 1.2% agarose gel
electrophoresis²⁹.

2.5 Densitometry

After electrophoresis, the agarose gel was photographed using UVP Bioimaging systems
at 302nm and individual lanes were analyzed by densitometry.

2.6 Flow Cytometry

PBMCs were stained with C 1.7 mAb (anti-2B4), anti-human CRACC mAb (anti-CS1),
anti-LLT1, anti-NKp30, and anti-Nkp46 conjugated to different fluorophores (Becton Dickinson,
San Diego, CA) to determine the cell surface expression of the receptors. The multi-colored
stained PBMC will be analyzed on a Beckman Coulter FC 500 flow cytometer. PBMCs obtained

from healthy individuals served as control for comparison of expression of 2B4, CS1, LLT1, NKp30 and NKp46.

2.7 Statistical Analysis

To analyze the differences in immune receptor expression between specimen collected at the time of diagnosis and specimen collected 29 days after diagnosis and after chemotherapy treatment, a paired sample t-test and non-parametric Mann-Whitney test were used using GraphPad Prism. Differences were considered significant at values of $p < 0.05$.

Chapter 3

mRNA Expression of Immune Receptors on PBMCs in Patients with Acute Lymphoblastic Leukemia Children

Purpose of the Aim

The purpose of this aim was to determine the expression of NK cell receptors 2B4, CS1, LLT1, NKp30 and NKp46 at the mRNA level in children with ALL as compared to healthy subjects. Determining the receptor expression at the gene level may also help in predicting surface protein expression. The mRNA expression was examined on PBMCs collected from ALL patients at the time of diagnosis as well as healthy donors. The difference in the expression of these receptors could suggest the involvement of these receptors in disease pathophysiology.

Receptor Expression at the mRNA Level

2B4, CS1 and LLT1 are receptors cloned in our laboratory. Along with NKp30 and NKp46, these receptors are known to play a role in NK cell function. They can either be

inhibitory or activating which determines the NK cell cytotoxicity towards a target cell. Previous studies have shown the involvement of these receptors in the disease pathogenesis of various cancers, but there are very few studies that have studied their role in acute lymphoblastic leukemia in children. Therefore, mRNA expression of each receptor was examined to determine if there is any significant difference between expressions in ALL patients as compared to healthy controls. If so, this difference could be a factor to the development and progression of the disease.

In order to perform the comparison, newly diagnosed ALL patients were enrolled in the study as well as age, gender and ethnicity matched healthy subjects. The demographics and clinical characteristics of each patient and healthy donors are given in Table 1. PBMCs from both ALL patients and healthy donors were separated from whole blood by the procedure mentioned previously²². RT-PCR was performed using different primers specific for each receptor and expression was determined by gel electrophoresis and densitometry.

Analysis of mRNA expression in the PBMC of ALL patients revealed overexpression of 2B4 in patients 1, 3, 5 and 9 as compared to healthy subjects (Figure 2a). Patients 2, 4, 6, 7, 8, 10 and 11 either showed no expression or little expression of 2B4. As for NKp46 (Figure 6a) patients 1, 2, 7, 8 and 10 either showed no expression or very little expression. The results for NKp30 were inconclusive due to the high variability of NKp30 mRNA expression of the healthy controls (Figure 5a). For the CS1 receptor, some patients (patient 3 and 5) showed over expression of CS1 while others showed a decrease in the expression (Figure 3a). Interestingly, both ALL and healthy subjects showed expression of different isoforms of the LLT1 receptor with variation in their expression level (Figure 4a). To verify these observations, optical density

of each receptor expression were obtained through densitometry and these values were normalized against their corresponding healthy control values. This aided in determining the fold change of each receptor expression for each patient as compared to the healthy control (figure 2b,3b,4b,4c,5b and 6b).

These results confirm that there is indeed an alteration in receptor expression at the mRNA level on PBMCs of ALL patients as compared to healthy controls. Furthermore, it was also demonstrated that there is a variation in expression of receptors between each individual patient.

S. No.	Subject Code	Age	Sex	Race/Ethnicity	Immuno-phenotype	WBC counts Normal Range (4.5 – 13.0 x 10 ³ /ul)
1	UNTALL10	7	M	Caucasian	T Cell	50.0
2	UNTALL24	3	F	Mix-C-H-AA	B Precursor	35.5
3	UNTALL4	4	M	Hispanic	B Precursor	2.9
4	UNTALL29	3	F	African American	B Precursor	3.6
5	UNTALL12	10	M	Hispanic	B Precursor	5.88
6	UNTALL3	5	F	Hispanic	B Precursor	2.7
7	UNTALL14	3	F	Caucasian	B Precursor	12.6
8	UNTALL15	15	M	Caucasian	B Precursor	23.15
9	UNTALL9	5	M	Caucasian	B Precursor	60.4
10	UNTALL21	12	M	Caucasian	B Precursor	27.9
11	UNTALL25	11	M	Caucasian	B Precursor	5.4

Table 1: List of ALL patient demographics, immunophenotype and WBC counts.

2B4

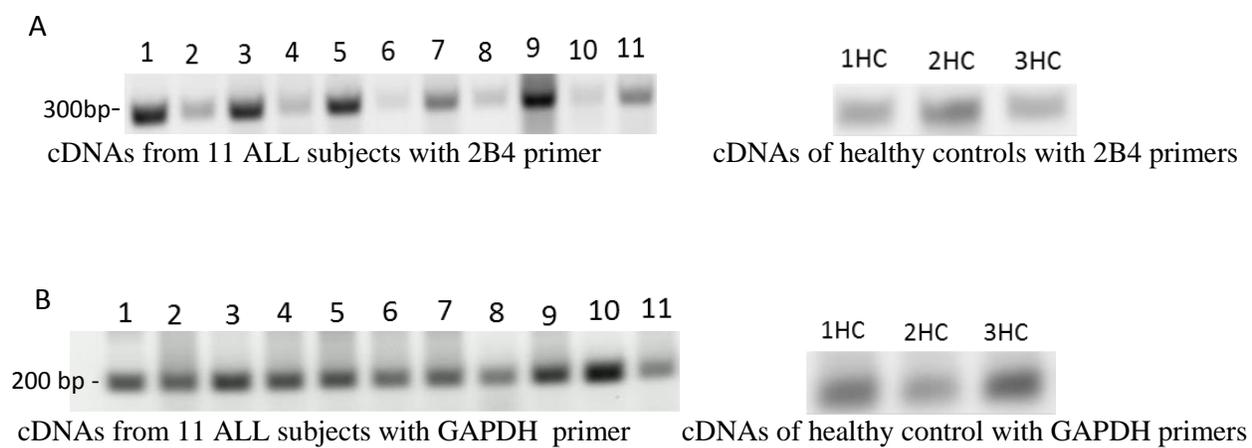


Figure 2a: RT-PCR analysis of NK cell receptor 2B4. (A) RT-PCR analysis of 2B4 expression in 11 ALL subjects and 3 healthy control (HC). NK92 cell line was also used as control (not shown). (B) ALL subjects and healthy controls with GAPDH primer as loading control.

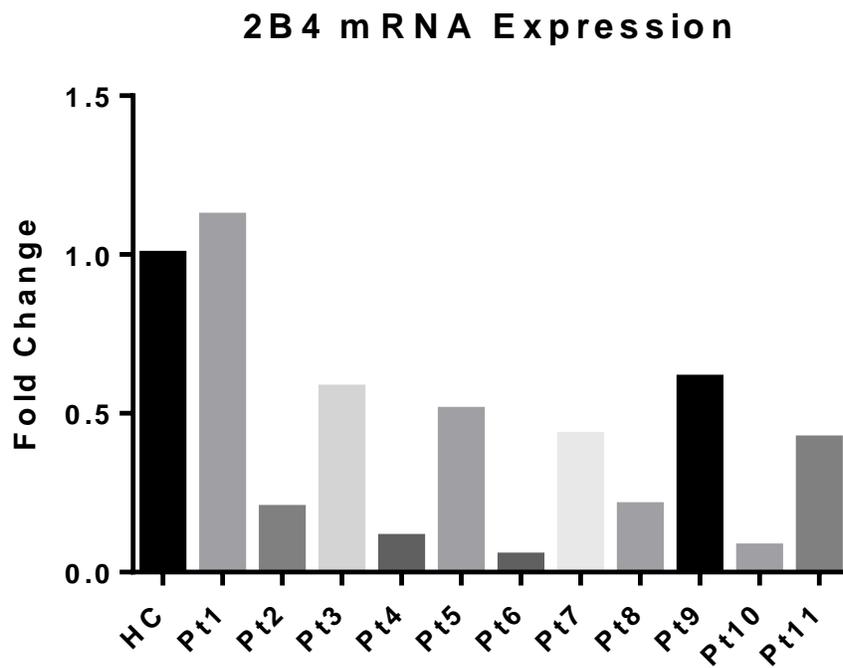


Figure 2b: Optical density of the 2B4 expression of 11 patients (Pt) normalized to healthy controls (HC)

CS1

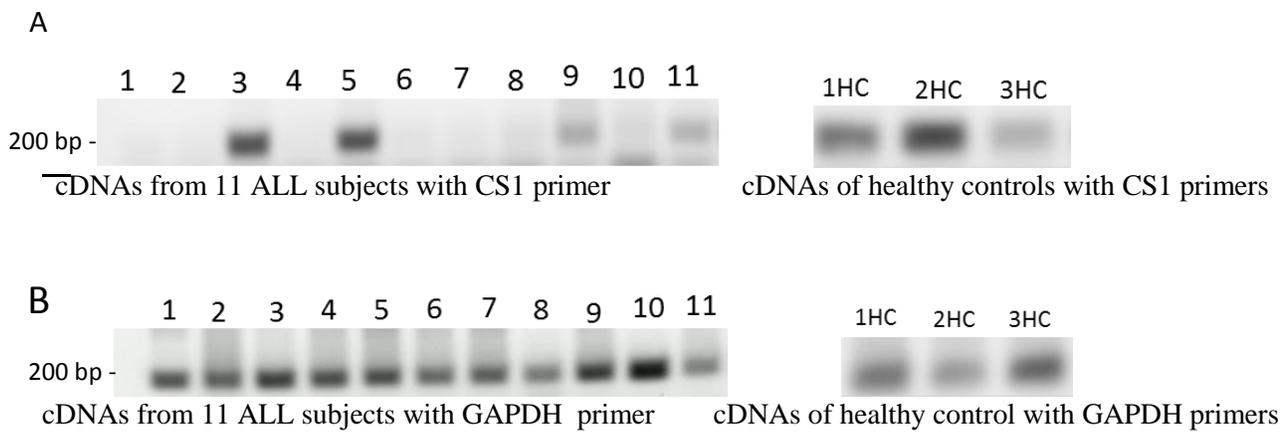


Figure 3a: RT-PCR analysis of NK cell receptor CS1. (A) RT-PCR analysis of CS1 expression in 11 ALL subjects and 3 healthy control (HC). NK92 cell line was also used as control (not shown). (B) ALL subjects and healthy controls with GAPDH primer as loading control.

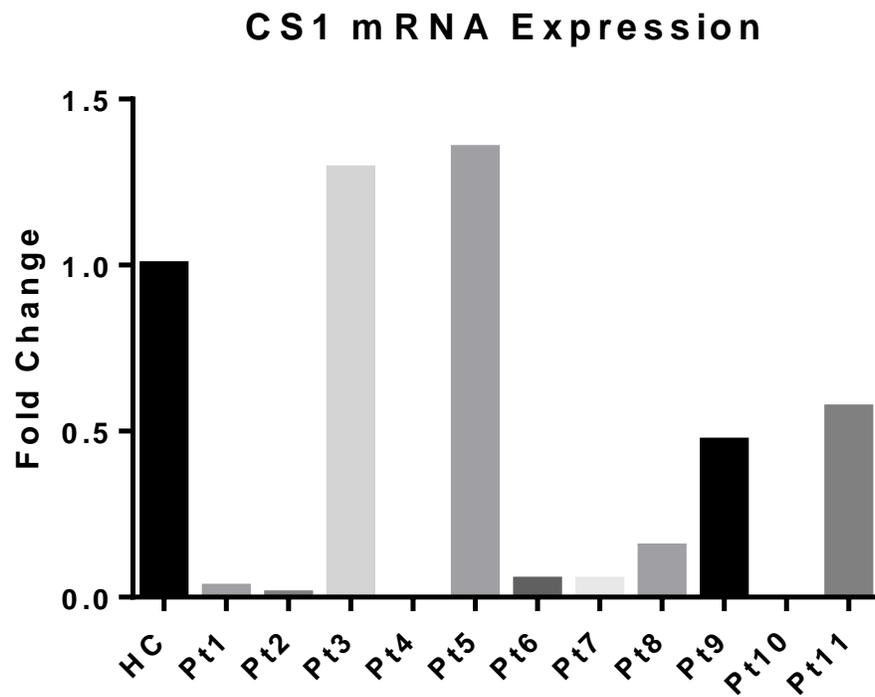


Figure 3b: Optical density of the CS1 expression of 11 patients (Pt) normalized to healthy controls (HC)

LLT1

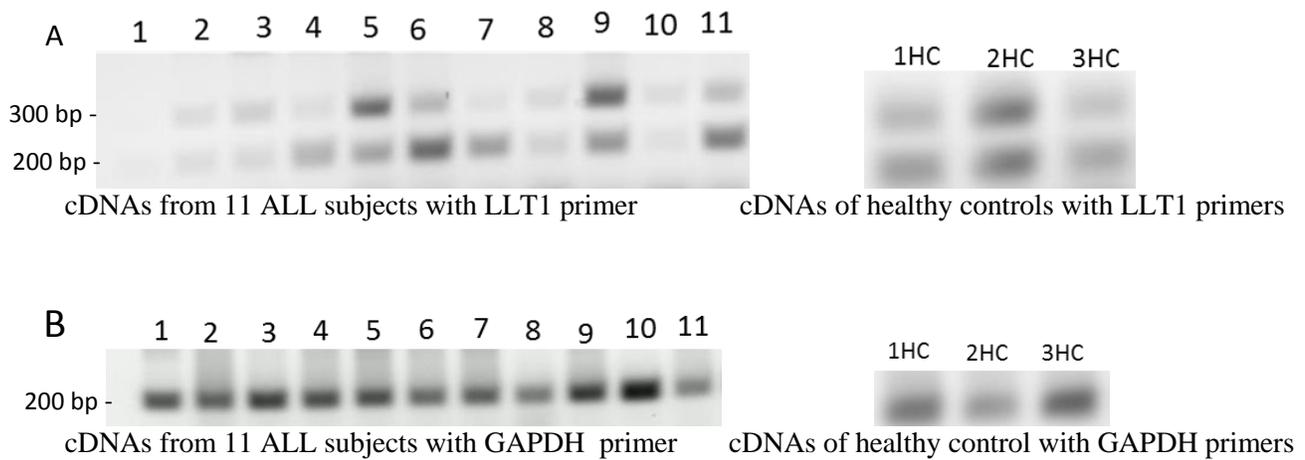


Figure 4a: RT-PCR analysis of NK cell receptor LLT1. (A) RT-PCR analysis of LLT1 expression in 11 ALL subjects and 3 healthy control (HC). NK92 cell line was also used as control (not shown). (B) ALL subjects and healthy controls with GAPDH primer as loading control.

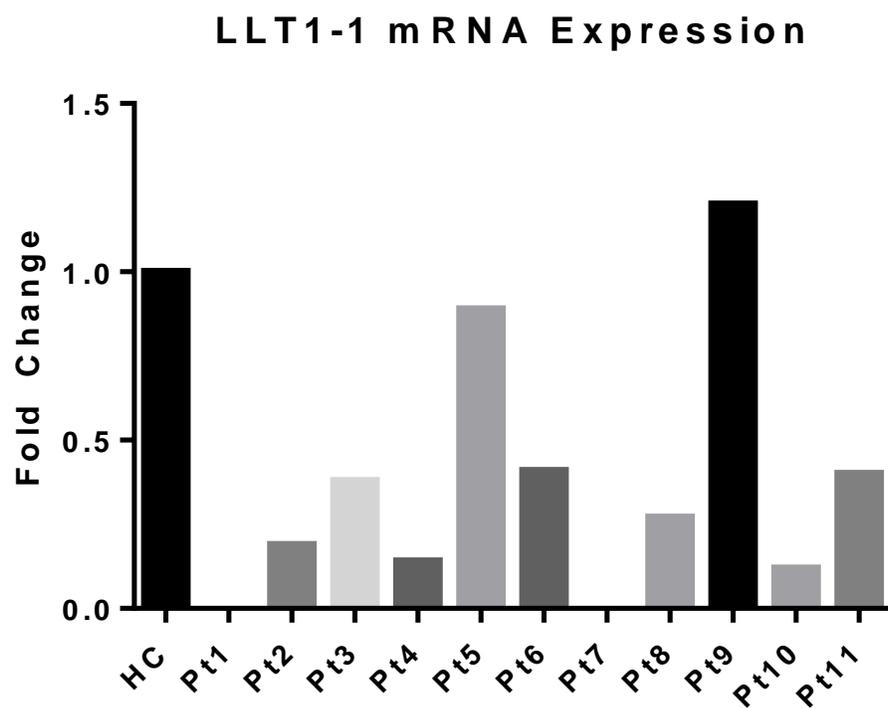


Figure 4b: Optical density of the LLT1 (isoform 1) expression of 11 patients (Pt) normalized to healthy controls (HC)

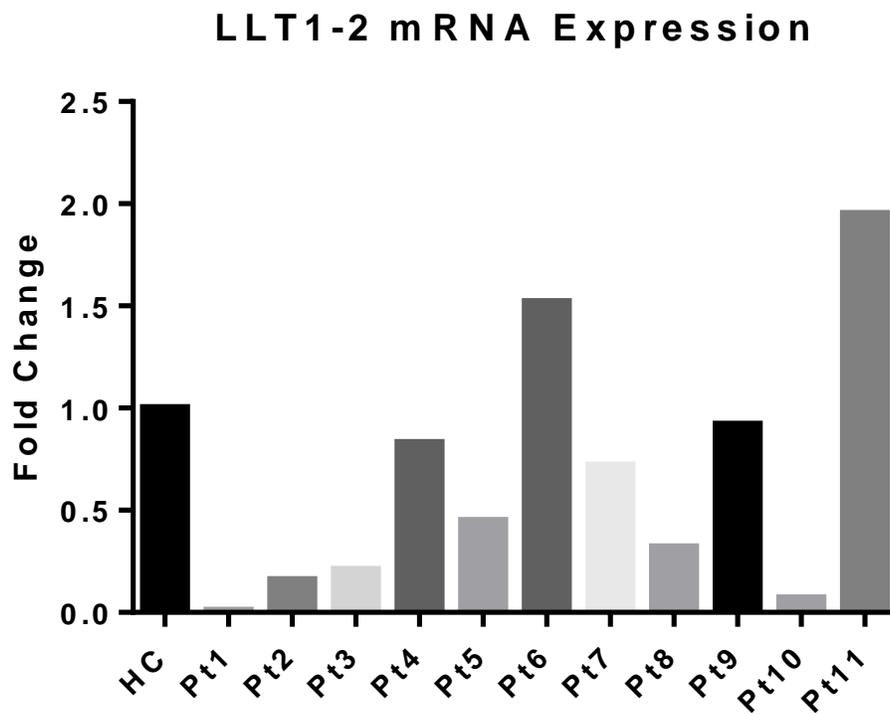


Figure 4c: Optical density of the LLT1 (isoform 2) expression of 11 patients (Pt) normalized to healthy controls (HC)

NKp30

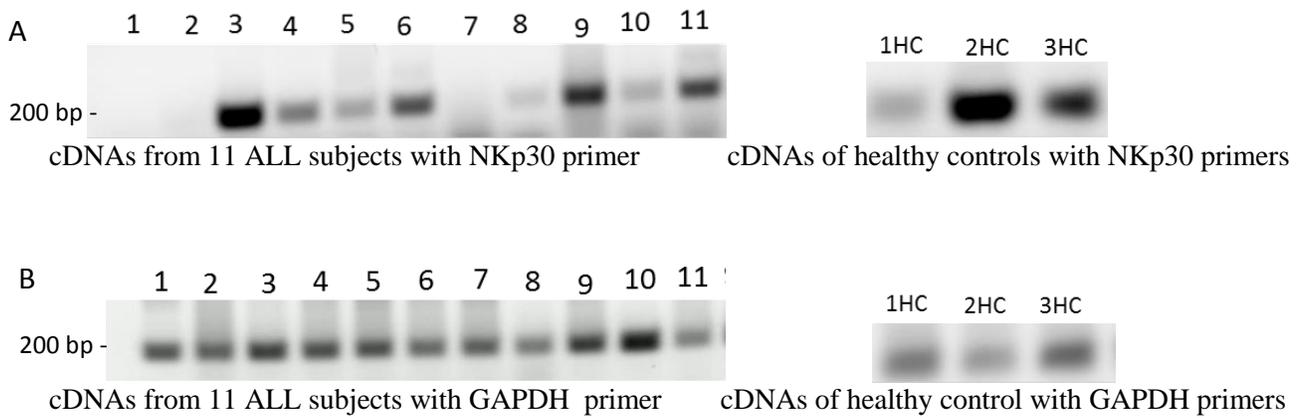


Figure 5a: RT-PCR analysis of NK cell receptor NKp30. (A) RT-PCR analysis of NKp30 expression in 11 ALL subjects and 3 healthy control (HC). NK92 cell line was also used as control (not shown). (B) ALL subjects and healthy controls with GAPDH primer as loading control.

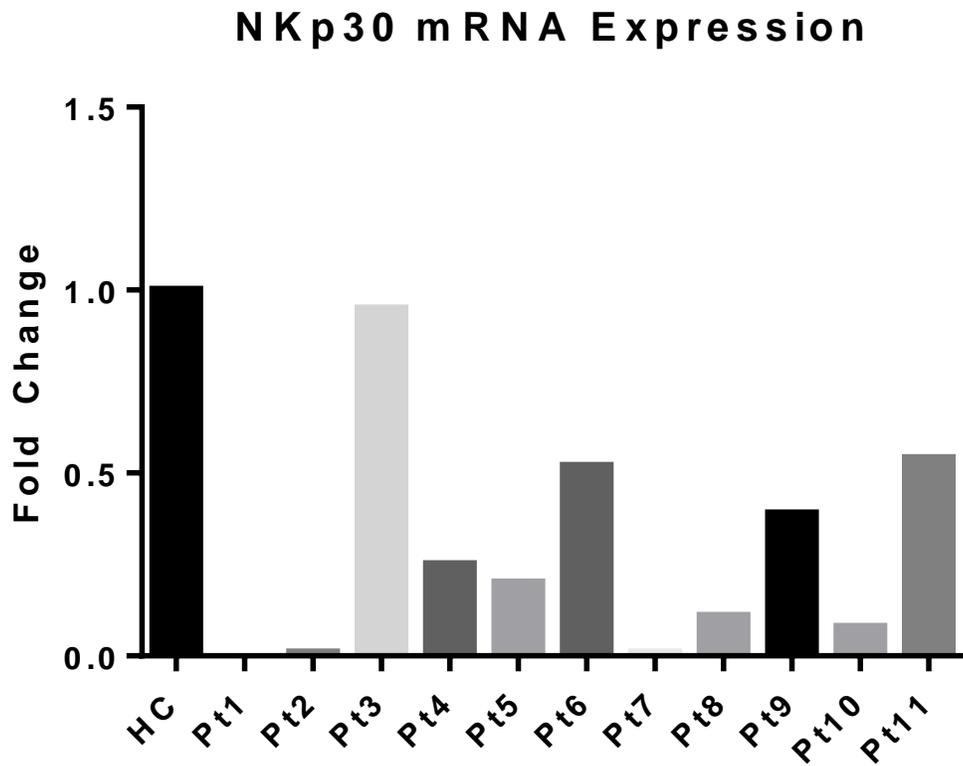


Figure 5b: Optical density of the NKp30 mRNA expression of 11 patients (Pt) normalized to healthy controls (HC)

NKp46

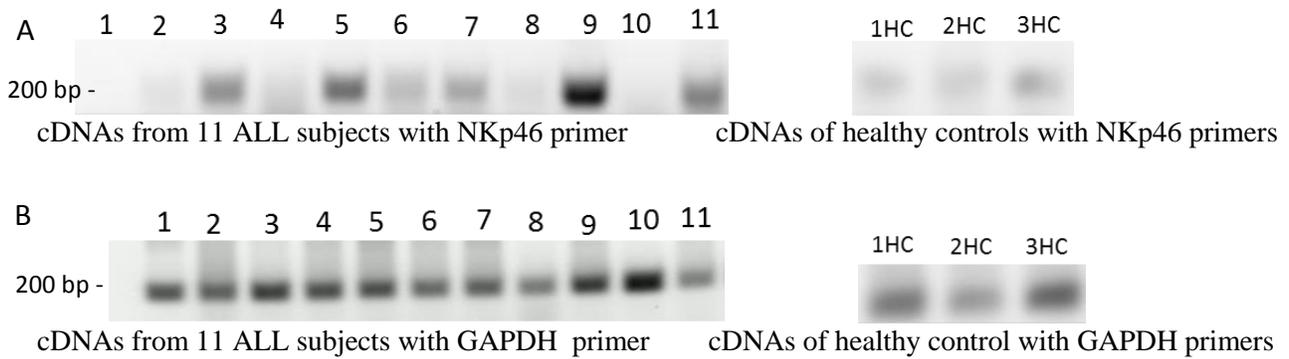


Figure 6a: RT-PCR analysis of NK cell receptor NKp46. (A) RT-PCR analysis of NKp46 expression in 11 ALL subjects and 3 healthy control (HC). NK92 cell line was also used as control (not shown). (B) ALL subjects and healthy controls with GAPDH primer as loading control.

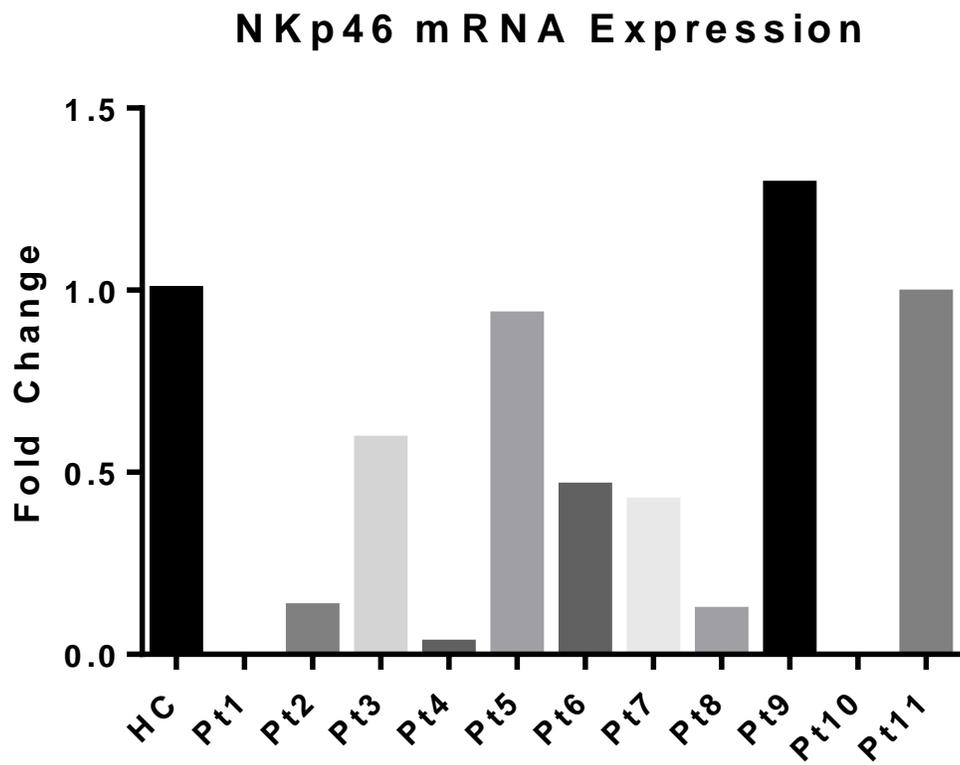


Figure 6b: Optical density of the NKp46 expression of 11 patients (Pt) normalized to healthy controls (HC)

Chapter 4

Cell Surface Protein Expression of NK Cell Receptors on PBMCs in children with Acute Lymphoblastic Leukemia

Purpose of Aim

The purpose of this aim was to evaluate the expression of NK cell receptors at the cell surface protein level in patients with ALL. Surface protein expressions were examined on PBMCs isolated from the whole blood of ALL patients both at the time of diagnosis and 29 days after diagnosis and chemotherapy treatment. Specimens from healthy subjects were also analyzed to serve as control. Alterations in NK cell receptor surface protein expression could influence the function of NK cells and other immune cells as they interact with leukemic or blast cells.

Receptor Expression at Surface Protein Level

Surface proteins are a vital part in the way cell interacts with their environment which includes communication with other cells. NK cell receptors 2B4, CS1, LLT1, NKp30 and NKp46 are known cell surface receptors that are implicated in the regulation of NK cells and

other immune cells^{13,22,27,29}. Although, we observed altered mRNA expression of receptors in the ALL patients, it is imperative to investigate whether there is alteration in the expression of these receptors at the surface protein level

To determine if variations exist in surface protein receptor expression of ALL subjects, PBMCs collected from patients both at the time of diagnosis and after 29 days after chemotherapy treatment were analyzed using Beckman Coulter FC500 flow cytometry. Forward scatter (FS) and side scatter (SS) were used to define populations of lymphocytes, monocytes, and granulocytes. The gates were set up based on size and granularity to differentiate the lymphocytes from the rest of the cells. We then further gated on specific cell markers to differentiate T cells (CD3-FITC), B cells (CD19-PE-Texas Red), NK cells (CD56-APC), and monocytes (CD14-APC Cy7). PBMCs were also stained with C-1.7 mAb (anti-2B4), anti-human CRACC mAb (anti-CS1), anti-LLT1, anti-NKp30 and anti-NKp46 conjugated to PE fluorophore (Becton Dickinson, San Diego, CA) to determine the cell surface expression of these receptors. Similarly, PBMCs from healthy subjects were stained to serve as controls. Furthermore, we grouped the patients as either high risk or standard risk as per the risk classification. The criteria for risk classification is based on the age, WBC counts, central nervous system (CNS) status, hyperdiploidy, hypodiploidy, TEL/AML1, trisomy 4, 10 & 17, minimal residual disease (MRD), BCR/ABL and MLL translocations^{34,35} as listed on table 2.

Cell surface protein expression was determined on 11 ALL patients and comparisons were made between healthy subjects and ALL patients at the time of diagnosis and 29 days after chemotherapy treatment. Due to limited patient samples, we did not observe any statistically significant differences in the cell surface protein expression but certain trends were detected.

Total percentage of T cells expressing 2B4, CS1 and NKp46 were observed to be down regulated on specimens collected at the time of diagnosis as compared to the healthy subjects (Figure 7a,8a,11a). There was also a down regulation on the percent expression of 2B4 and NKp46 on B cells on specimens collected after chemotherapy (Figure 12a,16a). NK cells seems to show similar receptor expression for all receptors both on specimens collected at diagnosis and 29 days after chemotherapy treatment when compared to the healthy subjects with the exception of LLT1 which appears to be up regulated on both type of specimens compared to the healthy subjects (Figure 17a,18a,19a,20a,21a). Monocytes showed a down regulation in the total percentage of cells expressing 2B4 and CS1 on specimens collected at diagnosis and after chemotherapy treatment (Figure 22a, 24a). On the other hand, LLT1 expression on monocytes was observed to be upregulated on specimens collected 29 days after chemotherapy treatment as compared to healthy subjects- (Figure 24a).

Mean fluoresce intensity (MFI) were also analyzed on all 11 patient for each receptor. MFI of 2B4+ cells were increased on T cells, B cells and monocytes at the time of diagnosis (figure 7b, 12b, and 17b). It also showed an increase on NK cells both at the time of diagnosis and after treatment as compared to the healthy controls (figure 22b). MFI of CS1+ cells, on the other hand, were increased on T cells, B cells and NK cells at specimen collected after treatment as compared to the healthy controls (figure 8b, 13b, and 18b) while monocytes were also increased at specimens collected at diagnosis and after treatment (figure 23b). MFI of both LLT1+ cells and NKp30+ cells were increased on all cell types both at diagnosis and after treatment (figure 9b, 10b, 14b, 15b, 19b, 20b, 24b and 25b). Lastly, MFI of NKp46+ cells showed increase expression on all cell types both at the time of diagnosis and after treatment

with the exception of NKp46 expression on B cells which appeared to be comparable to the healthy controls (figure 11b, 16b, 21b and 26b).

To further dissect our data, we analyzed each individual patient and focused on the ones that showed a marked change in surface protein expression between blood collections (blood collected at the time of diagnosis and 29 days after chemotherapy treatment). Summary of patient and healthy subjects surface protein expression in percent is presented in tables 3 to 6. Using criteria from table 2, we have observed that patients that show the most change are patients that are characterized as high risk patients. Patients 1, 8 and 10 shown in figures 27 to 31 demonstrates this pronounced change in expression of most receptors. Another observation is that most of these high risk patients also showed an increase in the percentage of CS1 expressing B cells on blood collected at diagnosis (Table 4). This is interesting considering that all but one of these patients are of B cell precursor type ALL.

These results demonstrate that there are indeed alterations on receptor expression at the surface protein level on ALL patients compared to healthy subjects. It also showed that treatment received by these patients may contribute in further alteration of these receptor expressions. It would be interesting to examine the mechanisms of these treatments and know exactly the impact they might have. Furthermore, patients categorized as high risk demonstrated a marked change in their surface protein expression when specimens collected at diagnosis and specimens collected 29 days after diagnosis and chemotherapy treatment were compared. This could further imply that these receptors do play a role in disease development.

Risk group classification A	Risk group classification B	Provisional criteria	Additional Features ²	IDs
Standard Risk	Standard Low	1<=age<10 <u>AND</u> WBC<50.000/ul <u>AND</u> CNS-1	(Hyperdiploid <u>AND</u> Trisomy 4 <u>AND</u> Trisomy 10) <u>OR</u> TEL/AML positive <u>AND</u> MRD <0.1	UNTALL4
	Standard Average	1<=age<10 <u>AND</u> WBC<50.000/ul <u>AND</u> CNS-2	(Does not meet Hyperdiploid <u>AND</u> Trisomy 4 <u>AND</u> Trisomy 10) <u>AND</u> TEL/AML negative <u>AND</u> MRD <0.1	UNTALL3, 24,29
	Standard High	1<=age<10 <u>AND</u> WBC<50.000/ul <u>AND</u> CNS-2	(Does not meet Hyperdiploid <u>AND</u> Trisomy 4 <u>AND</u> Trisomy 10) <u>AND</u> TEL/AML negative <u>AND</u> MRD ≥0.1	
High Risk	High + Fast Early Response	Age>=10 <u>OR</u> WBC>=50,000/ul <u>OR</u> CNS-3	MRD<0.1	UNTALL9,10,12, 14,15, 25
	High + Slow Early Response	Age>=10 <u>OR</u> WBC>=50,000/ul <u>OR</u> CNS-3	MRD ≥0.1	UNTALL21

Table 2: Risk classification adapted to categorize ALL patients as high risk or low risk^{34,35}

CD3 - 2B4 Surface Expression

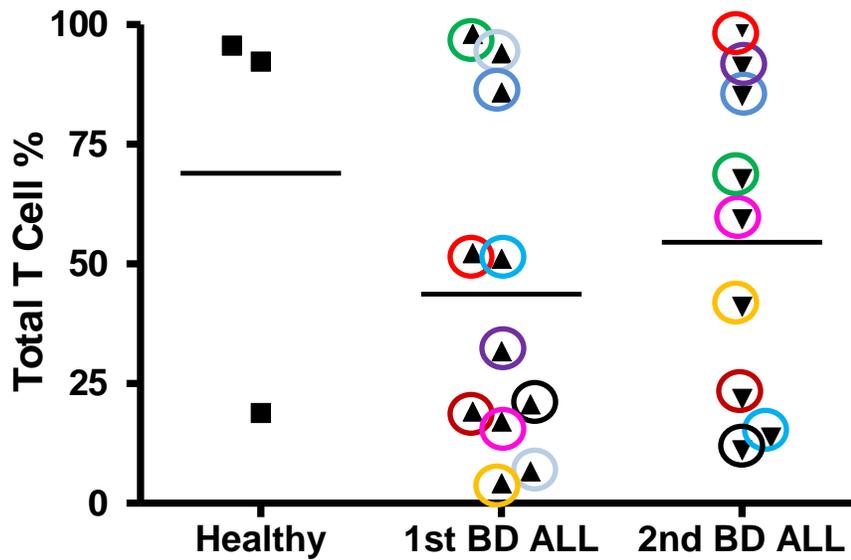


Figure 7a: Cell surface expression of 2B4. 2B4 expression on T cells (CD3) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

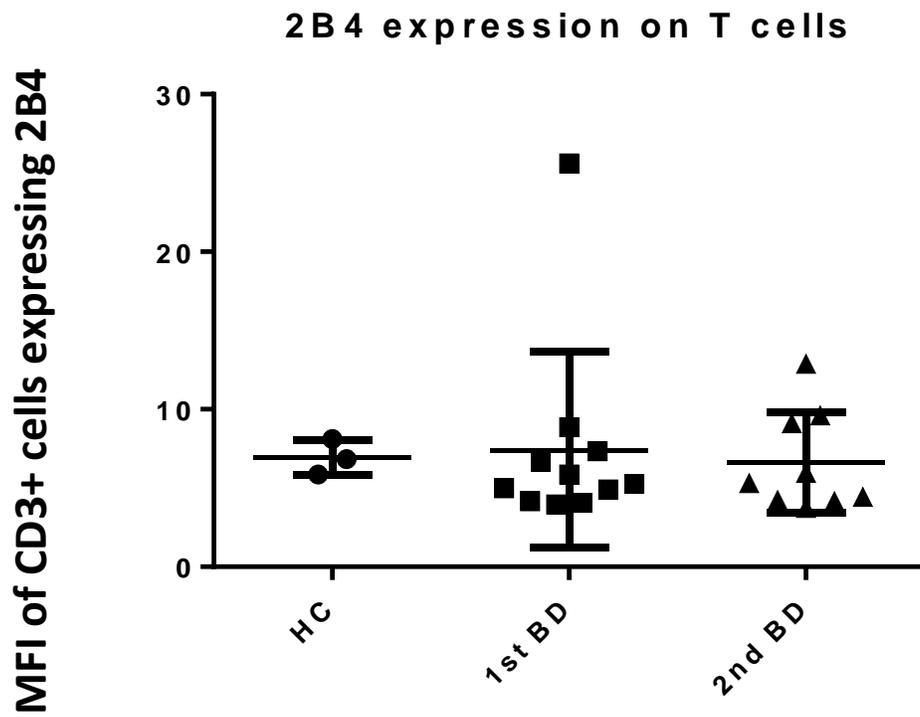


Figure 7b: Mean fluorescence intensity of CD3+ cells expressing 2B4 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD3 - CS1 Surface Expression

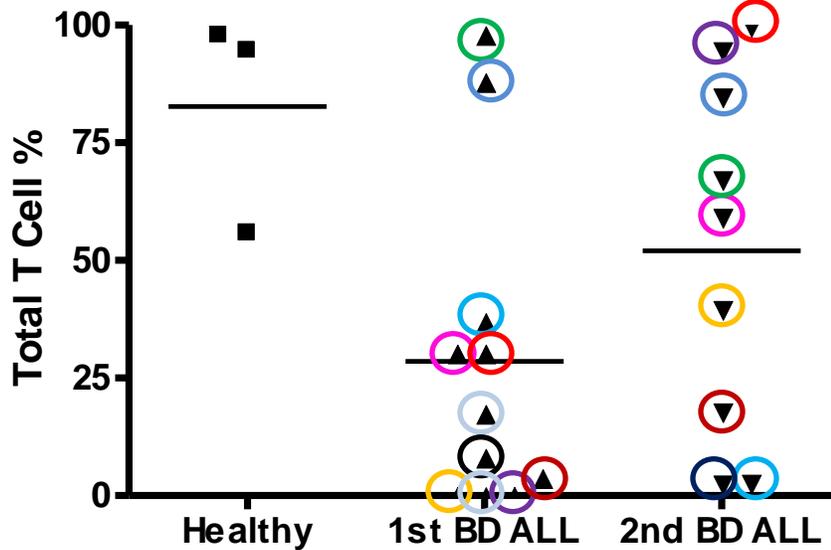


Figure 8a: Cell surface expression of CS1. CS1 expression on T cells (CD3) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

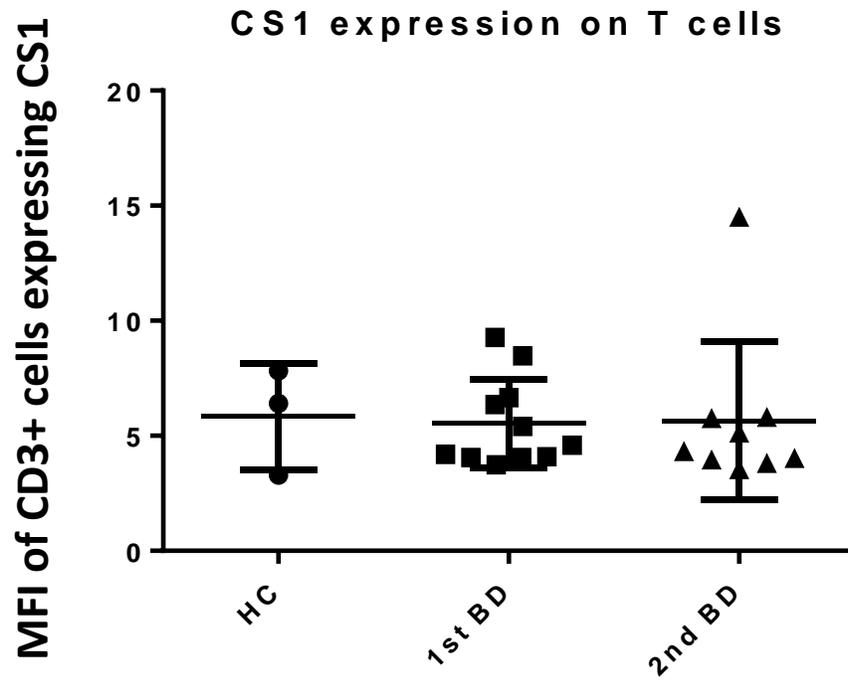


Figure 8b: Mean fluorescence intensity of CD3+ cells expressing CS1 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD3 - LLT1 Surface Expression

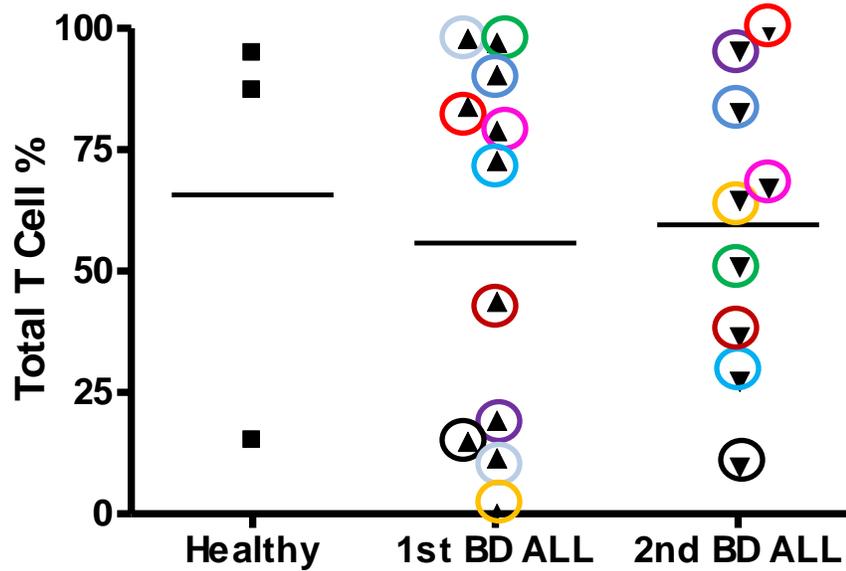


Figure 9a: Cell surface expression of LLT1. LLT1 expression on T cells (CD3) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

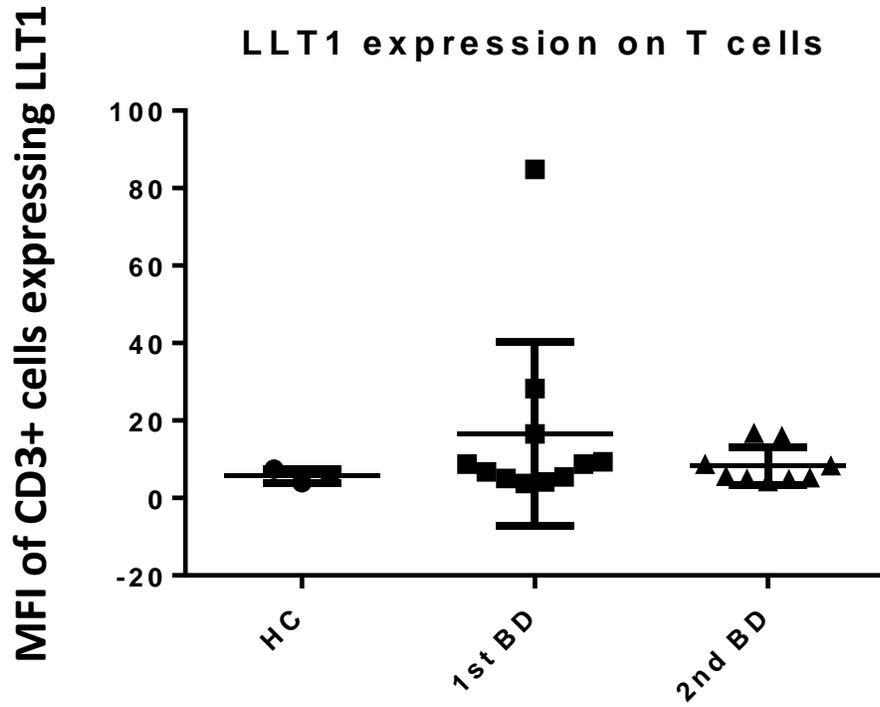


Figure 9b: Mean fluorescence intensity of CD3+ cells expressing LLT1 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD3 - NKp30 Surface Expression

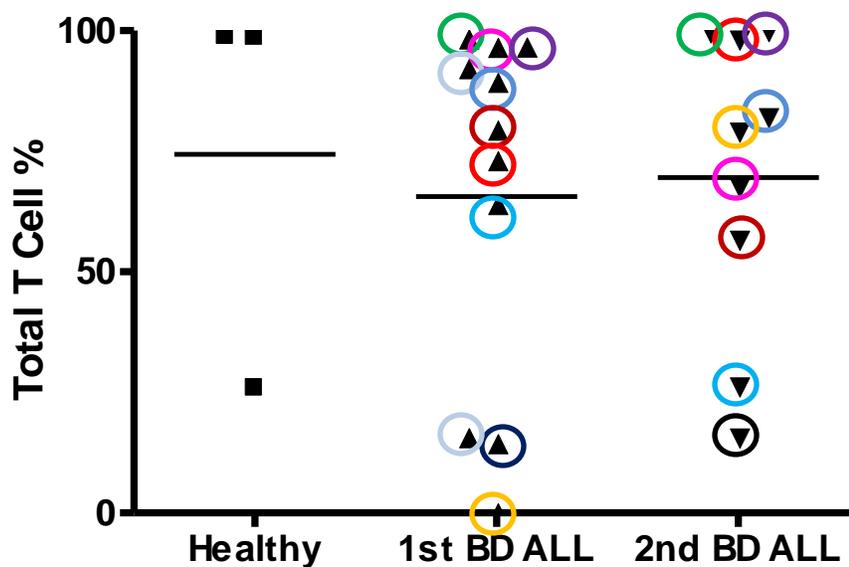


Figure 10a: Cell surface expression of NKp30. NKp30 expression on T cells (CD3) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

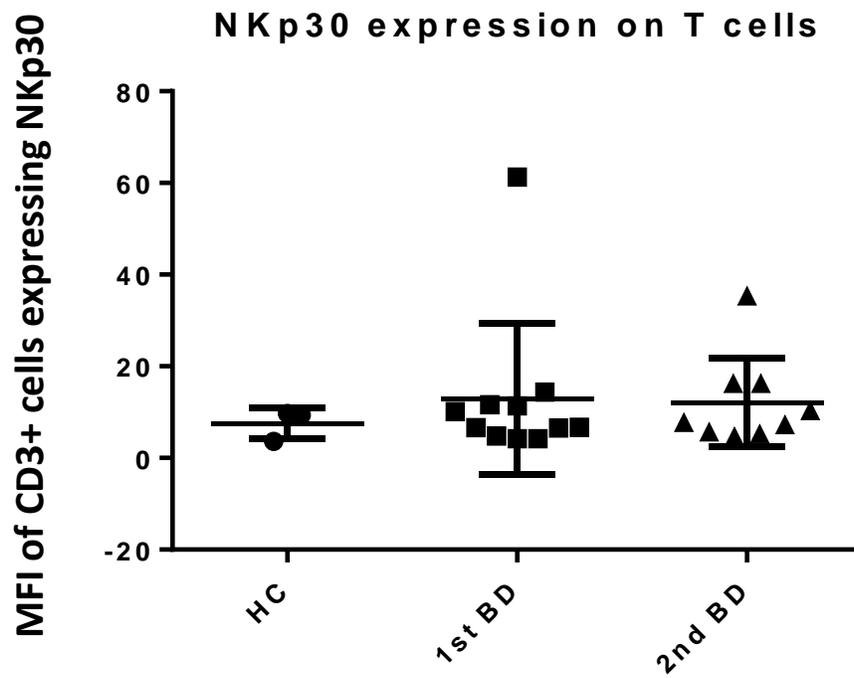


Figure 10b: Mean fluorescence intensity of CD3+ cells expressing NKp30 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD3 - NKp46 Surface Expression

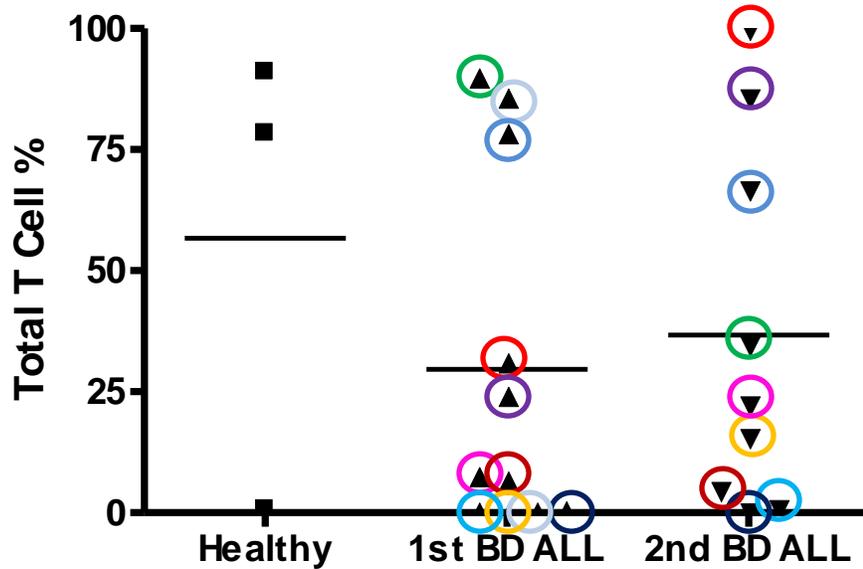


Figure 11a: Cell surface expression of NKp46. NKp46 expression on T cells (CD3) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

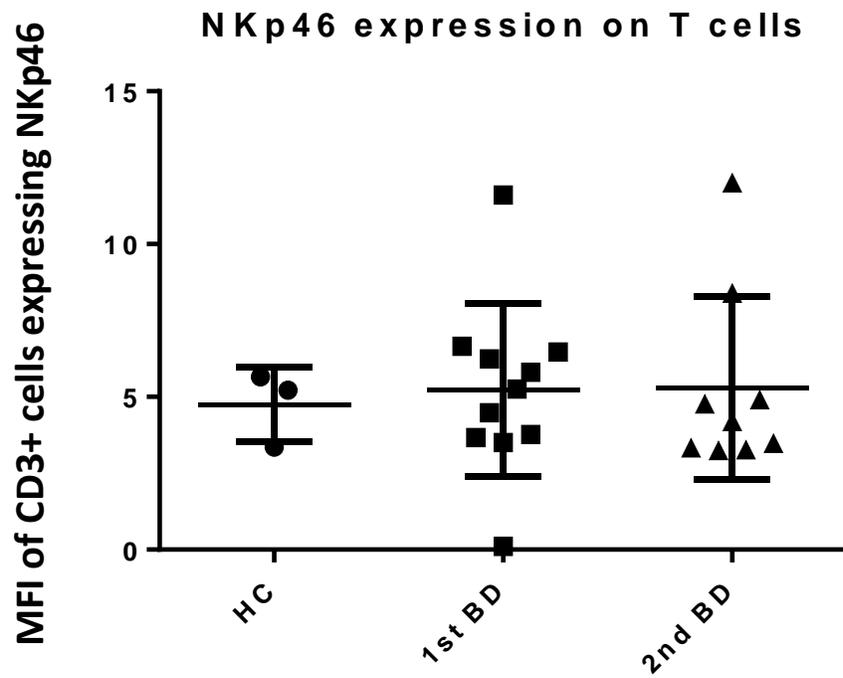


Figure 11b: Mean fluorescence intensity of CD3+ cells expressing NKp46 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

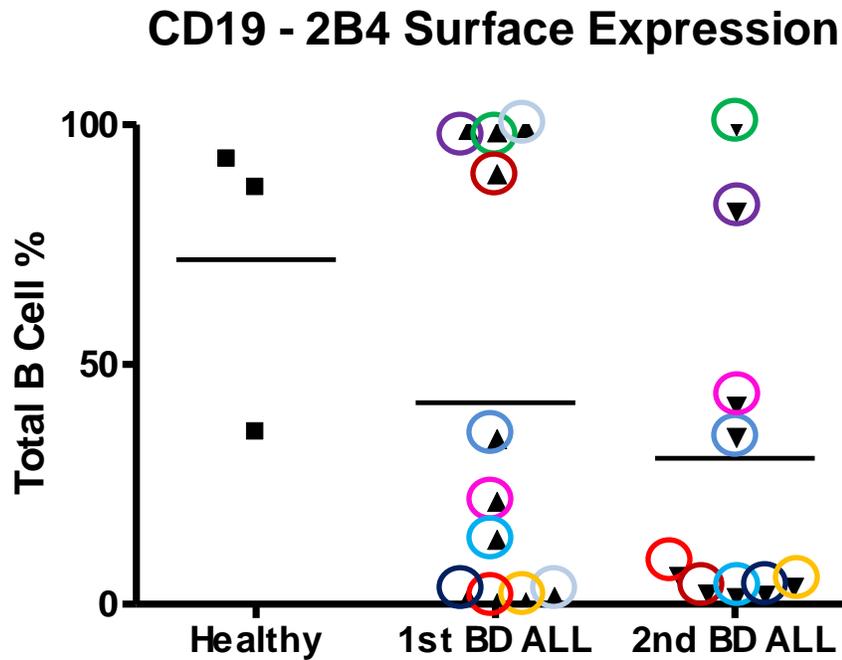


Figure 12a: Cell surface expression of 2B4. 2B4 expression on B cells (CD19) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

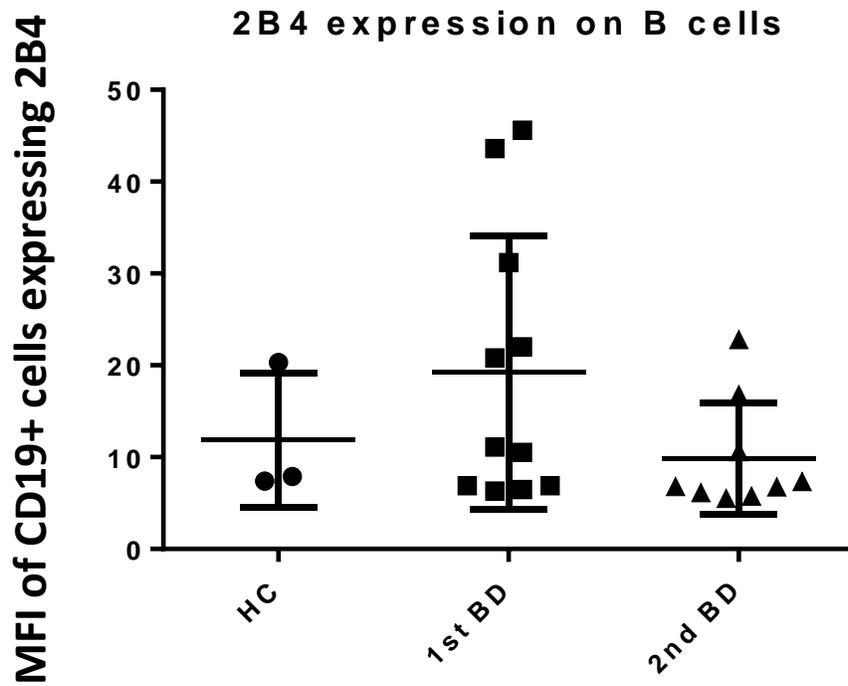


Figure 12b: Mean fluorescence intensity of CD19+ cells expressing 2B4 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD19 - CS1 Surface Expression

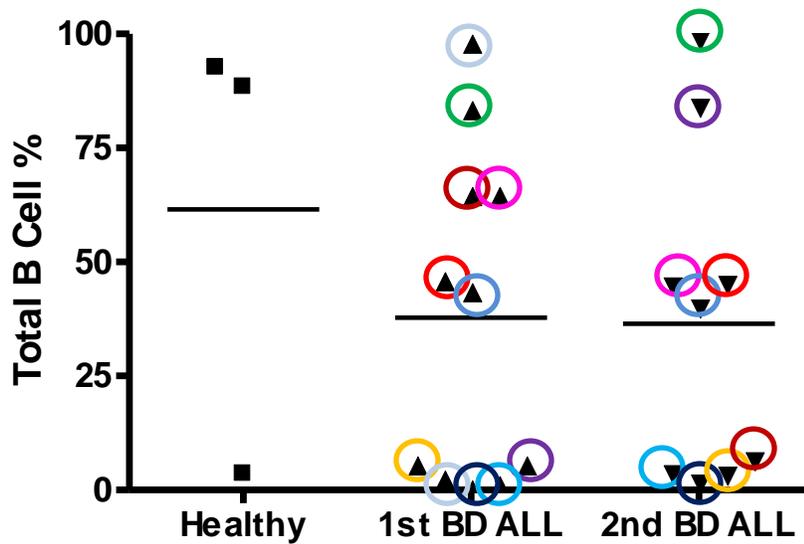


Figure 13a: Cell surface expression of CS1. CS1 expression on B cells (CD19) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

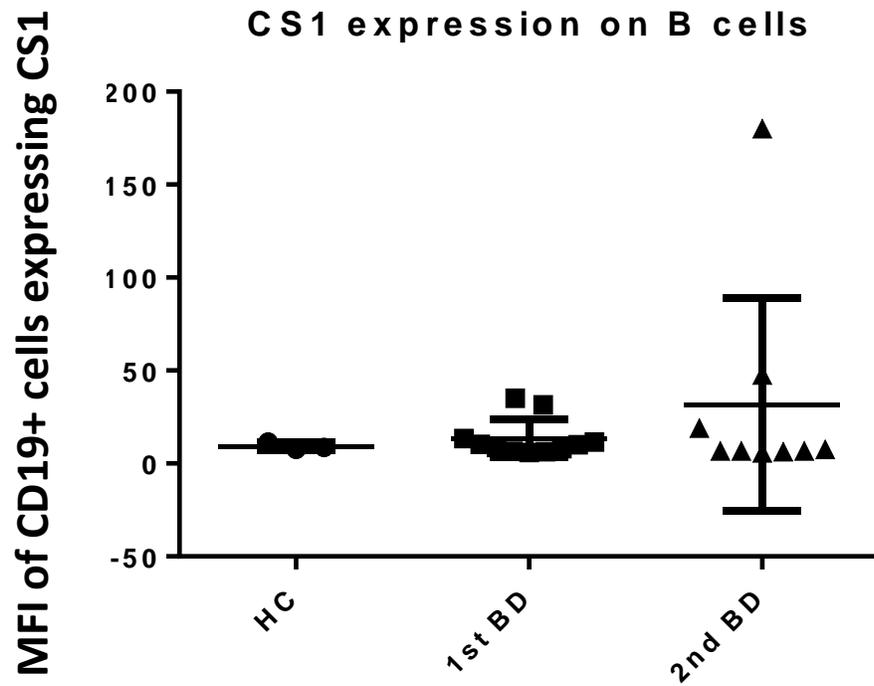


Figure 13b: Mean fluorescence intensity of CD19+ cells expressing CS1 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD19 - LLT1 Surface Expression

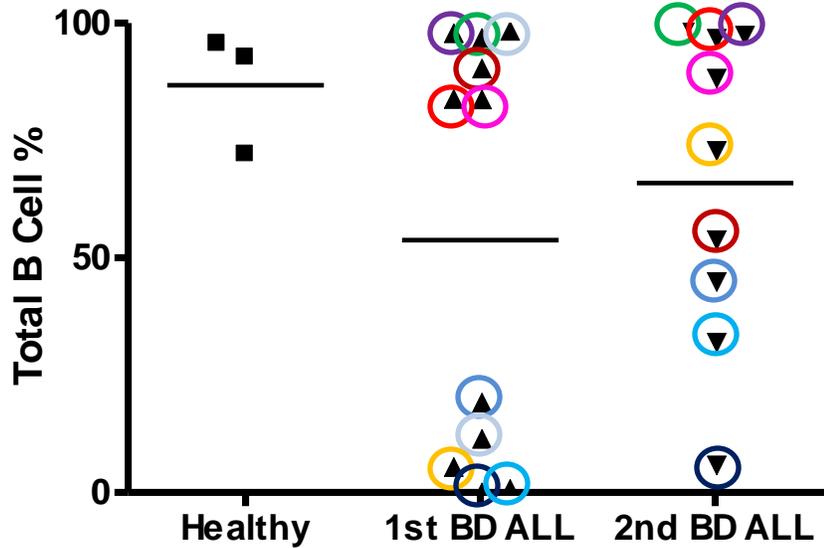


Figure 14a: Cell surface expression of LLT1. LLT1 expression on B cells (CD19) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

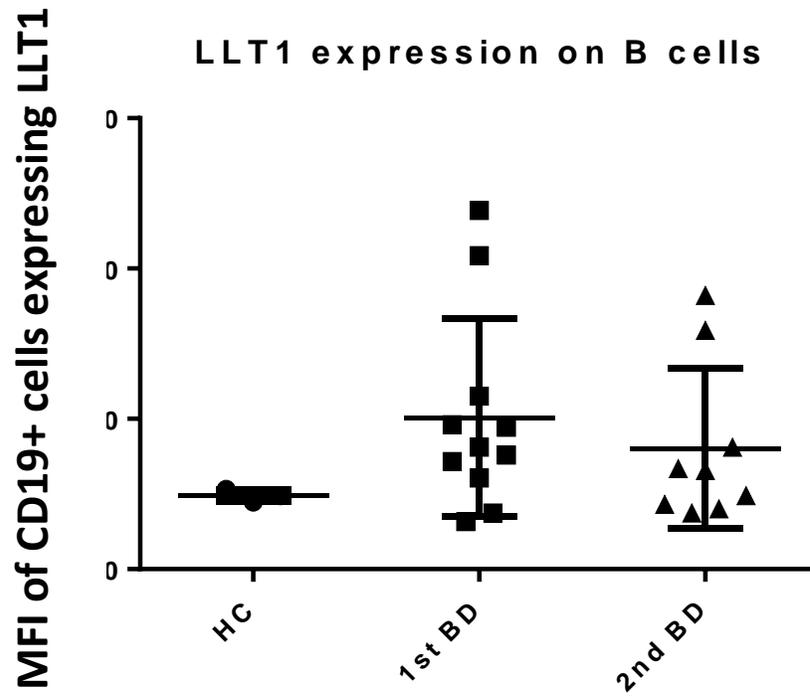


Figure 14b: Mean fluorescence intensity of CD19+ cells expressing LLT1 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD19 - NKp30 Surface Expression

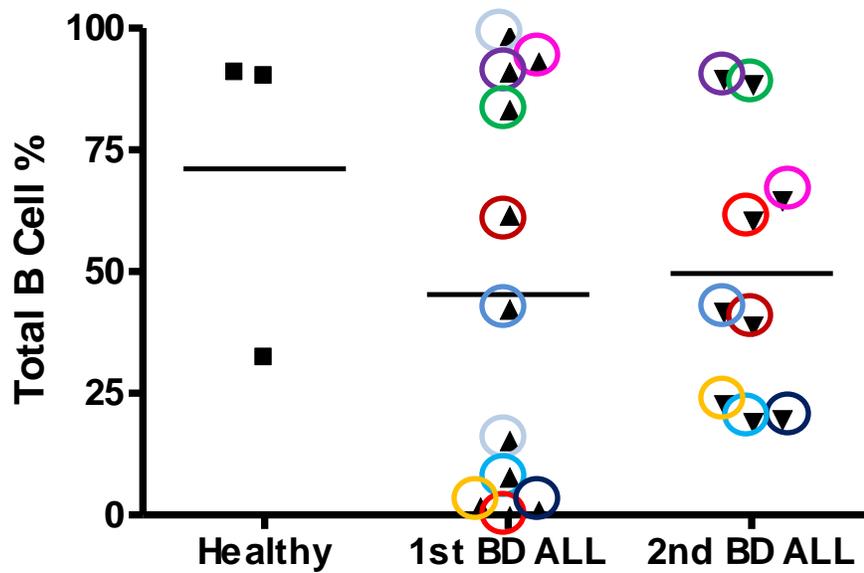


Figure 15a: Cell surface expression of NKp30. NKp30 expression on B cells (CD19) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

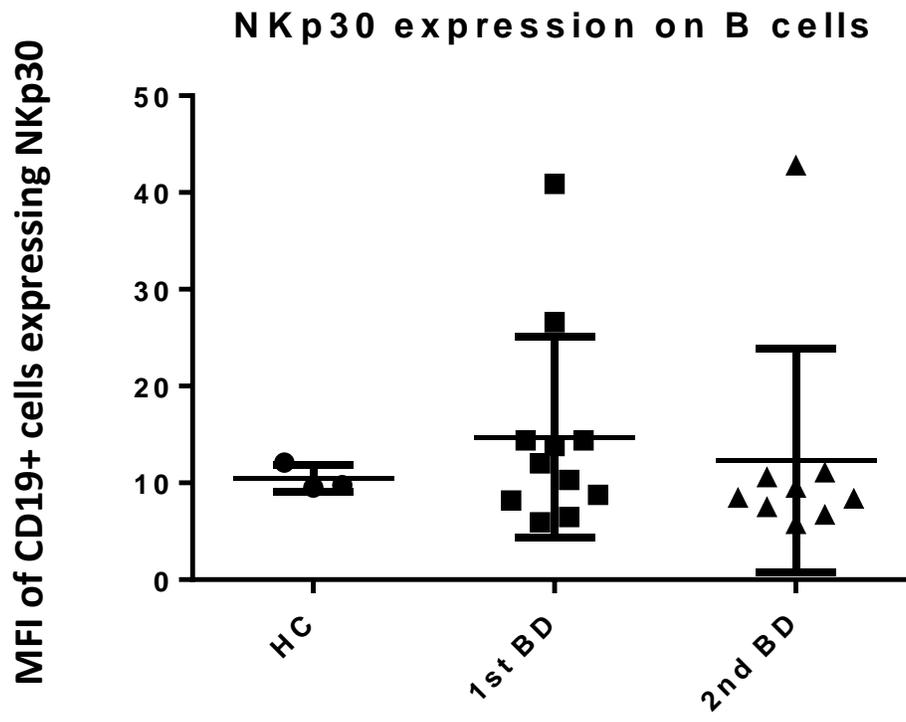


Figure 15b: Mean fluorescence intensity of CD19+ cells expressing NKp30 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD19 - NKp46 Surface Expression

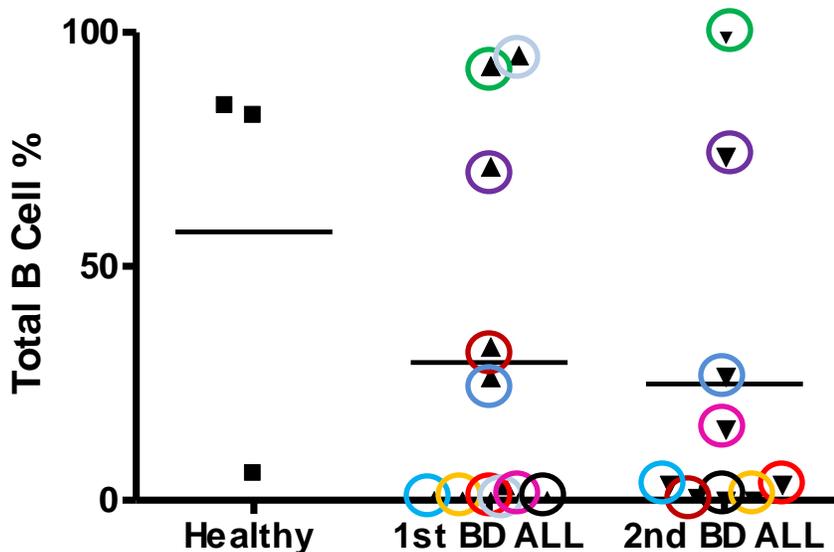


Figure 16a: Cell surface expression of NKp46. NKp46 expression on B cells (CD19) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

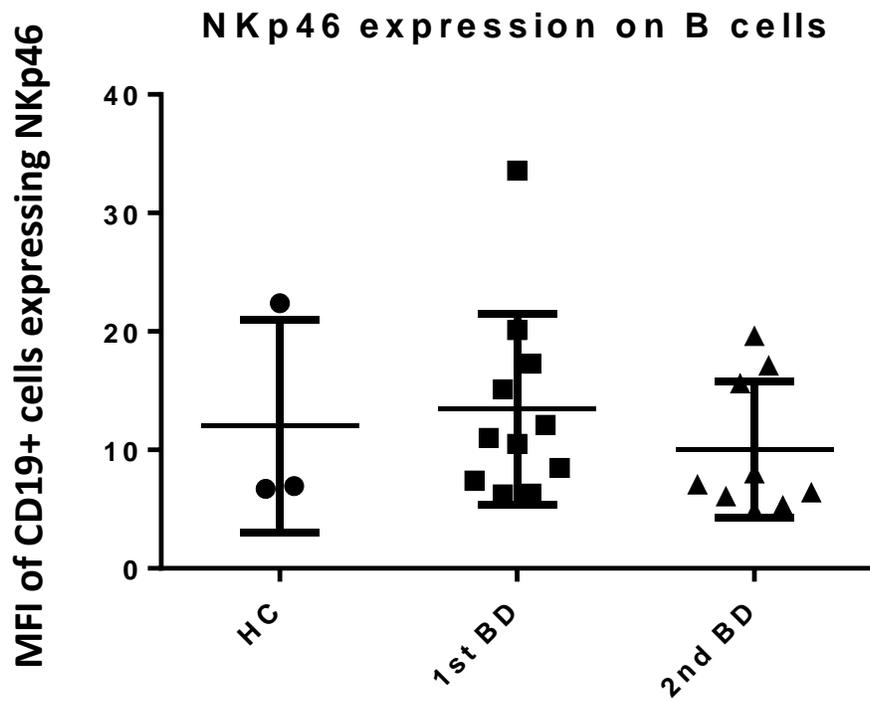


Figure 16b: Mean fluorescence intensity of CD19+ cells expressing NKp46 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD56 - 2B4 Surface Expression

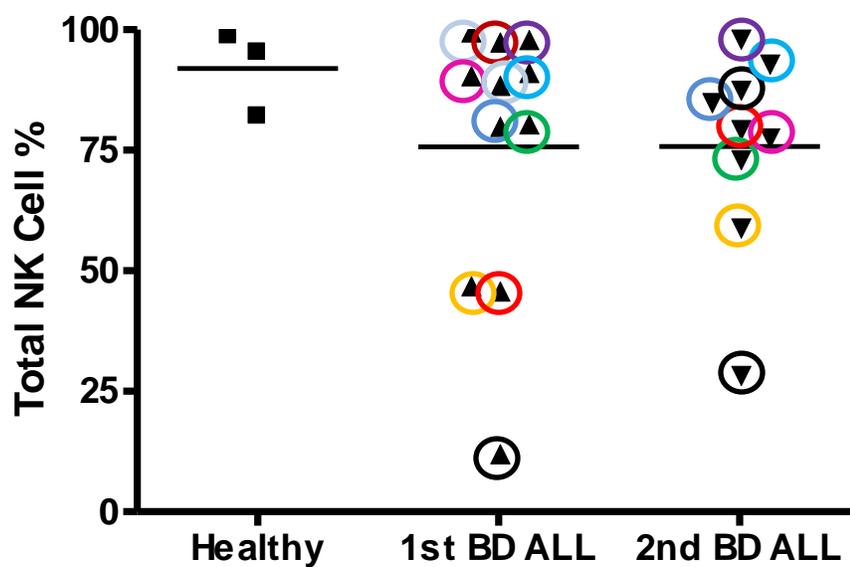


Figure 17a: Cell surface expression of 2B4. 2B4 expression on NK cells (CD56) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

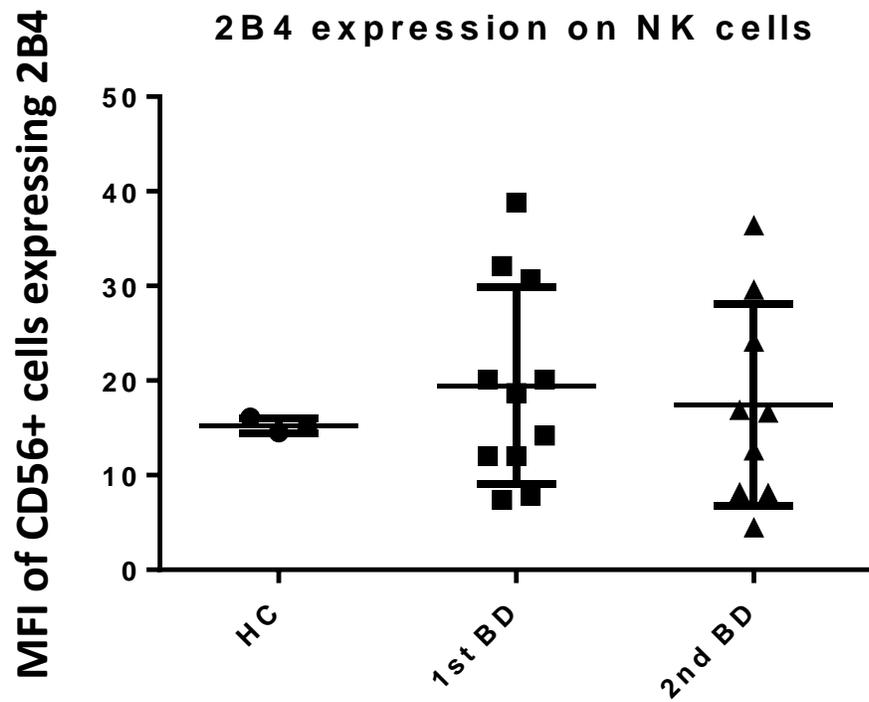


Figure 17b: Mean fluorescence intensity of CD56+ cells expressing 2B4 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD-56 - CS1 Surface Expression

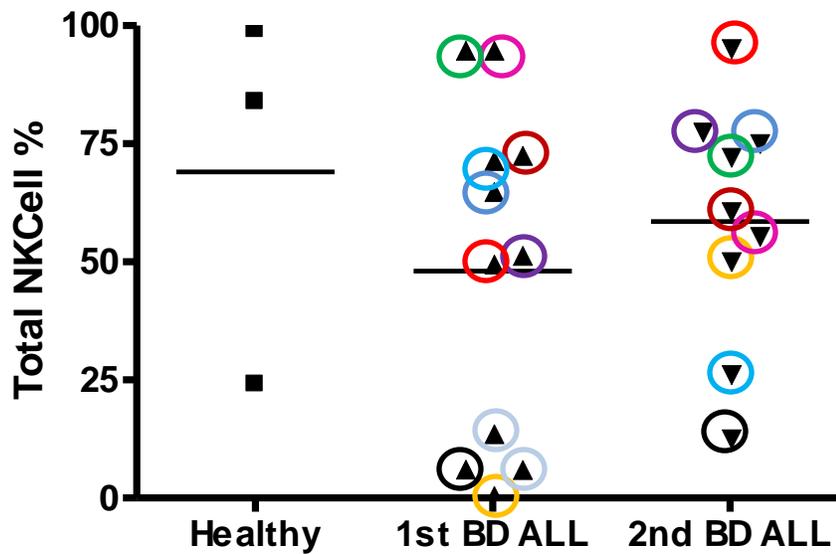


Figure 18a: Cell surface expression of CS1. CS1 expression on NK cells (CD56) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

CD56 - LLT1 Surface Expression

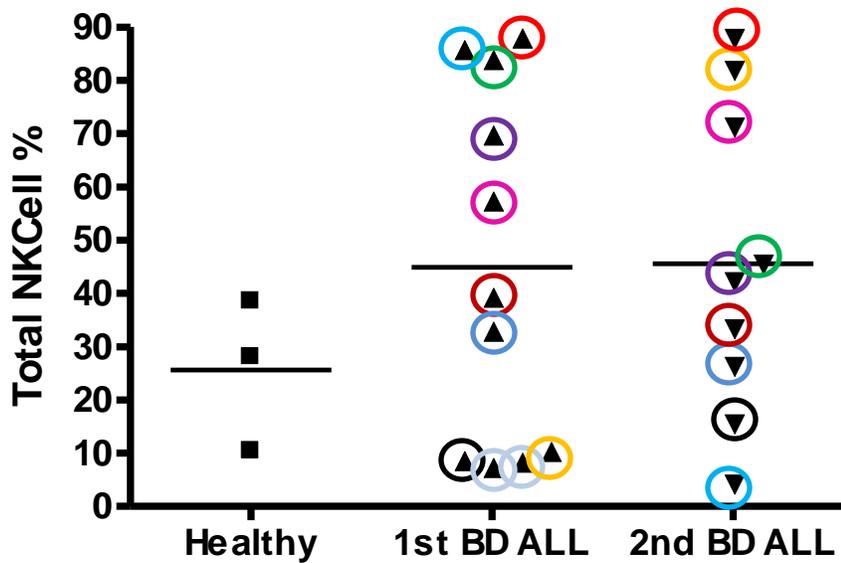


Figure 19a: Cell surface expression of LLT1. LLT1 expression on NK cells (CD56) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

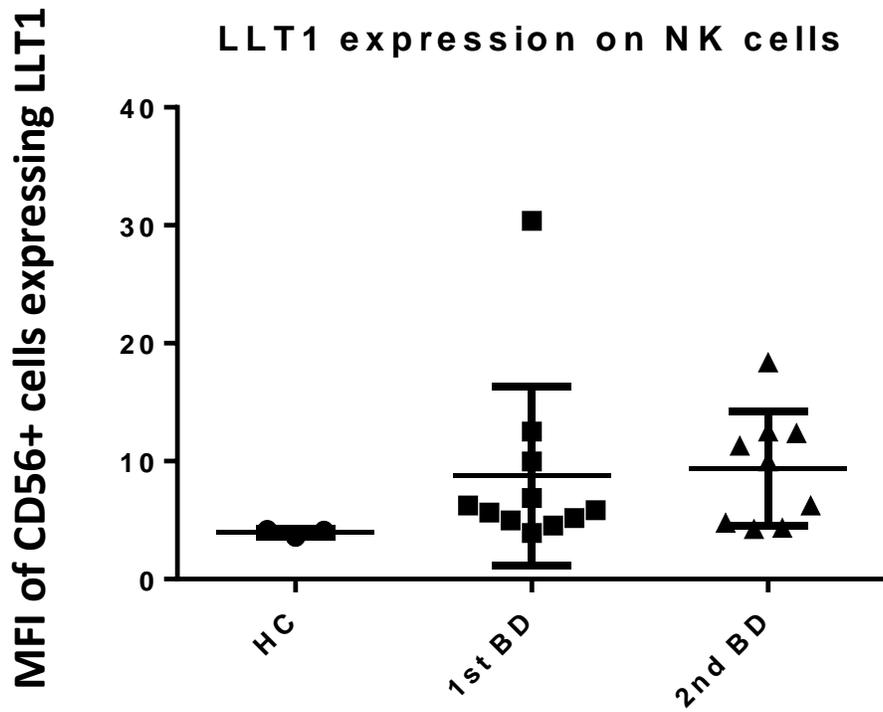


Figure 19b: Mean fluorescence intensity of CD56+ cells expressing LLT1 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD56 - NKp30 Surface Expression

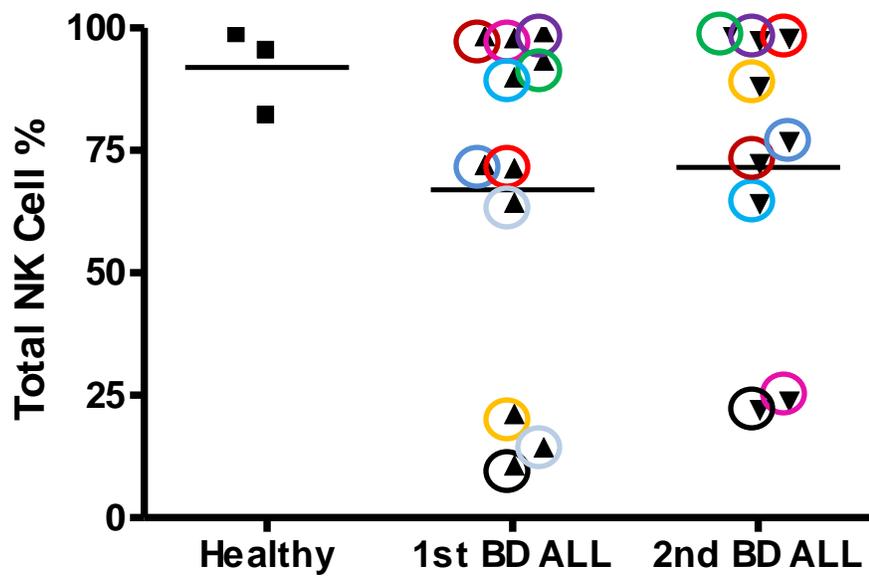


Figure 20a: Cell surface expression of NKp30. NKp30 expression on NK cells (CD56) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

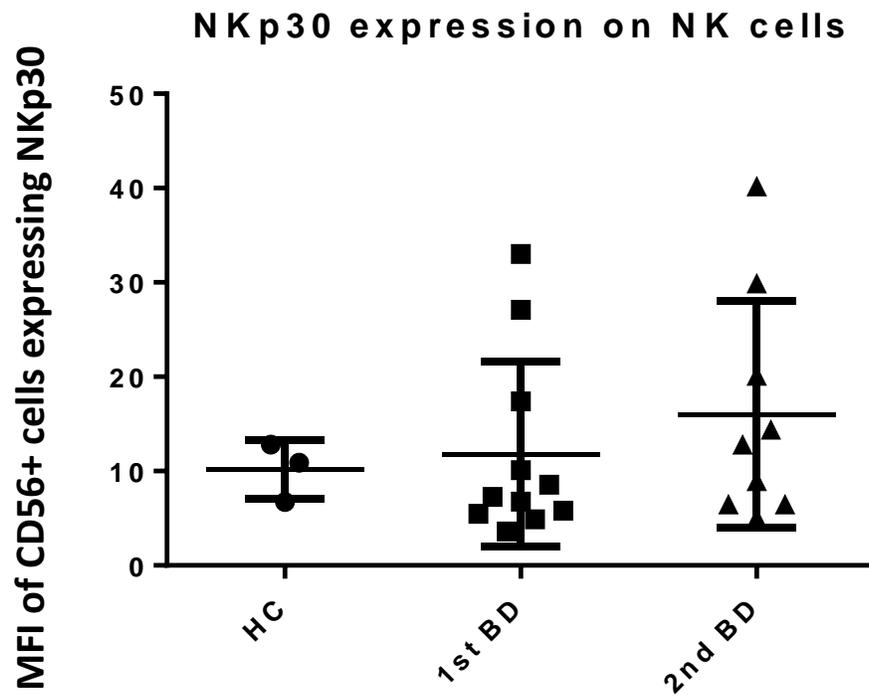


Figure 20b: Mean fluorescence intensity of CD56+ cells expressing NKp30 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD56 - NKp46 Surface Expression

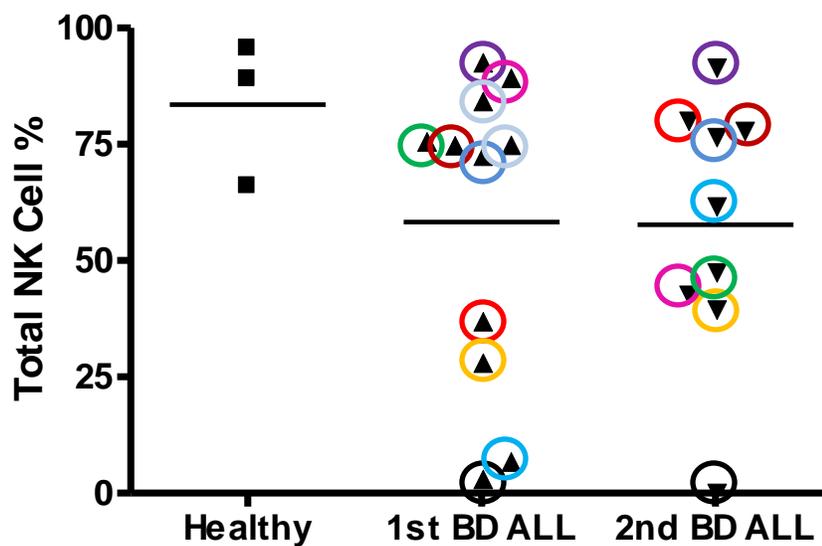


Figure 21a: Cell surface expression of NKp46. NKp46 expression on NK cells (CD56) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

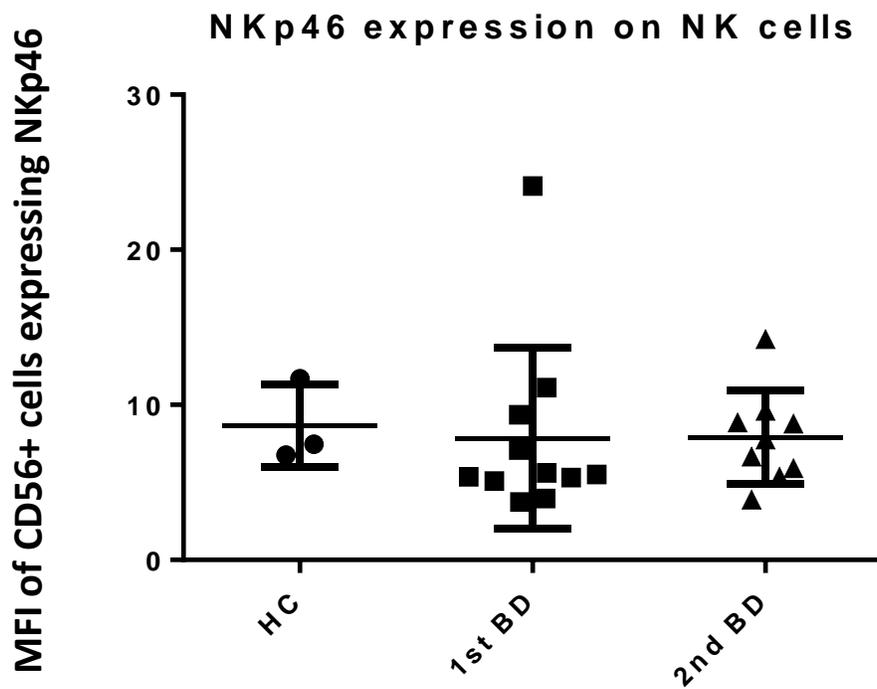


Figure 21b: Mean fluorescence intensity of CD56+ cells expressing NKp46 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD14 - 2B4 Surface Expression



Figure 22a: Cell surface expression of 2B4. 2B4 expression on monocytes (CD14) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

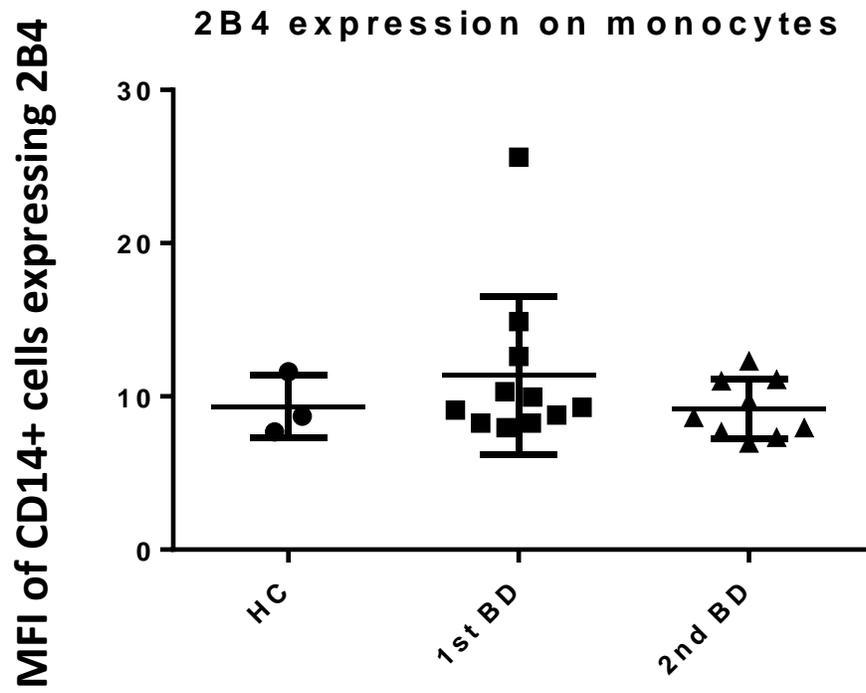


Figure 22b: Mean fluorescence intensity of CD14+ cells expressing 2B4 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD14 - CS1 Surface Expression

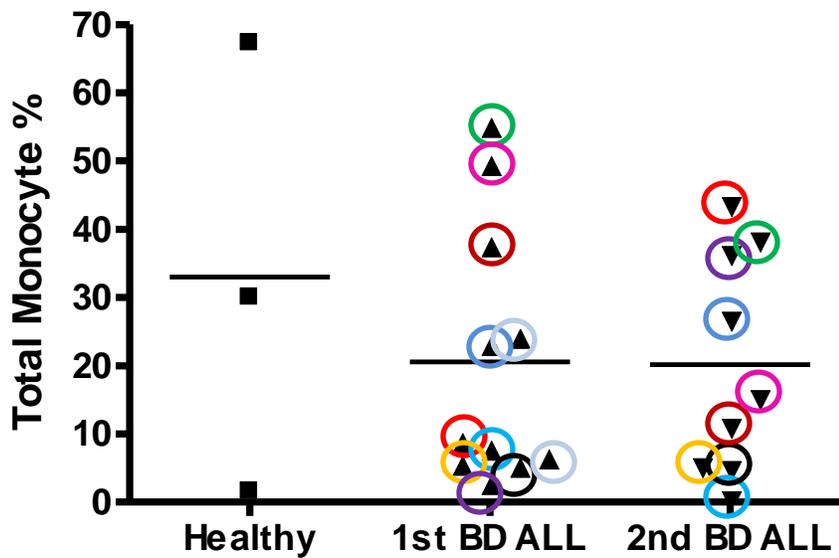


Figure 23a: Cell surface expression of CS1. CS1 expression on monocytes (CD14) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

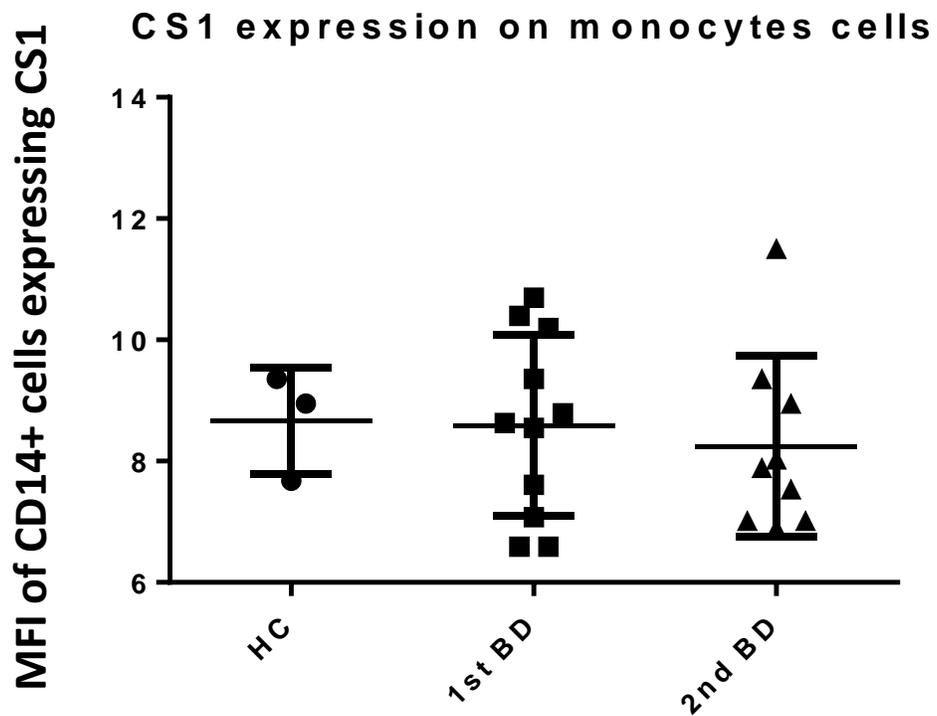


Figure 23b: Mean fluorescence intensity of CD14+ cells expressing CS1 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD14 - LLT1 Surface Expression

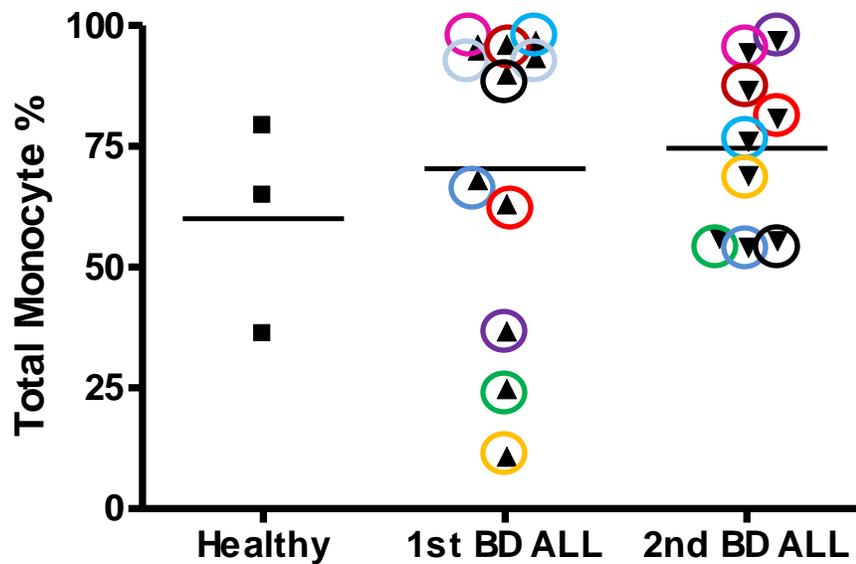


Figure 24a: Cell surface expression of LLT1. LLT1 expression on monocytes (CD14) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

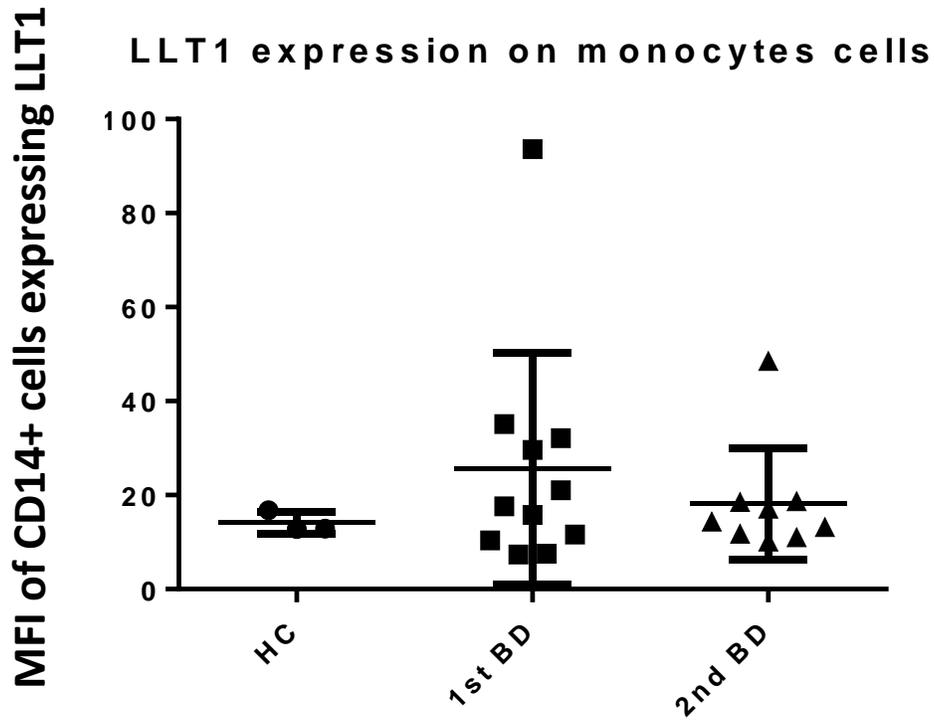


Figure 24b: Mean fluorescence intensity of CD14+ cells expressing LLT1 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD14 - NKp30 Surface Expression

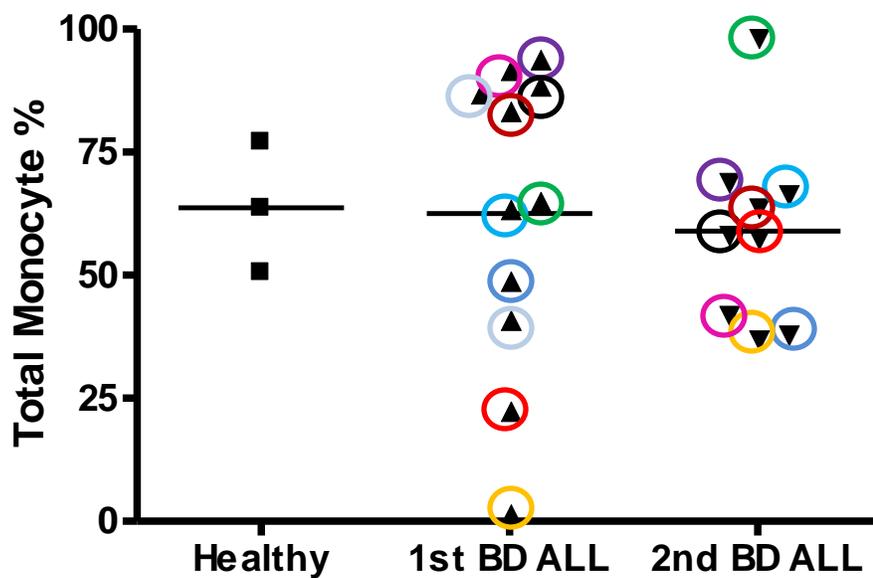


Figure 25a: Cell surface expression of NKp30. NKp30 expression on monocytes (CD14) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

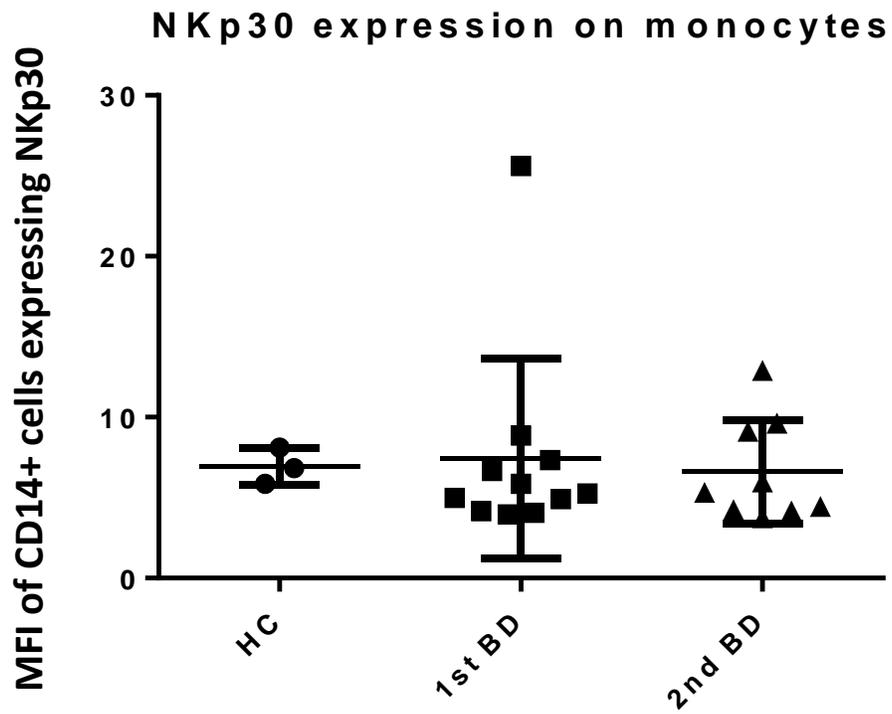


Figure 25b: Mean fluorescence intensity of CD14+ cells expressing NKp30 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

CD14 - NKp46 Surface Expression

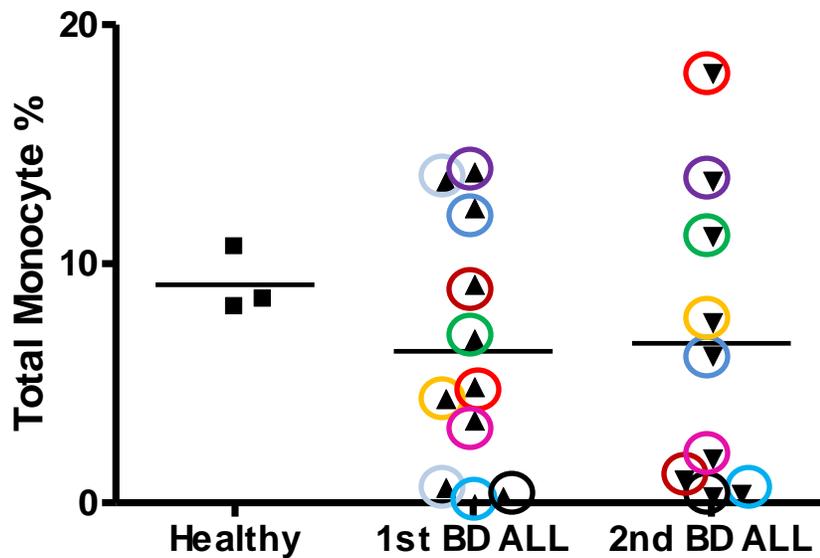


Figure 26a: Cell surface expression of NKp46. NKp46 expression on monocytes (CD14) from healthy subjects (Healthy), specimen collected from ALL patients at diagnosis (1st BD ALL) and specimen collected 29 days after diagnosis (after chemotherapy treatment) (2nd BD ALL). Each patient is assigned the following color: Pt1-purple, Pt2-gray, Pt3-red, Pt4-blue, Pt5-gray, Pt6-orange, Pt7-pink, Pt8-maroon, Pt9-green, Pt10-lt blue and Pt11-black.

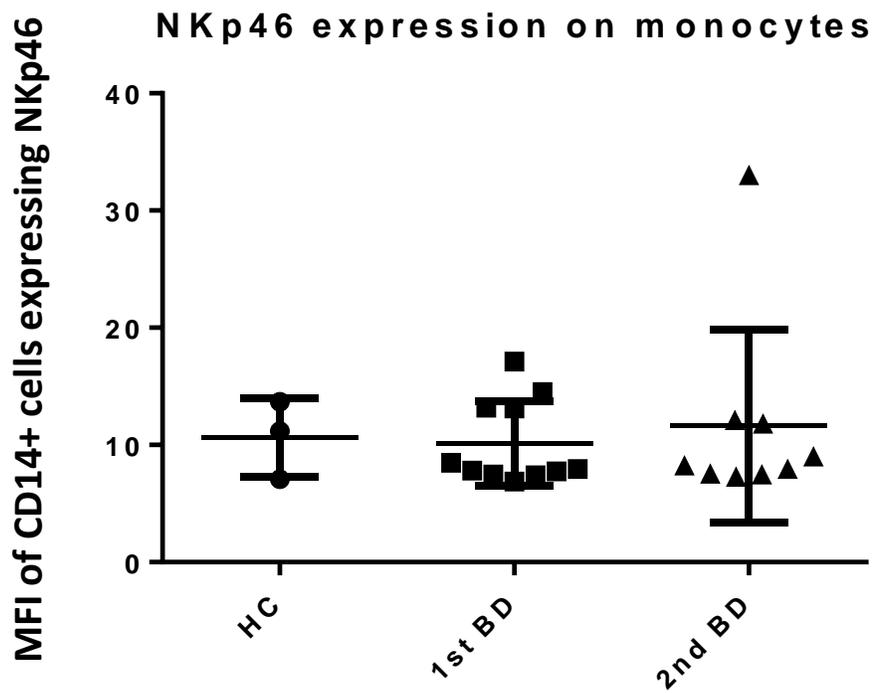


Figure 26b: Mean fluorescence intensity of CD14+ cells expressing NKp46 of 11 patients at the time of diagnosis (1st BD) and after treatment (2nd BD) compared to healthy controls (HC)

ALL Patient 1 - Surface protein expression on CD3 (T-cells)

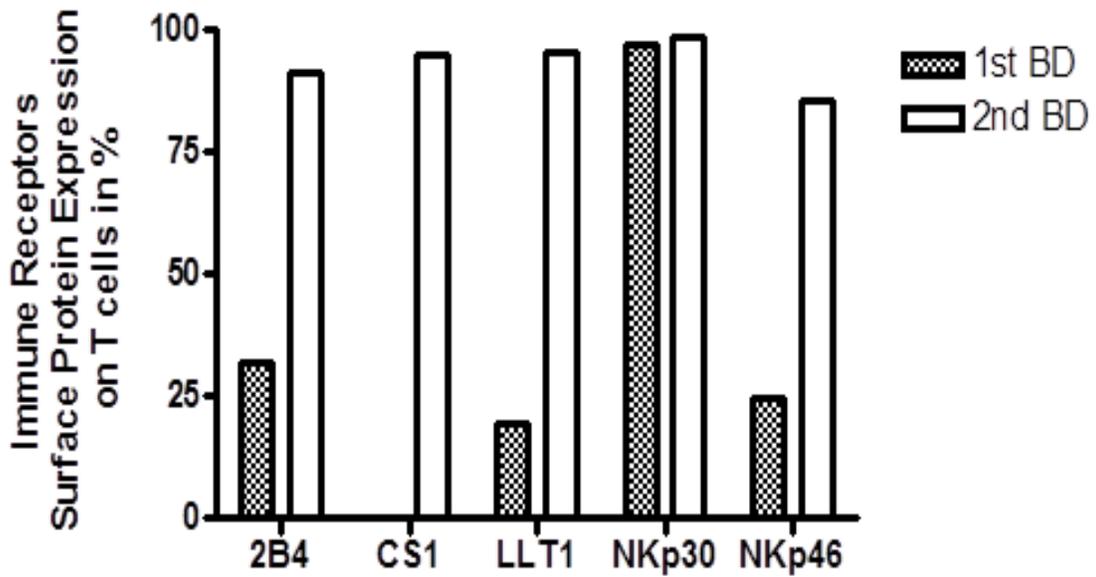


Figure 27: Patient 1 show pronounced change in receptor expression on T cells between time of diagnosis and after treatment

ALL Patient 8 - Surface protein expression on CD19 (B-cells)

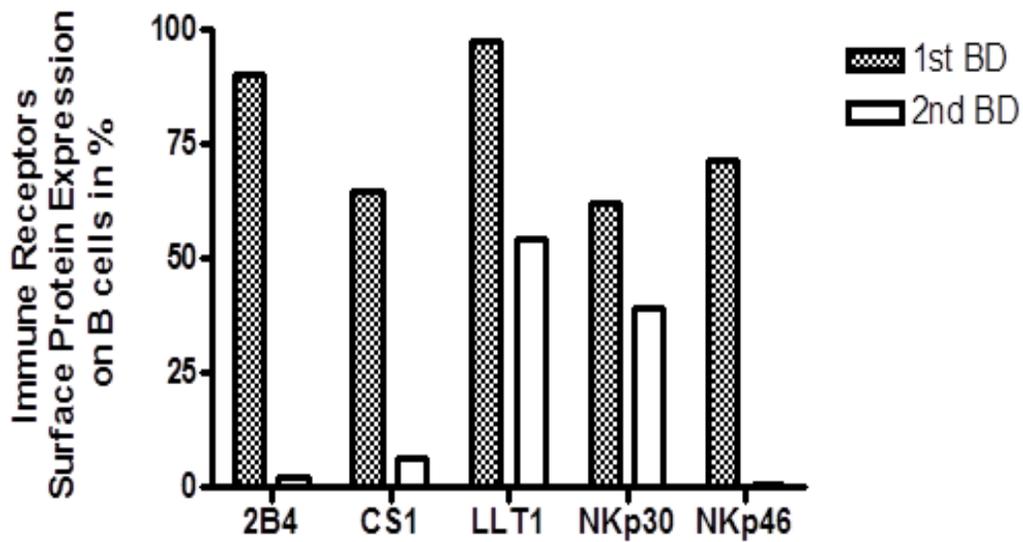


Figure 28: Patient 8 show pronounced change in receptor expression on B cells between time of diagnosis and after treatment

ALL Patient 10 - Surface protein expression on CD56 (NK-cells)

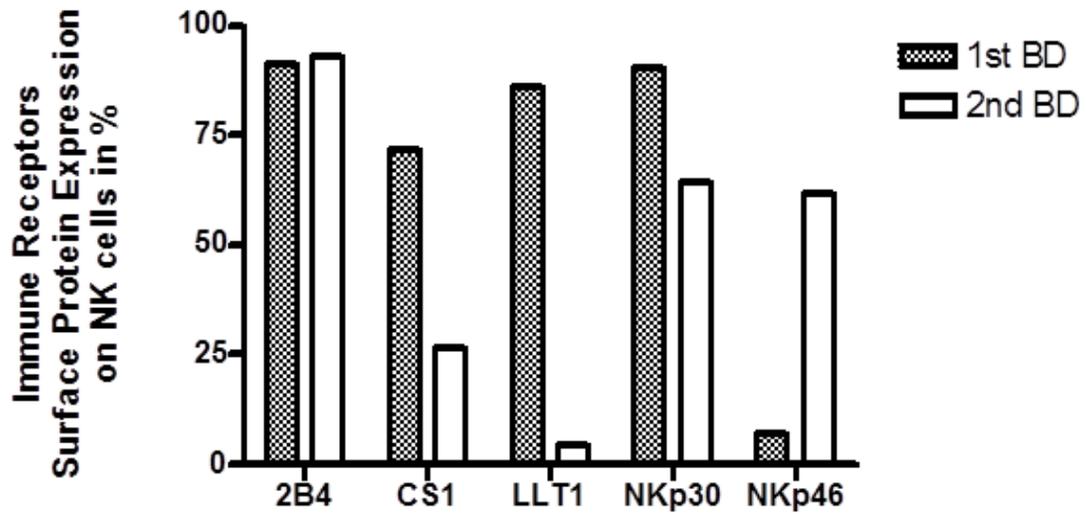


Figure 29: Patient 10 show pronounced change in receptor expression on NK cells between time of diagnosis and after treatment

ALL Patient 10 - Surface protein expression on CD3 (T-cells)

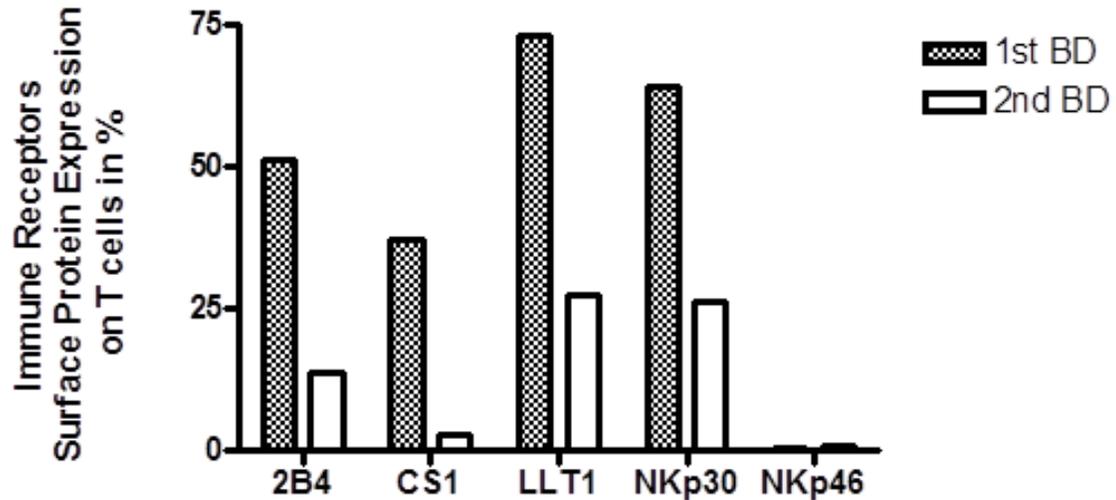


Figure 30: Patient 10 show pronounced change in receptor expression on T cells between time of diagnosis and after treatment

ALL Patient 10 - Surface protein expression on CD19 (B-cells)

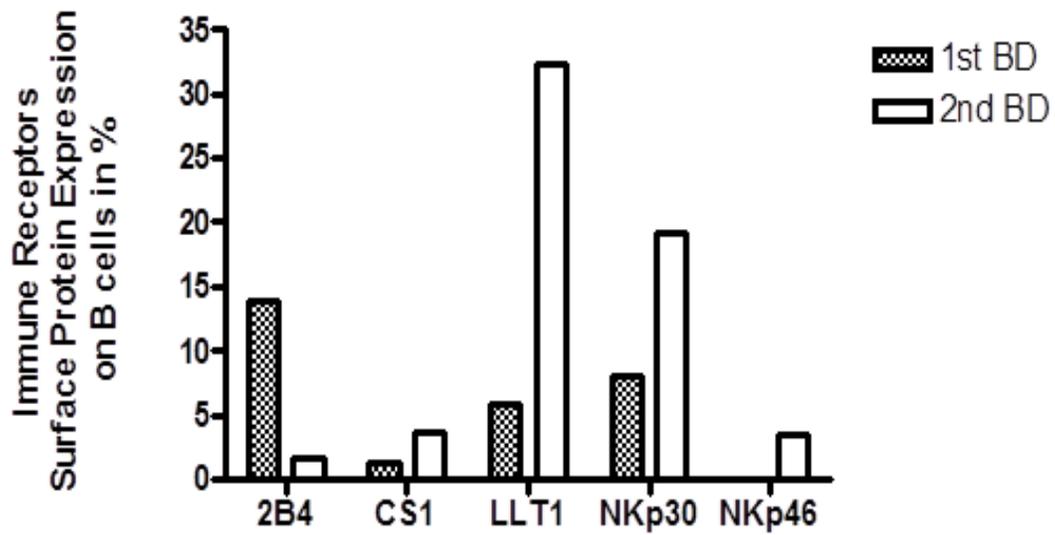


Figure 31: Patient 10 show pronounced change in receptor expression on B cells between time of diagnosis and after treatment

CD3 – T cells

CD3 – T cells											
ALL Patient	2B4		CS1		LLT1		NKp30		NKp46		Risk classification
	1st BD	2nd BD	1st BD	2nd Bd							
1	31.80%	91.30%	0.10%	94.60%	19.50%	95.50%	96.80%	98.30%	24.30%	85.70%	High. risk
2	6.70%		0.00%		11.80%		15.90%		0.40%		Std. risk
3	52.30%	99.50%	17.50%	99.80%	84.20%	99.90%	73.30%	100.00%	31.20%	99.30%	Std. risk
4	85.90%	85.20%	88.00%	84.80%	90.70%	82.90%	89.50%	82.20%	78.50%	66.70%	Std. risk
5	94.00%		8.20%		98.20%		92.40%		85.90%		High risk
6	4.20%	41.10%	0.10%	39.60%	0.40%	64.80%	0.30%	79.20%	0.00%	15.50%	Std. risk
7	17.10%	59.40%	30.40%	59.20%	79.20%	67.30%	96.90%	67.70%	7.70%	22.30%	High risk
8	19.20%	21.90%	30.40%	17.90%	44.00%	36.70%	79.60%	56.80%	6.80%	4.60%	High risk
9	98.10%	67.80%	98.00%	67.20%	97.40%	51.10%	98.50%	99.80%	90.00%	34.70%	High risk
10	51.10%	13.80%	37.00%	2.70%	73.00%	27.50%	64.20%	26.40%	0.40%	0.90%	High risk
11	20.70%	11.10%	3.90%	2.50%	15.20%	9.90%	14.60%	15.80%	0.60%	0.20%	High risk
Hlty 1	18.9%		5.3%		15.1%		25.9%		0.7%		
Hlty 2	92.3%		97.8%		94.9%		98.5%		91.0%		
Hlty 3	95.6%		94.5%		87.2%		98.8%		78.4%		

Table 3: Summary of each receptor surface protein expression on T cells (CD3) and risk classification.

CD19 – B cells

ALL Patient	2B4		CS1		LLT1		NKp30		NKp46		Risk classification
	1st BD	2nd BD	1st BD	2nd BD	1st BD	2nd BD	1st BD	2nd BD	1st BD	2nd BD	
1	99.70%	82.00%	5.60%	84.00%	99.00%	97.70%	91.30%	89.70%	93.10%	73.60%	High. risk
2	1.90%		2.50%		14.70%		15.50%		0.20%		Std. risk
3	0.50%	6.00%	46.00%	45.20%	94.10%	97.00%	0.00%	60.60%	0.20%	3.50%	Std. risk
4	34.80%	35.00%	43.60%	40.00%	38.50%	45.20%	42.50%	41.90%	33.10%	26.60%	Std. risk
5	99.30%		98.10%		99.80%		98.80%		98.10%		High risk
6	0.70%	3.80%	5.60%	3.40%	11.60%	73.10%	1.90%	22.90%	0.10%	0.10%	Std. risk
7	21.70%	41.50%	64.70%	44.90%	84.10%	88.50%	93.30%	64.80%	3.50%	15.40%	High risk
8	90.00%	2.40%	64.70%	6.50%	97.20%	54.10%	61.90%	39.00%	71.60%	0.70%	High risk
9	98.80%	100.00%	83.50%	98.80%	98.70%	100.00%	83.50%	88.60%	95.40%	100.00%	High risk
10	13.80%	1.60%	1.30%	3.70%	5.80%	32.30%	8.00%	19.20%	0.00%	3.50%	High risk
11	1.60%	2.10%	0.40%	1.60%	1.10%	6.10%	1.10%	19.80%	0.00%	0.10%	High risk
Hlty 1	35.9%		3.5%		42.0%		17.6%		5.6%		
Hlty 2	86.9%		92.6%		95.6%		90.9%		84.3%		
Hlty 3	92.8%		88.4%		92.7%		90.1%		82.2%		

Table 4: Summary of each receptor surface protein expression on B cells (CD19) and risk classification.

CD56 – NK cells											
	2B4		CS1		LLT1		NKp30		NKp46		Risk classification
ALL Patient	1st BD	2nd BD									
1	97.70%	98.30%	51.60%	77.70%	69.90%	42.40%	99.50%	97.60%	92.90%	91.70%	High. risk
2	88.70%		13.90%		7.50%		64.70%		75.10%		Std. risk
3	46.00%	79.50%	49.80%	95.40%	88.20%	88.10%	71.70%	98.10%	37.20%	80.30%	Std. risk
4	80.20%	85.10%	65.10%	75.30%	33.10%	26.50%	72.40%	77.00%	72.80%	76.80%	Std. risk
5	99.80%		6.40%		8.50%		14.70%		84.50%		High risk
6	47.10%	59.10%	0.80%	50.30%	10.50%	82.10%	21.50%	88.30%	28.30%	39.70%	Std. risk
7	90.70%	77.80%	95.00%	55.60%	57.60%	71.40%	98.30%	24.00%	89.50%	42.90%	High risk
8	98.20%	87.70%	72.80%	60.90%	39.50%	33.60%	98.70%	72.50%	75.00%	78.10%	High risk
9	80.70%	73.30%	95.00%	72.40%	84.10%	45.70%	93.70%	99.60%	75.90%	47.70%	High risk
10	91.40%	93.10%	71.70%	26.40%	86.00%	4.40%	90.30%	64.40%	7.00%	61.90%	High risk
11	12.30%	28.50%	6.30%	12.80%	8.80%	15.70%	11.00%	22.30%	3.30%	0.30%	High risk
Hlty 1	97.3%		24.0%		5.1%		82.0%		66.0%		
Hlty 2	99.6%		99.1%		28.0%		98.6%		95.6%		
Hlty 3	98.3%		83.9%		10.4%		95.3%		89.0%		

Table 5: Summary of each receptor surface protein expression on NK cells (CD56) and risk classification.

CD14- Monocytes											
	2B4		CS1		LLT1		NKp30		NKp46		Risk classification
ALL Patient	1st BD	2nd BD									
1	47.70%	42.10%	2.80%	36.30%	37.00%	97.10%	94.00%	69.00%	13.90%	13.50%	High. risk
2	9.90%		6.50%		93.60%		87.10%		0.70%		Std. risk
3	29.70%	32.90%	8.90%	43.50%	63.40%	81.00%	22.60%	57.50%	4.90%	18.00%	Std. risk
4	33.40%	42.20%	23.10%	26.70%	68.40%	54.40%	49.00%	38.00%	12.40%	6.20%	Std. risk
5	70.50%		24.20%		96.40%		41.10%		13.50%		High risk
6	15.80%	26.90%	5.60%	5.20%	11.10%	69.20%	1.70%	37.00%	4.40%	7.60%	Std. risk
7	24.00%	54.90%	49.50%	15.20%	96.40%	94.60%	91.90%	42.00%	3.50%	1.90%	High risk
8	29.60%	28.10%	37.60%	11.00%	95.20%	86.80%	83.50%	63.90%	9.20%	1.00%	High risk
9	17.90%	74.50%	55.10%	38.30%	25.10%	56.20%	65.20%	98.40%	6.90%	11.20%	High risk
10	27.90%	7.30%	7.80%	0.40%	97.10%	76.30%	63.60%	66.50%	0.00%	0.40%	High risk
11	29.80%	16.80%	5.30%	4.80%	90.20%	55.70%	88.70%	58.30%	0.30%	0.30%	High risk
Hlty 1	47.6%		1.6%		27.8%		26.9%		10.7%		
Hlty 2	45.2%		67.3%		79.2%		63.6%		8.2%		
Hlty 3	72.1%		30.0%		64.8%		77.0%		8.5%		

Table 6: Summary of each receptor surface protein expression on Monocytes (CD14) and risk classification.

Chapter 5

Discussion

NK cell receptors 2B4, CS1, LLT1, NKp30 and NKp46 has been shown in previous studies to play a role in leukemia and other cancers^{14,21,22,25,26,28}, but their role in acute lymphoblastic leukemia in children is yet to be elucidated. Therefore, our laboratory decided to ascertain if all or any of these receptors play a role in the disease development of ALL in children. This thesis is focused on determining if there are alterations in the expression of each receptor in ALL as compared to healthy controls.

Experiments were conducted in order to determine if there are any modifications or alterations in receptor expression at the mRNA level and at the surface protein level. PBMCs from 11 ALL patients and 3 healthy subjects were analyzed. PBMCs collected at the time of diagnosis were analyzed through RT PCR to detect the mRNA expression of each receptor while PBMCs collected at the time of diagnosis and 29 days after diagnosis and after chemotherapy treatment were analyzed through flow cytometry to determine the cell surface protein expression. The PBMCs from the healthy subjects were analyzed in the same manner to serve as controls.

Although we failed to acquire any statistically significant data, the data we have gathered suggests that ALL patients demonstrate, to some degree, variations in the expression of receptors both at the mRNA and surface protein level as compared to the healthy subjects.

NK cell receptor 2B4 showed variations in their expression at the mRNA level though no specific trend was observed (Figure 2a). At the cell surface protein level, there was an observed decrease on the expression of receptor 2B4 on T cells and monocytes from specimens collected at diagnosis (figure 7a and 22a). Reduced 2B4 expression in NK cells has been suggested to play a role in tumor immune escape (^{14,33}). In our patients, with the exception of patients 3, 6 and 11, the surface protein expression of 2B4 on NK cells in both specimens (at the time of diagnosis and after chemotherapy treatment) of children with ALL were comparable to the healthy subjects (figure 17a). This could suggest that there are other mechanisms by which the lymphoblasts evade the immune system.

The CS1 expression of the ALL patients demonstrated a decrease in the CS1 mRNA expression with the exception of some patients (patient 3 and 5) that showed over expression of CS1 at the mRNA level on PBMC (Figure 3a). At the surface protein level we observed a decrease in the percentage of cells expressing CS1 on T cells and monocytes from specimen collected at diagnosis. CS1 expression has been shown in previous studies to be absent in the vast majority of acute leukemias, B cell lymphomas and classic Hodgkin lymphomas ²³. In our finding, patients characterized as high risk were shown to have an increased percentage of B cells expressing CS1 (Table 4). Previous studies from our lab show that CS1 expression on human B cells promotes the proliferation of B cells and induces autocrine secretion of cytokines

²². This data seem to correlate with our previous studies and other studies on CS1 surface protein expression in multiple myeloma patients ³⁰ and since majority of the ALL patients were B precursor ALL subtype it may imply that increased expression of CS1 on the B lymphoblasts may help in the rapid proliferation of these blasts and exacerbate the disease progression. Therefore, a humanized anti-CS1 monoclonal antibody (elotuzumab) could be used as an immunotherapy for these patients. Additionally, CS1 can also be explored for its potential to be a biomarker due to its overexpression on high risk patients.

Patients and healthy subjects alike expressed two different isoforms of LLT1. Patient expression of the different LLT1 isoforms were more varied in intensities when compared with the healthy subjects which demonstrated a more uniform expression (Figure 4a). In the future, it would be interesting to study the function of these LLT1 isoforms. Examining the function of these isoforms independently from each other may explain the role they play in regards to the pathogenesis of ALL. In a previous study with malignant glioma cells, LLT1 expression increases with the World Health Organization (WHO) grade of malignancy²⁸. In our study we observed an upregulation of LLT1 surface protein expression on monocytes on specimens collected after chemotherapy treatment and again showed an up regulated expression on NK cells in both specimens collected at diagnosis and 29 days after chemotherapy treatment. This is interesting because some of the patients that showed an increase in expression were considered high risk. Future studies involving more patient samples may give further insights if the grade of malignancy does play a role in LLT1 receptor expression in ALL in children.

Both NKp30 and NKp46 showed variations in their mRNA receptor expression though no trend can be observed (Figure 5a and 6a). In the surface protein expression level, with the exception of a few patients, NKp30 surface protein expression appeared to be comparable with the healthy subjects. It has been previously shown that in gastrointestinal sarcoma, there is an over expression of the immunosuppressive isoform of NKp30²⁵. Although isoforms were not detected in our studies, in the future, it would be interesting to study if such isoforms of NKp30 exist in childhood ALL and what role do they play in regards to the pathogenesis of ALL. On the other hand, NKp46 showed a reduced expression on B cells on specimens collected after chemotherapy. This correlated with previous studies on patients with Chronic Lymphocytic Leukemia (CLL) and Acute Myelogenous Leukemia (AML) that showed a decrease in the surface protein expression of NKp46 which contributed to the disease pathogenesis^{31,32}.

By examining each individual patient more closely, we noticed that by comparing the surface protein expression of specimens collected at the time of diagnosis from specimens collected after chemotherapy treatment, a more pronounced shift in expression can be observed in certain individual patients. Using criteria presented in table 2 as reference, we have noticed that patients that show a more overstated change in expression are patients that are categorized as high risk patients. Further studies into the treatment received by these patients can help elucidate not only the effects of such treatments to each individual patients but also further explain the role of the receptors.

As with all research studies dealing with human samples, there were potential limitations in our study both in the aspect of data gathered and methods used. One of the main limitations is

the inconsistency in the amount of PBMCs from each individual patient. Each patient have varying amount of cells that made it difficult to repeat and/or standardize certain procedures. Lastly, the availability of healthy donors that are age, gender and ethnicity matched with the ALL patients is another limitation we encountered. This contributed to being unable to obtain statistically significant data when comparing patient receptor expression to healthy subjects.

Conclusion

In conclusion, receptors 2B4, CS1, LLT1, NKp30 and NKp46 showed altered expression in both the mRNA and surface protein level on immune cells of patients with childhood ALL. Altered expression of these receptors has been shown in previous studies to contribute to disease pathogenesis of leukemia and other cancers^{14,21,22,25,26,28}. Being able to show that there is an alteration in receptor expression in ALL in children could demonstrate their possible involvement in the development of the disease. Furthermore, demonstrating that high risk patients show a more evident change in receptor expression strengthens the idea that, at the very least, some of these receptors are truly involved in disease pathogenesis. In the future, since these receptors are known to play a role in NK cell tumor killing by enhancing the NK-mediated cytolytic activity^{22,25,27}, it would be of best interest if NK cells can be isolated from ALL patients and functional studies are conducted to observe their mechanism of action.

This additional understanding of the possible role of immune receptors in the pathophysiology of childhood ALL opens the door for future research into the function of these receptors and the mechanism of their action. This would then allow for the development of a

more specific, less invasive treatment option for children with ALL that will not only lessen both the short and long term side effects of the current treatments, but would also lower the chances for relapse.

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