Powers, Sheila B., Expression and Function of Immune Receptors in Pediatric Acute Lymphoblastic Leukemia. Master of Science (Biomedical Sciences), November, 2016.

Acute lymphoblastic leukemia (ALL) is a cancer that mainly affects children around the age of five. Due to current treatments, 80-90% of children achieve long-term remission. However, because of the current treatments, which include chemotherapy and CNS-directed radiation, these children may experience late effects which impact growth and development. Even though survival rate is high, quality of life can be greatly reduced for some of the survivors.

Natural Killer (NK) cells are cells of the innate immune system that are important in fighting cancer and virally-infected cells. They have been a subject of interest in ALL because ALL of the B cell lineage is particularly resistant to NK cell killing. NK cells get activated by their surface receptors and their ligands on target cells. In B cell ALL, the NK cells do not appear to get the proper activating signals and this has been determined to be one reason this cancer is able to thrive. Our lab has cloned three immune receptors, 2B4 (CD244), CS1 (CRACC, CD319) and LLT1 (CLEC2D), that are expressed on NK cells as well as other immune cells. These receptors have been shown to play an important role in NK cell activation. Two other receptors, NKp30 and NKp46, are well-known activating receptors that have also been implicated in ALL. In this project I compared the mRNA and surface protein receptor expression of immune receptors between healthy subjects and ALL subjects. Altered expression of immune receptors was observed in ALL subjects. In particular, a significant decrease in mRNA expression of 2B4, LLT1 and NKp30 was observed in ALL subjects at diagnosis compared to healthy subjects. mRNA expression of CS1 was increased significantly after

chemotherapy treatment. In contrast, NKp46 mRNA expression was significantly increased in ALL subjects as compared to healthy subjects. Cell surface protein expression of CS1 was significantly upregulated on T cells and monocytes and LLT1 on NK cells of ALL subjects at diagnosis. Interestingly, NKp30 was overexpressed on B cells, T cells, monocytes and NKp46 was overexpressed on T cells and monocytes but not on NK cells of ALL subjects at diagnosis. I also detected a significant increase of soluble CS1and BAT-3 in ALL subjects at diagnosis between the ages of 1-11 yrs. Also, soluble CD48 was significantly increased in ALL subjects after chemotherapy treatments. Future mechanistic studies may shed more light in the immune dysfunction in ALL ultimately contributing to better treatment options for patients with pediatric ALL.

EXPRESSION AND FUNCTION OF IMMUNE RECEPTORS IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

THESIS

Presented to the Graduate Council of the Graduate

School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Sheila B. Powers B.A.

Fort Worth, Texas

November 2016

ACKNOWLEDGEMENTS

I would like to thank Dr. Stephen Mathew for accepting me into his lab and allowing me to share in his research. I thank him for this incredible learning experience and his guidance for the past two years. I would also like to thank all of my committee members: Dr. Porunelloor Mathew, who was always available with advice and encouragement; Dr. Paul Bowman, without whom this project would not be possible; and Dr. Sid O'Bryant, for acting as my university member and offering a new perspective on my work. Everyone has complex schedules and I appreciate the time and effort they all put into my graduate experience.

I would like to thank Dr. Harlan Jones and Colette Ndjom for allowing me to use their lab for some of my experiments, as well as Dr. Xiangle Sun for her eternal patience with my flow cytometry work.

I am also very appreciative of Cook Children's Medical Center and the Patient Care Center of UNTHSC for supplying me with the healthy and patient samples I needed to complete this project.

I would also like to thank my labmates. Kelly Bowen taught me so much when I first joined the lab and I am eternally grateful to her. I also want to thank Joseph Malaer who was always an open ear for me and allowed me to bounce ideas off him. I want to thank Charisse Tan and Kim Aldy who started this project earlier. Finally I want to thank our research assistant, Bhavik Mohanlal, who assisted with preparation of experiments as well as moving the entire lab, twice.

I want to extend my gratitude to my coworkers of PBM Laboratories, Jennifer Archer especially, for going out of their way to make my life a little easier. I want to give a special thanks for my boss Bill Nelson for always giving me a day off when I needed it.

This project would not have been possible without the support of my best friend Maria Rivera, who helped me in so many ways. I also received unyielding support from my parents, who were always there with a home-cooked meal.

Finally, I thank my brother, Tim Powers, who is my touchstone.

TABLE OF CONTENTS

Chapter I

Introduction

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer in the developed world with peak incidence between the ages of 2 and 5 years. ALL is characterized by the overgrowth of lymphocyte precursor cells (either B cell or T cell) that are nonfunctioning, and crowd out other hematopoietic immune cells. The effect of this is anemia as well as increased susceptibility to infections that would eventually cause death if left untreated. With current treatments, such as chemotherapy and radiation, children with ALL have a high long term remission rate of 80-90%.[1,2,3,4] However, those who relapse have a much lower survival rate of 25-40% and one third of relapse cases involve the central nervous system, causing further complications.[5] The strategy of immunotherapy has amassed great interest in recent years. Immunotherapy strives to use the body's own immune system to fight disease. Immunotherapy is specific in the targeting of the disease so that healthy cells are typically not destroyed.

Because of this, the side effects are minimal, and this therapy can be utilized in conjunction with conventional therapies. Developing an immunotherapy relies heavily on understanding the mechanism and cellular environment of the disease. The ultimate goal of this project is to develop an immunotherapy strategy to reduce the rates of relapse and decrease side-effects of current treatments for those diagnosed with ALL.

Previous literature has shown that ALL of the B cell lineage is particularly resistant to killing by natural killer (NK) cells.[6] NK cells are immune cells of the innate immune system that target virally infected and cancer cells. The innate immune system is the first line of defense for the body against cancer and invading pathogens. NK cells are typically classified under innate immunity although they do have characteristics of adaptive immunity such as clonal expansion of antigen-specific NK cells, longevity, and a more vigorous response upon reinfection, indicating a memory-like response.[7] Consequently, NK cells have garnered much interest in research involving cancer.

Currently, NK cell dysfunction has been linked to many cancers, playing at least some part in onset or continuance of the cancer. Specifically, the NK cells do not appear to achieve the proper activating signals through their receptors. NK cell function is regulated by its surface receptors that are classified as either activating or inhibiting. When an NK cell is activated, it can either directly kill the target cell or release cytokines that can further activate immune cells. For this study, five immune receptors were evaluated. Three of these receptors, 2B4 (CD244), CS1 (CRACC, CD319), and LLT1 (CLEC2D), have been cloned in our laboratory and have been shown to play a role in other cancers and diseases such as Systemic Lupus Erythematosus(SLE), X-Linked Lymphoproliferative Disease(XLPD), as well as other leukemias.[6,8,9,10,11,12] The other two receptors, NKp30 and NKp46, are well-known activating

receptors of NK cells against cancer cells and have already been identified as receptors of interest in ALL.[6,13]

This project involved isolating peripheral blood mononuclear cells (PBMC) from subjects with ALL recruited from Cook Children's Medical Center, Fort Worth, TX. Immune receptor expression and function were evaluated in ALL subjects and compared to a healthy control group of subjects. The immune receptors were also examined before and after treatment to see if current treatments are having an impact on receptor expression and function. Overall, this study will enable us to further understand the role of these receptors in the immune dysregulation of childhood ALL.

1.1 Problem and Hypothesis

Immunotherapy has recently been an area of much interest in cancer research. Conventional chemotherapy tends to be nonspecific, meaning it kills cancer cells, but also is harmful to the patient's normal cells. In our laboratory, CS1 has been shown to be an attractive target for immunotherapy in multiple myeloma, because its ligation activates NK cell killing, and its overexpression on the cancer cells makes the treatment cancer specific, thus reducing side effects and improving survival rate.[9] The research done for CS1 as an immunotherapy target shows the benefits of utilizing the body's own immune system and, because side effects are minimal, it can be used along with conventional therapies. The ultimate goal of this project is to develop an immunotherapeutic strategy for ALL.

Previous literature has demonstrated that insufficient activation of NK cells contributes to B cell ALL.[5] The immune receptors under investigation in this study have also been shown to

play a role in the development or perpetuation of other diseases, such as 2B4 in XLPD and LLT1 in glioblastoma. Also, NKp30 and NKp46 have been implicated as targets of interest in other leukemias. It is therefore possible that altered expression of these immune receptors can play a role in ALL.

I hypothesize that children with acute lymphoblastic leukemia (ALL) have altered expression and function of immune receptors compared to healthy individuals.

I will test the hypothesis in the following specific aims:

Specific aim 1:

Determine expression of immune receptors in B cells, T cells, NK cells and monocytes in pediatric ALL patients. (Chapter III and IV)

Specific aim 2:

Evaluate the function of immune receptors in leukemic cells in pediatric ALL patients.

(Chapter V and VI)

1.2 Significance

Pediatric Acute Lymphoblastic Leukemia is the most common cancer in children. Although remission rates are upwards of 90%, those who relapse only have a survival rate of 25-40%.[1] Also, those who do survive can have long lasting side effects, such as growth defects due to chemotherapy or radiation treatment as well as having to endure painful procedures such as bone marrow aspirations. The long lasting side-effects, which can also include infertility and

lifetime learning disabilities, are particularly devastating considering that this cancer mostly affects young children. Currently, there is also no preventative diagnostic screening for ALL.

The overall goal of this study is to eventually develop an immunotherapy target for ALL. This would lead to treatment options that specifically target just leukemic cells and spares normal cells and cellular function. This would minimize side effects of current treatment and improve prognosis and quality of life.

1.3 Background

Acute Lymphoblastic Leukemia

Leukemia is characterized by overgrowth of abnormal white blood cells forming in the bone marrow. The cancer cells, which are usually precursors of normal cells so they have little to no function, crowd out normal blood cells, preventing healthy cells from developing and functioning properly, leading to anemia, bleeding and increased susceptibility to infection. Leukemia is classified as either lymphoblastic, which means the cancerous cells are from either T or B cells, and myeloid which means the cancerous cells precursors of are monocytes, red blood cells, or platelets. Leukemia is also broken down into acute, which is very fast-acting, or chronic, which may take several years to develop. Therefore, acute lymphoblastic leukemia is a fast-acting cancer of precursor B or T cells.

ALL is the most common cancer in children and is most common in Hispanics and Caucasians, with the least frequency in those of African descent. There is an 80 to 90% long term remission rate. However the relapse rate is 15-20%, and the rate of survival for those who relapse is only about 25-40%.[1]

The current treatment for leukemia begins with an initial course of chemotherapy lasting approximately 1 month. In cases that respond poorly, a bone marrow transplant may be needed. Radiation therapy is also directed at the CNS, since many relapse cases involve the CNS, though even at low doses, this can cause growth defects in childhood and fertility issues later in life. Recent clinical trials seek to minimize the number of patients receiving radiation therapy. Finally, the patient is put on remission maintenance chemotherapy for at least 2 years.[1]

In recent years, immunotherapy involving genetically modified T cells called CARS (chimeric antigen receptors) have gained considerable success against many forms of cancer, including ALL. Essentially, the T cells are modified to express receptors specific for a patient's particular cancer. Although this has gained a lot of success, its role remains investigated and many toxicities have come to light through this method. The most common side effect of T cell CAR therapy is cytokine release syndrome (CRS), which is caused by excessive T cell proliferation and cytokine release. Other side effects include neurological dysfunction, non-specific T cell killing, and anaphylaxis.[14,15] Because of this, there is still a great deal of improvement that can be made in regards to ALL treatment.

Natural Killer Cells

Natural killer cells comprise approximately 5-10% of the normal lymphocyte population. They provide rapid responses to tumor and virally-infected cells, which they can recognize in the absence of antibodies or the MHC-1 receptor in a mechanism known as the "missing-self hypothesis". Responses of NK cells are regulated by inhibitory and activating receptors. NK cells express receptors specific for ligands expressed on target cells.[16,17] A seminal study noted

that NK cells can kill certain tumor cells lacking MHC class I, yet spare the same tumors expressing MHC class I.[18] This ability to attack cells 'missing self' predicted the existence of inhibitory receptors on NK cells, and it revealed the mechanism whereby NK cells engage in surveillance for transformed or virus-infected cells that have downregulated expression of MHC class I in an effort to avoid recognition by $CD8^+$ cytotoxic T lymphocytes. When a target is 'stressed', ligands for activating receptors are induced, as in 'induced-self', permitting NK cell activation by overcoming MHC class I inhibition. When the NK cell is inhibited, the target cell, presumably a normal healthy cell, is not killed.[19]

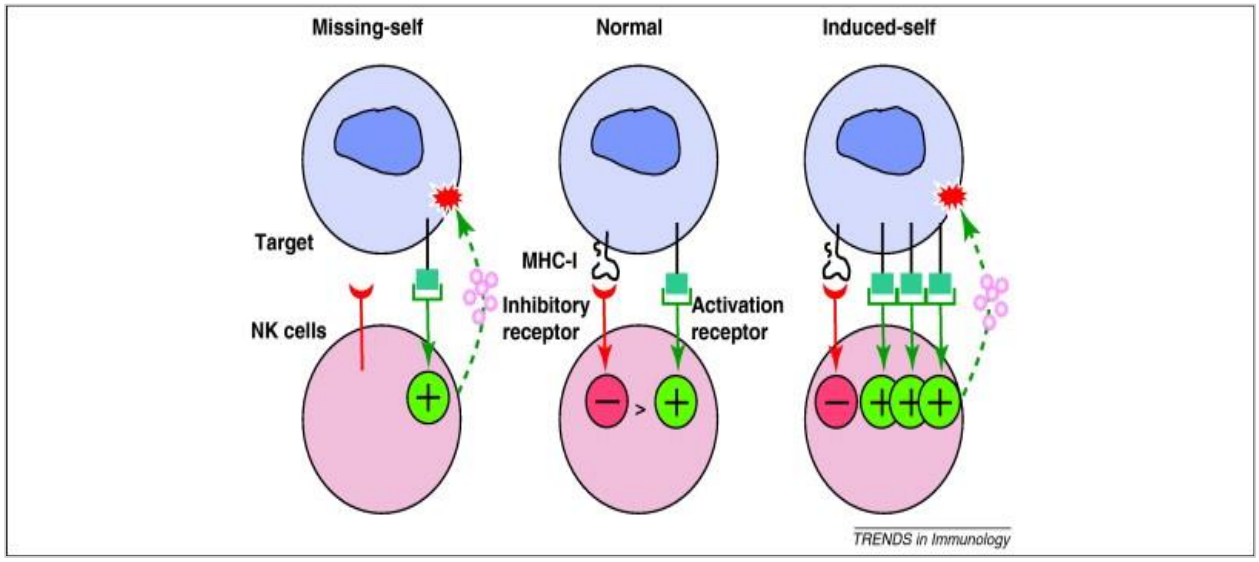

Figure 1: NK cell function is regulated by various surface receptors to either activate or inhibit the NK cellular function. Adapted from Elliot, J and Yokoyama, W. Trends in Immunology 2011.[19] Permission to use the figure was obtained from Trends in Immunology.

NK cell responses also include cytokine production, which is regulated in the same way by signaling through a combination of activating and inhibiting receptors.[15] NK cells can also kill target cells through ADCC (Antibody Dependent Cellular Cytotoxicity). When antibodies are bound to a target cell, a receptor on NK cells called CD16 binds the antibodies and activates

the NK cell to kill the target. In humans, several NK triggering receptors have been identified, including NKG2D, CD16, and the natural cytotoxicity receptors (collectively named NCRs) NKp30, NKp44 and NKp46.[17] Our laboratory has identified and cloned novel NK cell receptors 2B4 (CD244), CS1 (CRACC, CD319) and LLT1 (CLEC2D), which activate NK cells upon interaction with their ligands.[8,9,10] NKp30 and NKp46, two NCR receptors, have been shown to play a role in other cancers.[13] In this study, I will be investigating the role of these five immune receptors – 2B4, CS1, LLT1, NKp30 and NKp46 in pediatric ALL subjects.

2B4 (CD244)

2B4, which was originally identified, cloned and characterized in our laboratory, is expressed on NK cells, T cells, monocytes, basophils and eosinophils.[8] In both mice and humans, CD48 is the ligand for 2B4.[20,21] 2B4 has a dual role as it can be both activating and inhibiting, however, it is predominantly an activating receptor in humans.[22,23] It also co-stimulates other activating receptors.[24] The function of 2B4 is regulated by three factors: surface density, level of 2B4 binding, and abundance of SAP molecules inside the cell.[25] In XLPD (X-linked lymphoproliferative disease), the SAP adaptor molecule which binds 2B4 and is necessary for its activation, is defective.[12] This causes an inhibitory effect on the NK cell so it is not able to kill the target cell. Also, as previously researched in our lab, 2B4 is downregulated on NK cells in lupus[11]. The important role of 2B4 as a co-activating receptor was demonstrated when NKp30 and NKp46 activation was analyzed. Even when these receptors are engaged, in the absence of 2B4, the NK cell does not get activated.[12]

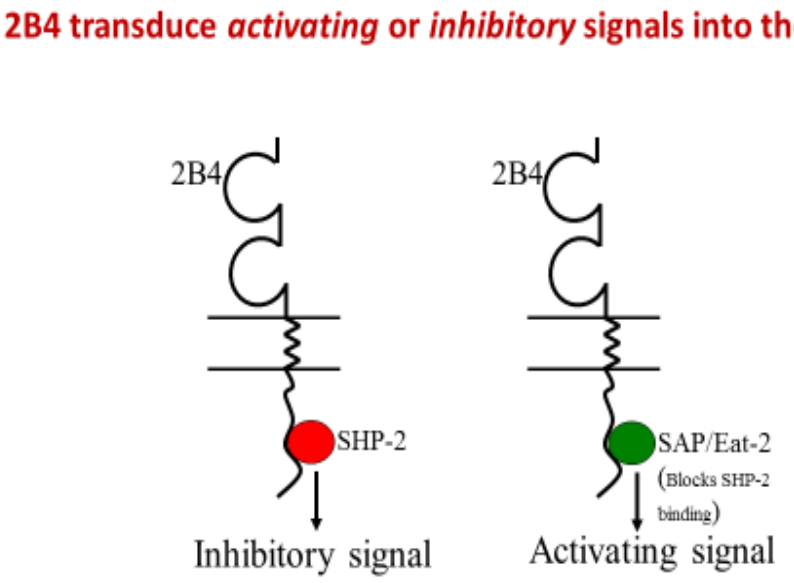

Figure 2: 2B4 activation is reliant on SAP molecules.

2B4 has also been implicated in immune evasion for some cancer cells. Recent studies have shown that monocytes in hepatocellular carcinoma have overexpression of the 2B4 ligand CD48. Upon ligation, the NK cell does get activated, however it appears it actually gets overstimulated, causing exhaustion and apoptosis of the NK cells.[26]

Elevated levels of soluble 2B4 ligand, CD48, were also detected in patients with arthritis and lymphoid leukemias[27]. This could also have the effect of overstimulating lymphocytes and causing exhaustion. Therefore, an overabundance of CD48 in serum could inhibit NK cell activation and killing.

CS1 (CRACC, CD319)

CS1, also called CRACC or CD319, was first identified and cloned in our laboratory[9]. It is expressed on activated B and T cells, NK cells and mature dendritic cells. CS1 is a self-ligand and has been shown to be highly expressed on the cancer cells in multiple myeloma as well as in a soluble form in the sera of multiple myeloma patients.[28] Anti-CS1 monoclonal antibody has shown great promise as a treatment for multiple myeloma by increasing NK cell killing of target cells.[29] CS1 also stimulates B cell proliferation and has been shown by previous research in our laboratory to be overexpressed in lupus[11,30].

Elotuzumab, also known by its trade name Empliciti, is the humanized anti-CS1 monoclonal antibody developed for treatment against multiple myeloma[31,32]. It helps NK cells kill multiple myeloma cells in two ways. It can bind on the CS1 receptor on the NK cell to stimulate activation, or it can bind to CS1 on the target cell. CD16 on the NK cell can then bind to the antibody, stimulating antibody dependent cellular cytotoxicity. Soluble CS1 was also shown to be overexpressed in the plasma of multiple myeloma patients.[28]

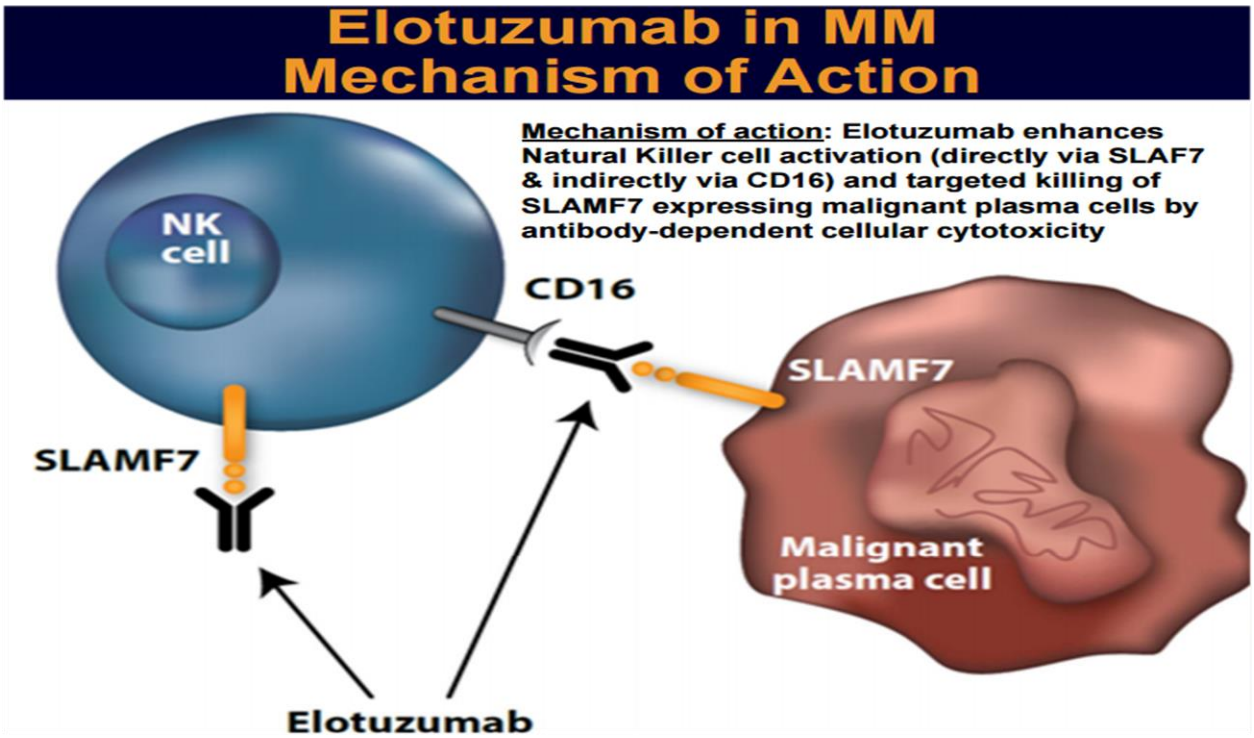

Figure 3: Empliciti improves NK cell killing in multiple myeloma by two mechanisms of action. Adapted from http://szpiczak.org[33]. Permission to use the figure was obtained from Dr. Paul Richardson, Harvard Medical School, Boston, MA.

LLT1 (CLEC2D)

LLT1 is another receptor that has been identified and cloned in our laboratory. It is expressed on T cells, B cells, NK cells and monocytes.[10] LLT1 activates IFN-gamma production, which in turn increases NK cell activity.[34] Its ligand is CD161, which is also expressed on NK cells and acts as an inhibiting receptor.[35] Human glioblastoma cells have been shown to upregulate LLT1 and when they come in contact with NK cell receptor CD161, the NK cell is inhibited.[36] There is also evidence that LLTI is upregulated in prostate cancer.[37] This makes it an appealing target for immunotherapy research.

NKp30 and NKp46

NKp30 and NKp46 are important activating receptors with a variety of ligands to combat viruses, bacteria, and cancer. They are part of a family of receptors called NCRs (natural cytotoxity receptors).[13,38] NKp30 has three isoforms, one of which has an inhibitory function and has been shown to play a key role in gastrointestinal sarcoma by inhibiting NK cell activation.[39] A soluble form of one of its ligands, BAT3, has been shown to impair NK cell cytotoxicity when it is overexpressed in plasma of patients who have chronic lymphocytic leukemia.[40] NKp46 has already been shown to be a receptor of interest in cancers and has been shown to have reduced expression and activity in other leukemias as well.[13,41]

Chapter II

Materials and Methods

2.1 Healthy and Patient Populations

Newly diagnosed ALL subjects, aged between 2 and 21 years old, were enrolled in the study at the Hematology & Oncology Clinic at Cook Children's Medical Center (CCMC), Fort Worth, TX, with informed consent/assent by Dr. Paul Bowman, MD and nursing staff as per IRB approval from UNTHSC and CCMC (UNTHSC IRB# 2008-094 & CCMC IRB# 2008-57). Also, healthy subjects under the age of 21, who attend regular medical visits at the pediatric clinic at UNTHSC Patient Care Center (PCC) were enrolled.

2.2 Blood Collection

After consent was obtained, the staff collected one blood sample (8 mLs) from the patient before any treatment. This is referred to as the first blood draw (1BD). Another blood sample was collected 29 days later, after the initial chemotherapy, which typically lasts 28 days. This is referred to as the second blood draw (2BD). Currently, 43 ALL subjects have been enrolled. Only one blood draw was collected from healthy subjects. Currently, 21 healthy subjects have been enrolled.

2.3 PBMC Isolation

Once the blood samples were obtained, peripheral blood mononuclear cells (PBMCs) were isolated by the Histopaque (Sigma Aldrich) method. ALL serum samples were also collected and stored at –80^{o}C. The PBMCs were then separated into two parts, one part was used for flow cytometric analysis of immune receptor expression and the second part was used to isolate mRNA for qRT-PCR analysis. Under circumstances where the patient specifically consents, some B cells were isolated to be made into an immortal cell line for future study.

2.4 qRT-PCR

The purpose of quantitative reverse transcriptase PCR was to evaluate the expression of total mRNA of each of the immune receptors of interest. This will not differentiate between the various immune cells. The mRNA was preserved with the use of RNA STAT60 reagent, and later converted to cDNA using Omniscript RT kit (Qiagen), which was then used in quantitative

PCR. Taqman mastermix and Taqman primers for 2B4, CS1, LLT1, NKp30 and NKp46 were used on the Eppendorf Realplex2 to perform the PCR reaction. The PCR data was evaluated using the ΔΔCT method. For this method, the difference in CT value (cycle of threshold) between the gene of interest, in this case the various receptors, and the housekeeping gene, in this case GAPDH, is compared to that of the healthy subjects. All "non-detects" are considered to have a CT value of 40, equivalent to the maximum PCR cycle.[42] Thus, I can determine the fold change of mRNA expression between the healthy controls, ALL subjects after the first blood draw, and ALL subjects after the second blood draw.

2.5 Flow Cytometry

The purpose of flow cytometry was to evaluate the cell surface expression of the receptors of interest. A Beckman Coulter FC500 was used and population of immune cells were gated by forward and side scatter to separate the lymphocytes and monocytes from other cells by size and granularity. Various fluorochrome markers were used to differentiate the immune cells: FITC conjugated anti-human CD3 monoclonal antibody (mAb) for T cells, PE-Texas red conjugated anti-human CD19 mAb for B cells, APC conjugated anti-human CD56 mAb for NK cells, and APC-Cy7 conjugated CD14 mAb for monocytes. Each immune receptor of interest and ligands were labeled with PE. Samples were stained with anti-2B4, anti-CS1, anti LLT1, anti-NKp30, anti-NKp46 mAb, anti-CD161 and anti-CD48 to determine percentage and quantitative cell surface expression of these receptors. If less than one percent of cells were acquired for a receptor, then that particular sample was taken out of further calculations as this was seen as not representative of the population. All the antibodies were ordered from Biolegend, San Diego, CA.

2.6 ELISA

The serum sample that was also saved during PBMC isolation was used to detect soluble protein expression of receptors by ELISA. One interesting aspect of CS1 in multiple myeloma was that soluble CS1 expression was observed in patient sera. CS1; CD48, the ligand for 2B4; and BAT3, a ligand for NKp30, are known to be found in soluble form in sera. ELISA was done on the patient sera and healthy subject sera to determine if these receptors are in under or overabundance in those with ALL. An indirect ELISA protocol was followed for all three receptors. CS1 was measured with purified anti-human CD319 from Biolegend, CD48 was measured with purified anti-human CD48 from Biolegend with purified mouse IgG2b as a control. BAT3 was measured with anti-h/m/rBAT3/BAG6 from R&D systems.

2.7 Cytotoxicity Assay

To analyze receptor function, I conducted killing assays utilizing the cell line Jurkat, which is a well-established T cell ALL cell line and the NK cell line NK92. Jurkat cells were first analyzed by flow cytometry to determine whether or not these cells expressed our receptors under investigation.

The percentage of killing was analyzed by flow cytometry. Affymetrix eBioscience Annexin V-APC staining kit was used to calculate early apoptosis of target cells and propidium iodine was used to calculate late apoptosis.[43]

2.8 Statistical Analysis

To analyze the differences in immune receptor expression between healthy subjects and ALL subject samples, an unpaired t test and non-parametric Mann-Whitney test were calculated by GraphPad Prism 7. To analyze differences in immune receptor expression between first and second blood draw of ALL subject samples, an unpaired t test and non-parametric Mann-Whitney test were used. Significance was determined if p values were less than 0.05.

Chapter III

Analysis of mRNA Expression of Immune Receptors between Healthy Subjects and Patients with Pediatric Acute Lymphoblastic Leukemia

Purpose of the Aim

The purpose of this aim was to determine if there is a difference of expression in immune receptors 2B4, CS1, LLT1, NKp30, and NKp46 at the mRNA level between healthy subjects and ALL subjects before and after treatment. As mentioned previously, these receptors have been shown to play a role in the progression of other diseases and cancers. The mRNA expression of these receptors was evaluated to determine if there is altered expression in ALL and if this is the case, could this be a factor in altered function of effector cells and progression of ALL.

In order to achieve this aim, qRT-PCR was performed on RNA from the isolated PBMCs of healthy and patient subjects. Altered expression of mRNA may give insight into altered

protein expression of the immune receptors. It is important to note that the overall fold change of the immune receptors represents total mRNA isolated and does not differentiate between the cell types.

As mentioned previously, PBMCs were isolated from healthy subjects and patient subjects who had been diagnosed with ALL. Quantitative RT-PCR was then preformed using different specific primers for each receptor of interest. Fold change of expression between healthy subjects and patient subjects was calculated using the ΔΔCT method, which compares the CT value, defined as the number of cycles required for the fluorescence to reach threshold, of the target gene and the housekeeping gene of one sample group to a control sample group.

Table 1 and 2 show the demographics of the healthy subjects and ALL subjects respectively. Initially, all of the subjects of each group were compared. Figures 1, 3 and 4 show a significant decrease in mRNA expression of 2B4, LLT1 and NKp30 in the ALL subjects before treatment. Figure 2 reveals that CS1 is increased significantly after treatment from 1BD to 2BD. Figure 5 shows that NKp46 was significantly increased in ALL subjects compared to healthy subjects.

Healthy subjects and patients were then matched by age into two groups, 0-11 years old and 12-21 years old. Each subgroup showed the same trend as the total albeit with decreased significance, however there was one notable exception. In figure 15, in the age group of 12-21, NKp46 was decreased in the ALL patient samples first blood draw compared to the healthy subjects. The mRNA expression of NKp46 also had the most standard deviation. This is also true for figure 25, which shows just male subjects. Previous literature has shown that NKp46 does get downregulated in other leukemias although the effect of this is inconclusive.[13] Subjects were separated into male and female groups, and continued the same trends.

These results show there is altered mRNA expression of these receptors between healthy subjects and ALL subjects. Also in the case of CS1, mRNA expression is altered after the patient undergoes treatment for ALL. The effect of this, and whether it is beneficial or not, is yet to be determined.

Table 1: Demographic characteristics of healthy subjects.

S.No.	Sample ID	Age	Sex	Race/Ethnicity
UNTHLTHY1	ADC	4	F	Hispanic
UNTHLTHY 2	MAS	19	M	Hispanic
UNTHLTHY 3	KRR	8	F	African American
UNTHLTHY 4	JHD	5	M	Caucasian
UNTHLTHY 5	AO	3	M	Hispanic
UNTHLTHY 6	JG	4	M	Hispanic
UNTHLTHY 7	IRC	11	M	Hispanic White
UNTHLTHY 8	TCR	8	F	African American
UNTHLTHY 9	CEM	18	F	Hispanic
UNTHLTHY 10	EN	21	M	Hispanic
UNTHLTHY 11	CJM	19	F	African American
UNTHLTHY 12	ROB	19	M	African American
UNTHLTHY 13	ARD	17	M	Caucasian
UNTHLTHY 14	NAB	21	M	Caucasian
UNTHLTHY 15	KTC	20	F	Caucasian
UNTHLTHY 16	CE	20	M	Caucasian
UNTHLTHY 17	RCM	20	M	African American
UNTHLTHY 18	2018	19	M	Hispanic
UNTHLTHY 19	2019	19	F	Hispanic
UNTHLTHY 20	2020	19	F	Hispanic
UNTHLTHY 21	2021	19	F	African American

Table 2: Demographic characteristics of ALL subjects.

S.No.	Sample ID	Age	Sex	Race/Ethnicity	ALL subtype
UNTALL-1	JJT	18	M	Hispanic	B Precursor
UNTALL-2	MHA	14	F	Caucasian	T Cell
UNTALL-3	MC	5	F	Hispanic	B Precursor
UNTALL-4	BTR	4	M	Hispanic White	B Precursor
UNTALL-5	MKC	10	F	African American	T Cell
UNTALL-6	JDB	17	M	Caucasian	B Precursor
UNTALL-7	MG	9	F	Hispanic White	B Precursor
UNTALL-8	KLP	13	F	Caucasian	B Precursor
UNTALL-9	RST	5	M	Caucasian	B Precursor
UNTALL-10	AJD	7	M	Caucasian	T Cell
UNTALL-11	JSH	16	M	Caucasian	B Precursor
UNTALL-12	JLD	10	F	African American	B Precursor
UNTALL-13	KMD	12	F	Caucasian	B Precursor
UNTALL-14	NLS	3	F	Caucasian	B Precursor
UNTALL-15	RK	15	M	Caucasian	B Precursor
UNTALL-16	YRR	3	F	Caucasian	B Precursor
UNTALL-17	GAC	3	F	Hispanic White	B Precursor
UNTALL-18	BAT	8	F	Caucasian	B Precursor
UNTALL-19	JEV	12	F	Hispanic White	B ALL w/ BCR/ABL positive ALL (Ph+).
UNTALL-20	MAB	5	F	Caucasian	B Precursor
UNTALL-21	TLW	12	M	Caucasian	B Precursor
UNTALL-22	SLR	2	F	Caucasian	B Precursor
UNTALL-23	GAG	12	M	Caucasian	B Precursor
UNTALL-24	ARR	3	F	Mix-C-AA-H	B Precursor
UNTALL-25	TOJM	11	M	Caucasian	B Precursor
UNTALL-26	LMS	2	F	Hispanic	B Precursor
UNTALL-27	MRR	8	F	Hispanic	B Precursor
UNTALL-28	HMP	19	F	Caucasian	B Precursor
UNTALL-29	ERM	3	F	African American	B Precursor
UNTALL-30	JVG	5	M	Caucasian	B Precursor
UNTALL-31	SAG	6	F	Caucasian	B Precursor
UNTALL-32	ER	4	M	Hispanic	B Precursor
UNTALL-33	XR	12	M	Hispanic	B Precursor
UNTALL-34	JTS	2	M	Caucasian	B Precursor
UNTALL-35	LJ	10	F	Caucasian	B Precursor
UNTALL-36	NS	10	M	Caucasian	B Precursor
UNTALL-37	1037	13	M	Hispanic	B Precursor
UNTALL-38	1038	5	M	Hispanic	B Precursor
UNTALL-39	1039	3	M	Caucasian	B Precursor
UNTALL-40	1040	5	F	Caucasian	B Precursor
UNTALL-41	1041	5	F	Caucasian	B Precursor
UNTALL-42	1042	5	M	Caucasian	B Precursor
UNTALL-43	1043	8	M	Hispanic	B Precursor

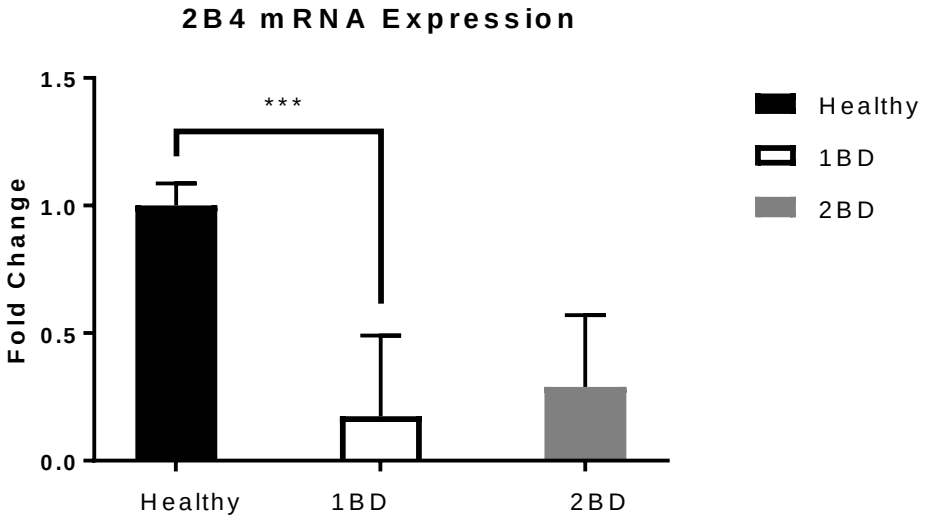

Figure 1: Comparison of 2B4 mRNA expression in total PBMCs between healthy subjects and ALL subjects. Healthy n=19, ALL subjects sample from 1st blood draw n=38, ALL subjects sample from 2nd blood draw n=18. *** indicates a p value of less than 0.0005. Bars represent the median, and the range was calculated using median absolute deviation.

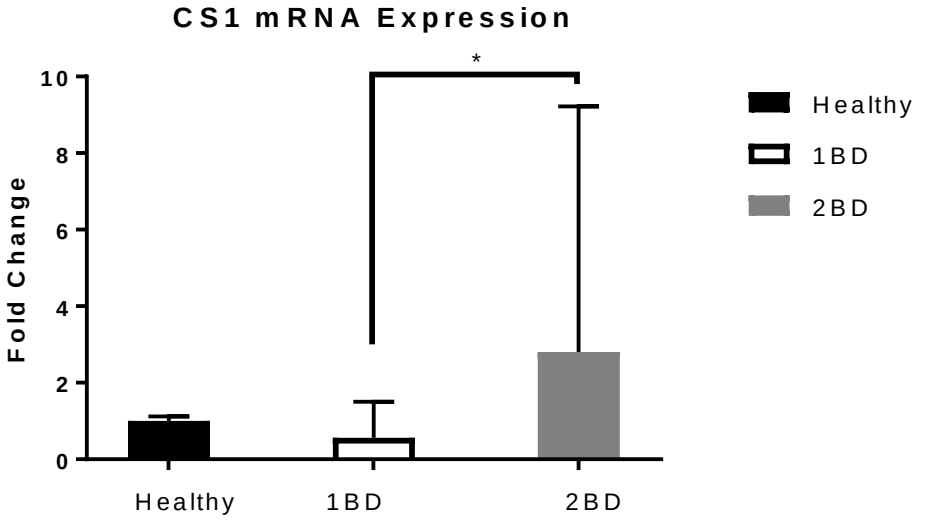

Figure 2: Comparison of CS1 mRNA expression in total PBMCs between healthy subjects and ALL subjects. Healthy n=19, ALL subjects sample from 1st blood draw n=38, ALL subjects sample from 2bd blood draw, n=18. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

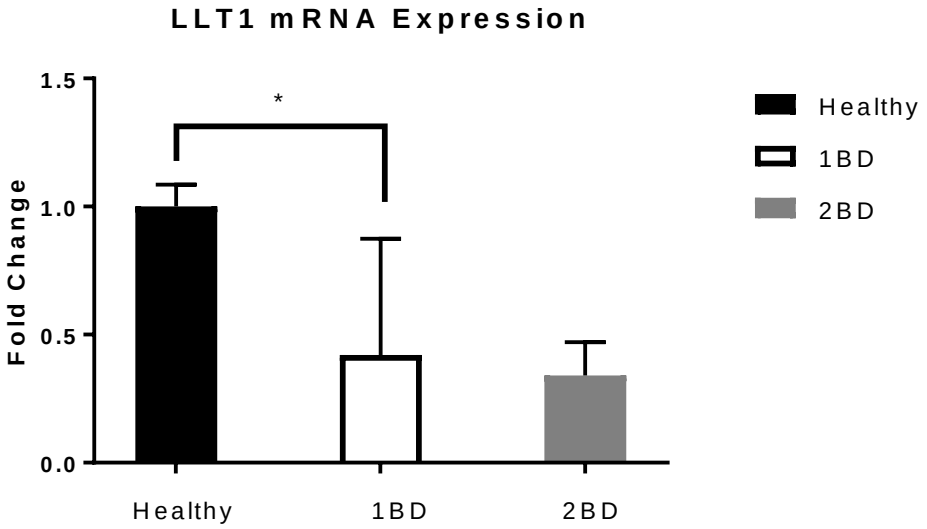

Figure 3: Comparison of LLT1 mRNA expression in total PBMCs between healthy subjects and ALL subjects. Healthy n=19, ALL subjects sample from 1st blood draw n=38, ALL subjects sample from 2bd blood draw n=18. *** indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

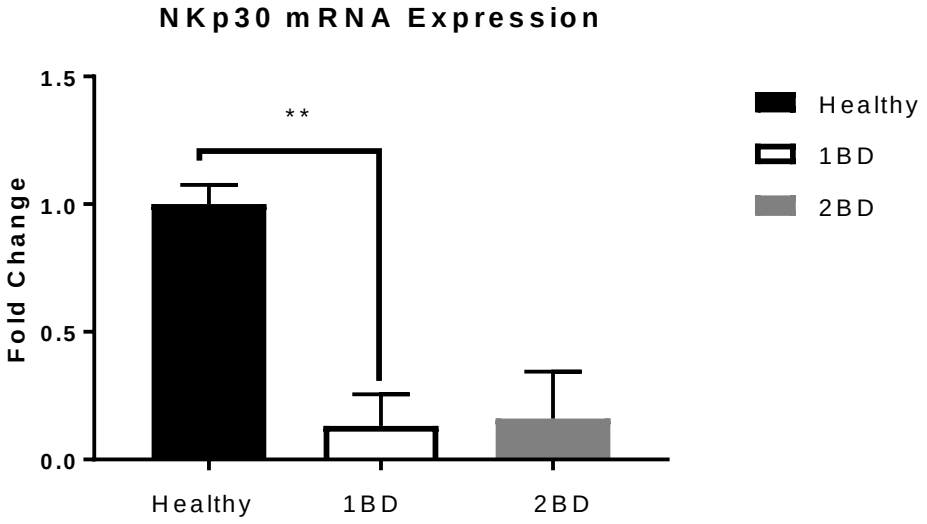

Figure 4: Comparison of NKp30 mRNA expression in total PBMCs between healthy subjects and ALL subjects. Healthy n=19, ALL subjects sample from 1st blood draw n=38, ALL subjects sample from 2nd blood draw n=18. ** indicates a p value of less than 0.005. Bars represent the median, and the range was calculated using median absolute deviation.

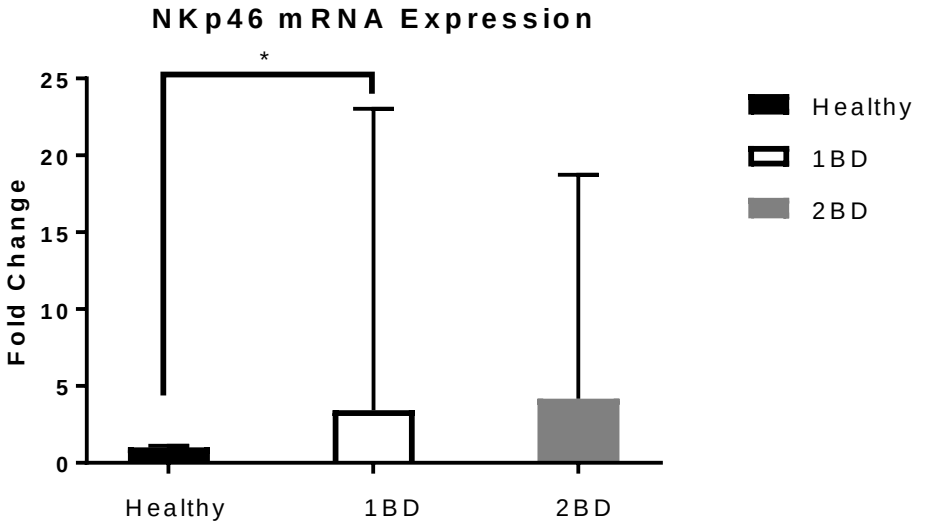

Figure 5: Comparison of NKp46 mRNA expression in total PBMCs between healthy subjects and ALL subjects. Healthy n=19, ALL subjects sample from 1st blood draw n=38, ALL subjects sample from 2nd blood draw n=18. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

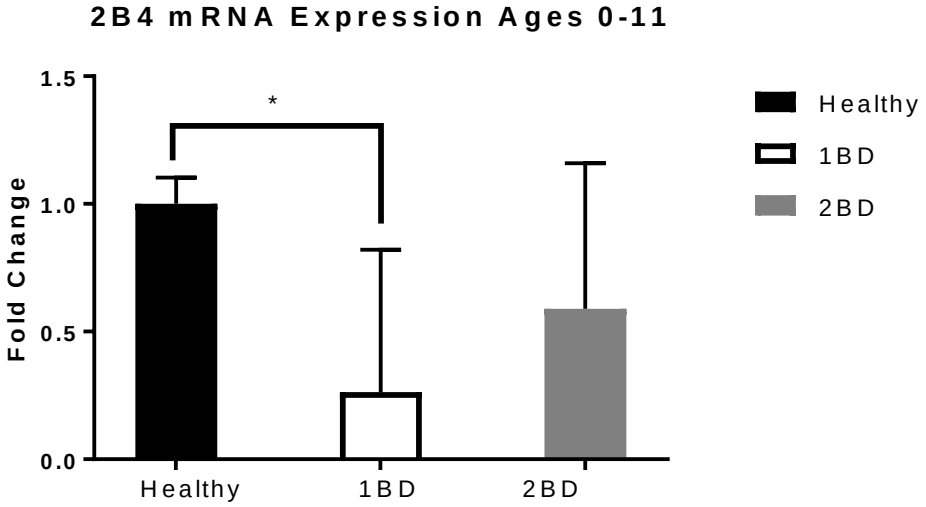

Figure 6: Comparison of 2B4 mRNA expression in total PBMCs between healthy patient subjects and ALL patients ages 0-11 years old. Healthy n=5, ALL subjects samples from 1st blood draw n=24, ALL subjects sample from 2nd blood draw n=12. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

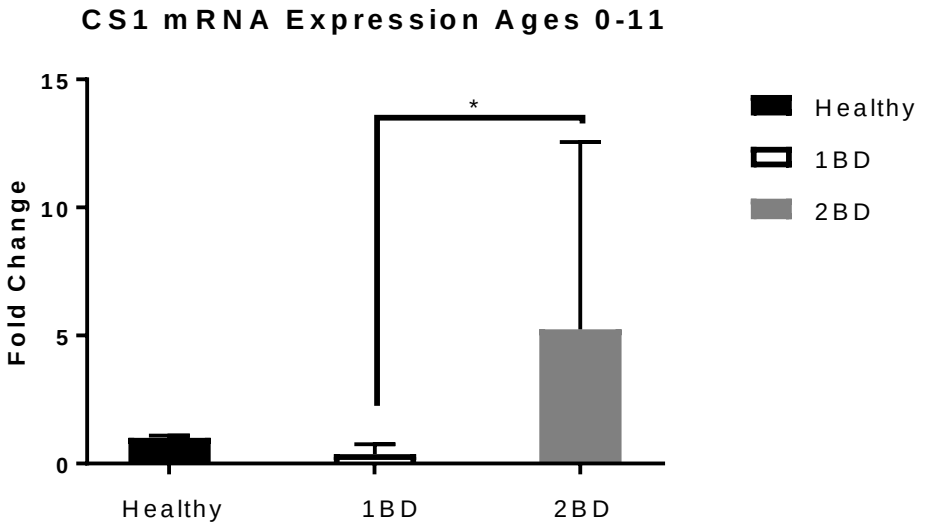

Figure 7: Comparison of CS1 mRNA expression in total PBMCs between healthy subjects and ALL subjects ages 0-11 years old. Healthy n=5, ALL subjects sample from 1st blood draw n=24, ALL subject samples from 2nd blood draw n=12. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

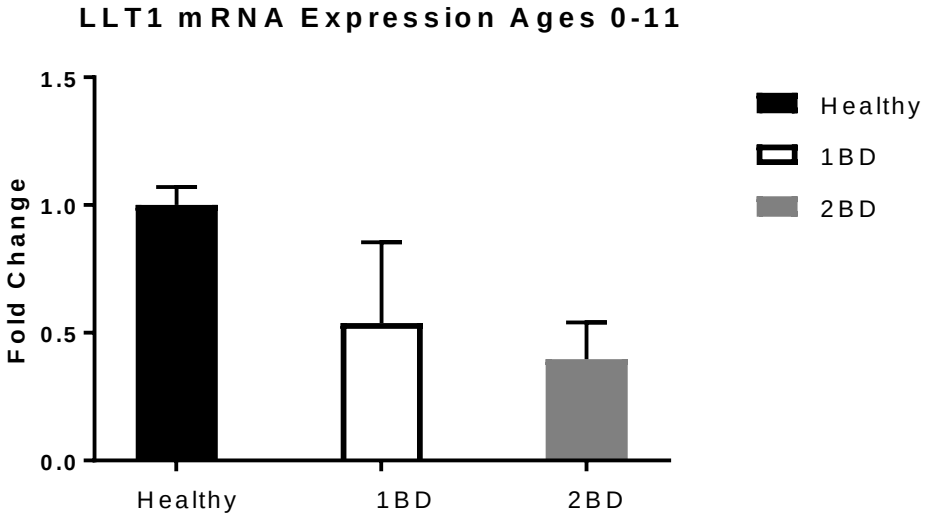

Figure 8: Comparison of LLT1 mRNA expression in total PBMCs between healthy subjects and ALL subjects ages 0-11 years old. Healthy n=5, ALL subjects sample from 1st blood draw n=24, ALL subjects sample from 2nd blood draw n=12. Bars represent the median, and the range was calculated using median absolute deviation.

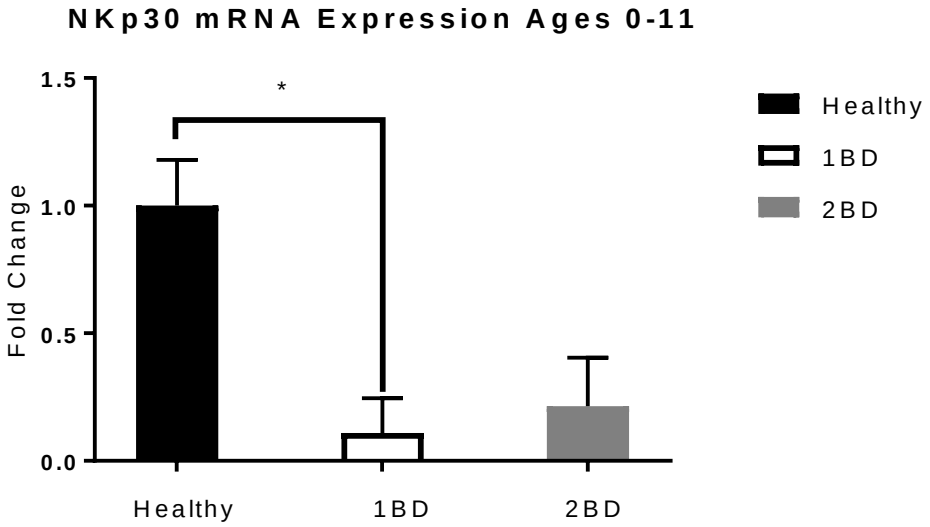

Figure 9: Comparison of NKp30 mRNA expression in total PBMCs between healthy subjects and ALL subjects ages 0-11 years old. Healthy n=5, ALL subjects sample from 1st blood draw n=24, ALL subjects sample from 2nd blood draw n=12. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

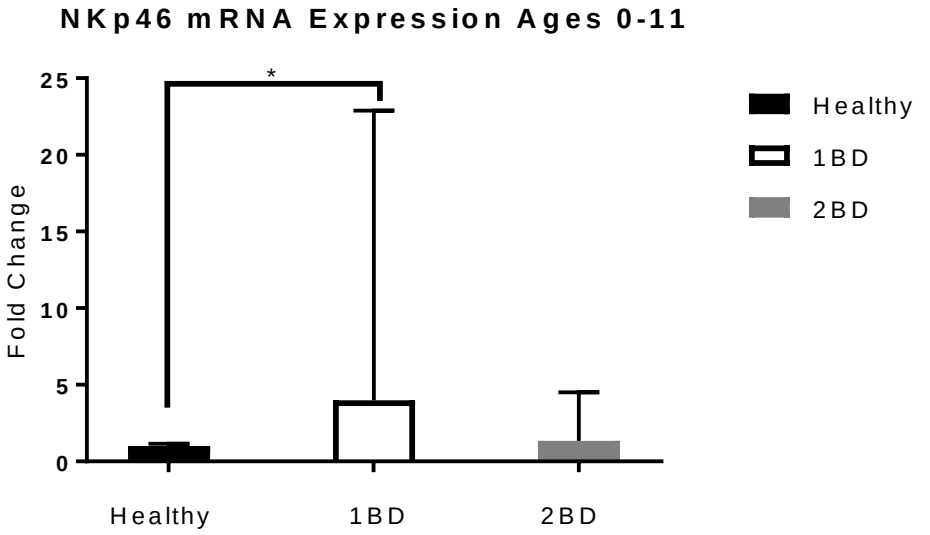

Figure 10: Comparison of NKp46 mRNA expression in total PBMCs between healthy subjects and ALL subjects ages 0-11 years old. Healthy n=5, ALL subjects samples from 1st blood draw n=24, ALL subjects sample from 2nd blood n=12. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

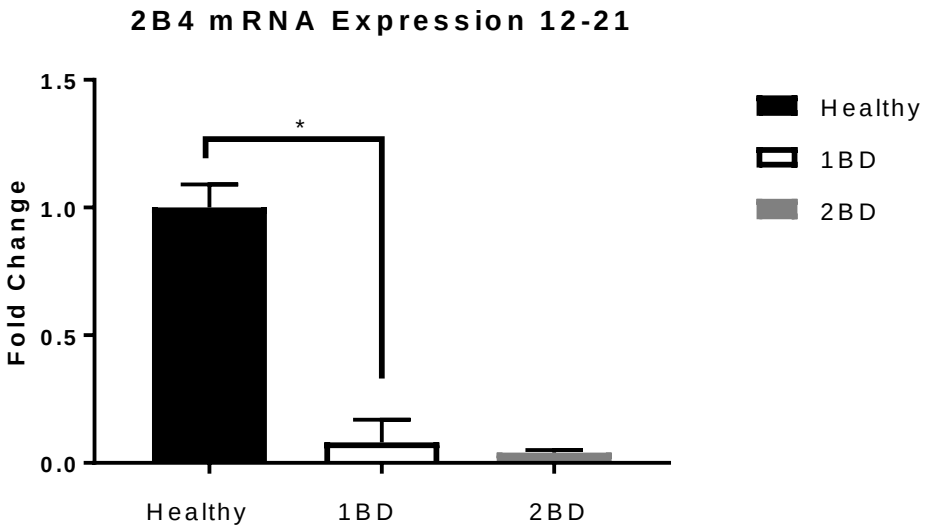

Figure 11: Comparison of 2B4 mRNA expression in total PBMCs between healthy subjects and ALL subjects ages 12-21 years old. Healthy n=11, ALL subjects samples from 1st blood draw n=9, ALL subjects samples from 2nd blood draw n=5. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

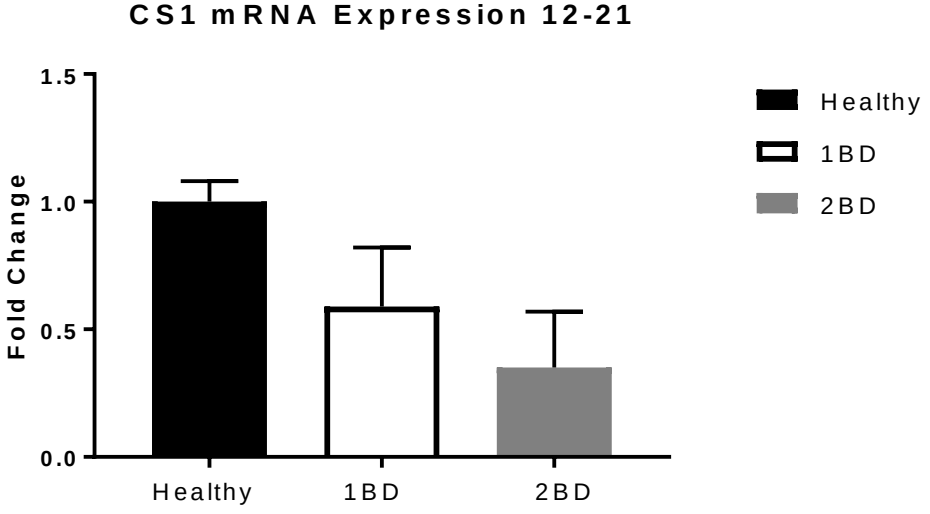

Figure 12: Comparison of CS1 mRNA expression in total PBMCs between healthy subjects and ALL subjects ages 12-21 years old. Healthy n=11, ALL subjects samples from 1st blood draw n=9, ALL subjects samples from 2nd blood draw n=5. Bars represent the median, and the range was calculated using median absolute deviation.

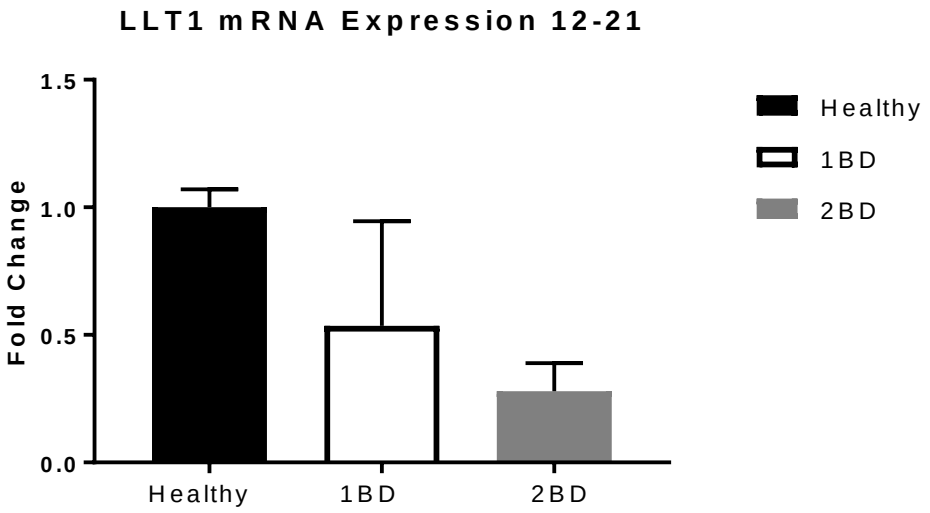

Figure 13: Comparison of LLT1 mRNA expression in total PBMCs between healthy subjects and ALL subjects ages 12-21 years old. Healthy n=11, ALL subjects samples from 1st blood draw n=9, ALL subjects samples from 2nd blood draw n=5. Bars represent the median, and the range was calculated using median absolute deviation.

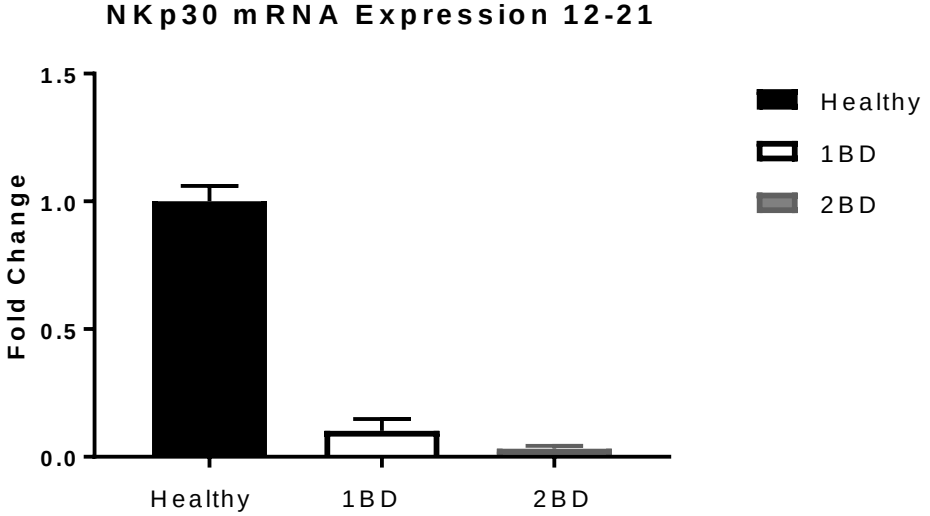

Figure 14: Comparison of NKp30 mRNA expression in total PBMCs between healthy subjects and ALL subjects ages 12-21 years old. Healthy n=11, ALL subjects samples from 1st blood draw n=9, ALL subjects samples from 2nd blood draw n=5. Bars represent the median, and the range was calculated using median absolute deviation.

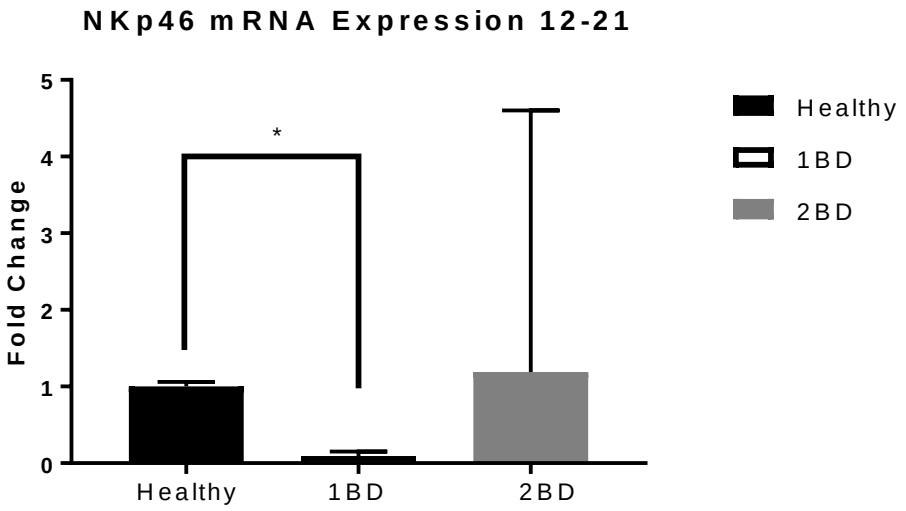

Figure 15: Comparison of NKp46 mRNA expression in total PBMCs between healthy subjects and ALL subjects ages 12-21 years old. Healthy n=11, ALL subjects samples from 1st blood draw n=9, ALL subjects samples from 2nd blood draw n=5. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

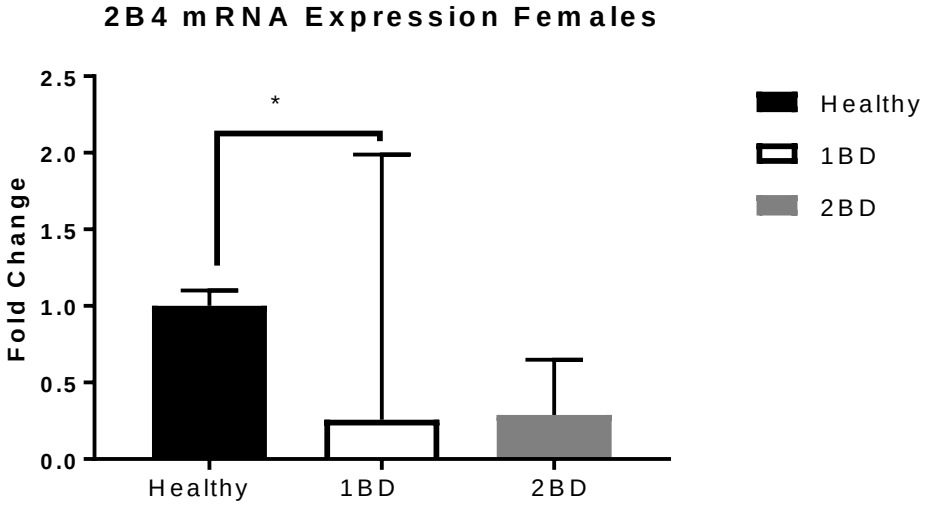

Figure 16: Comparison of 2B4 mRNA expression in total PBMCs between female healthy subjects and female ALL subjects. Healthy n=8, ALL subjects samples from 1st blood draw n=22, ALL subjects samples from 2nd blood draw n=8. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

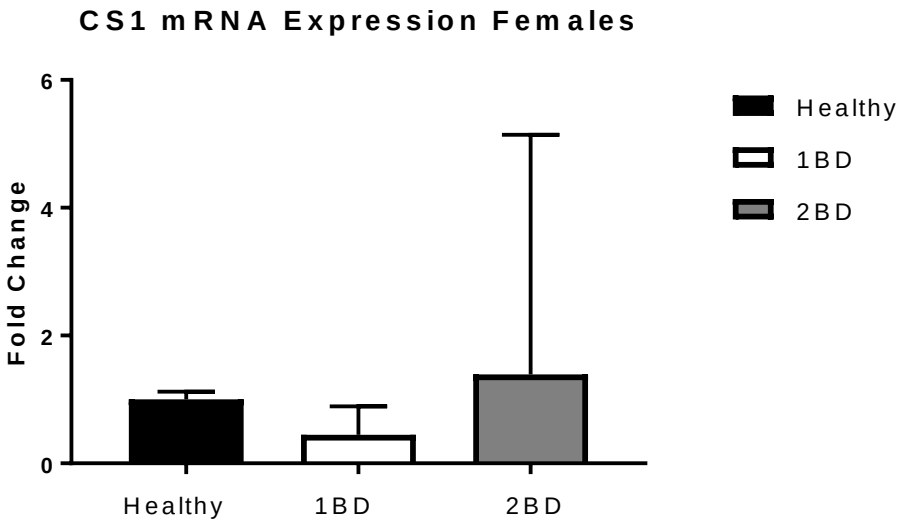

Figure 17: Comparison of CS1 mRNA expression in total PBMCs between female healthy subjects and female ALL subjects. Healthy n=8, ALL subjects samples from 1st blood draw n=22, ALL subjects samples from 2nd blood draw n=8. Bars represent the median, and the range was calculated using median absolute deviation.

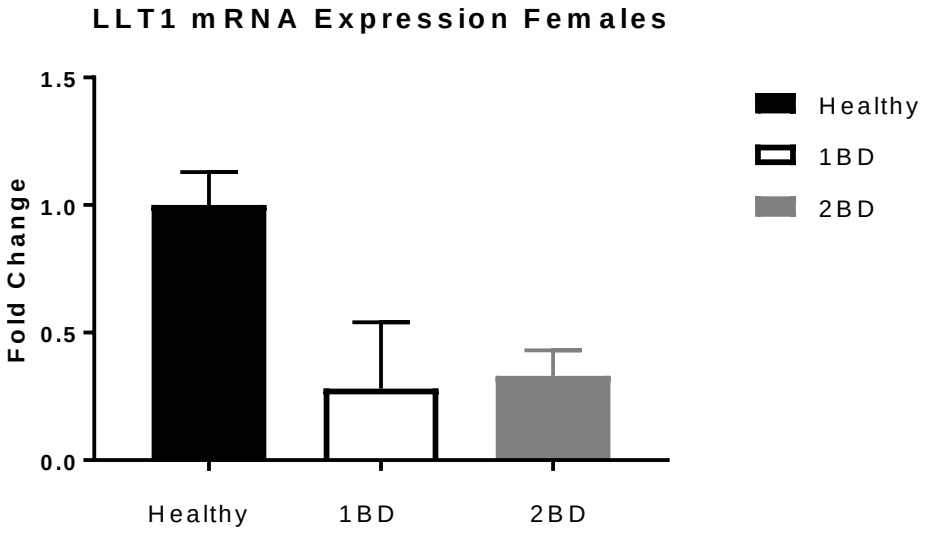

Figure 18: Comparison of LLT1 mRNA expression in total PBMCs between female healthy subjects and female ALL subjects. Healthy n=8, ALL subjects samples from 1st blood draw n=22, ALL subjects samples from 2nd blood draw n=8. Bars represent the median, and the range was calculated using median absolute deviation.

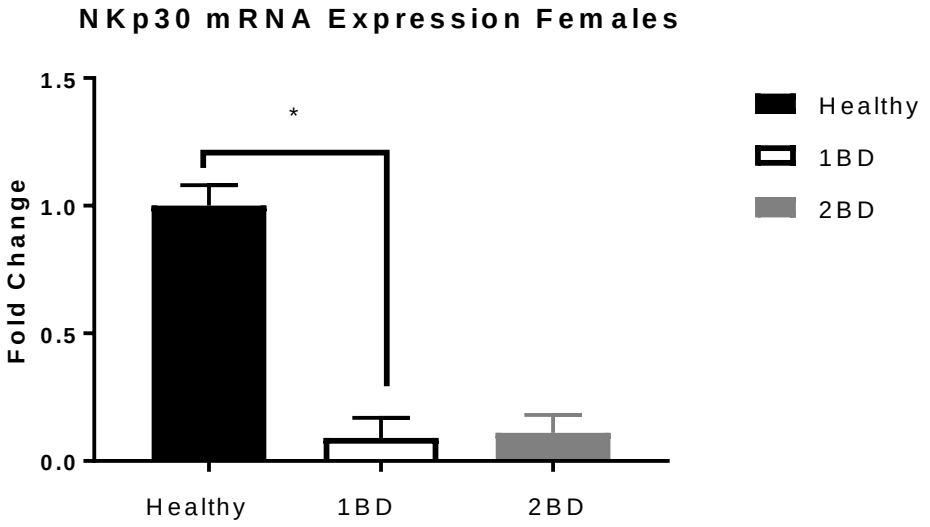

Figure 19: Comparison of NKp30 mRNA expression in total PBMCs between female healthy subjects and female ALL subjects. Healthy n=8, ALL subjects samples from 1st blood draw n=22, ALL subjects samples from 2nd blood draw n=8. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

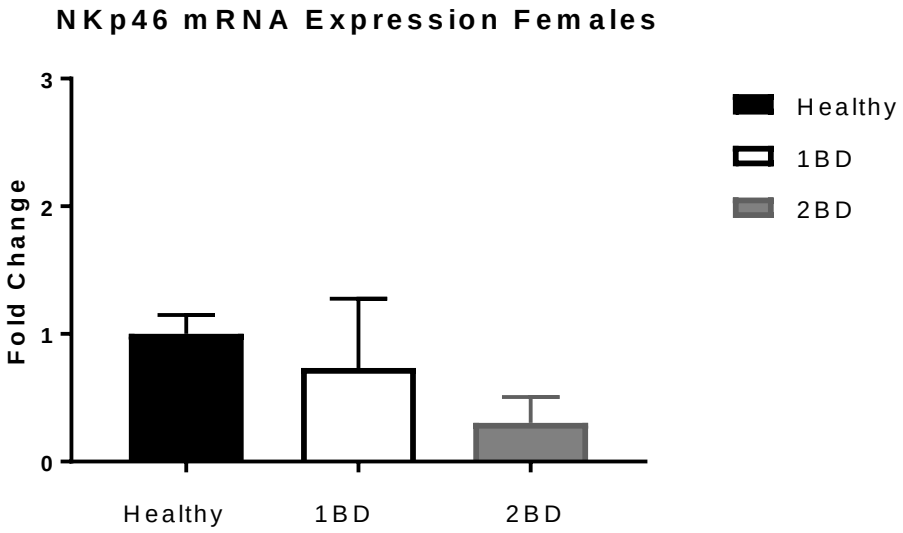

Figure 20: Comparison of NKp46 mRNA expression in total PBMCs between female healthy subjects and female ALL subjects. Healthy n=8, ALL subjects samples from 1st blood draw n=22, ALL subjects samples from 2nd blood draw n=2. Bars represent the median, and the range was calculated using median absolute deviation.

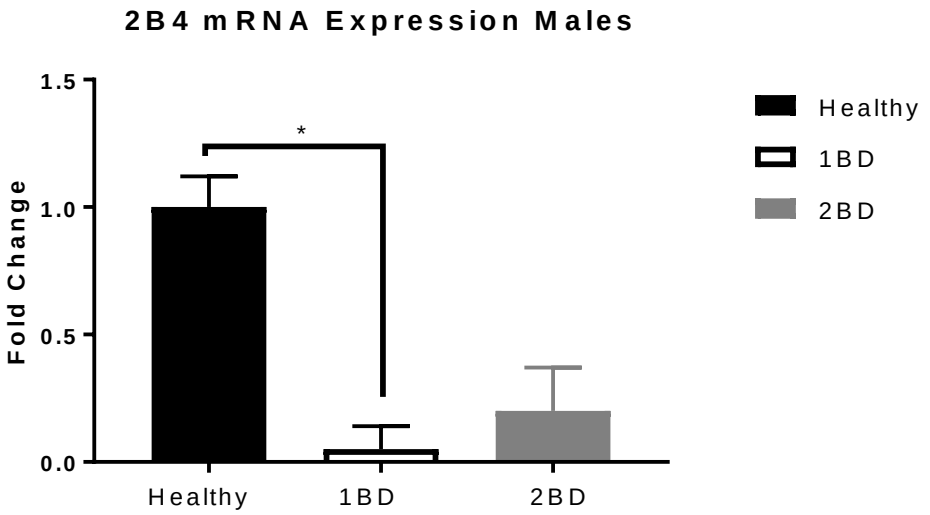

Figure 21: Comparison of 2B4 mRNA expression in total PBMCs between male healthy subjects and male ALL subjects. Healthy n=11, ALL subjects samples from 1st blood draw n=18, ALL subjects samples from 2nd blood draw n=9. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

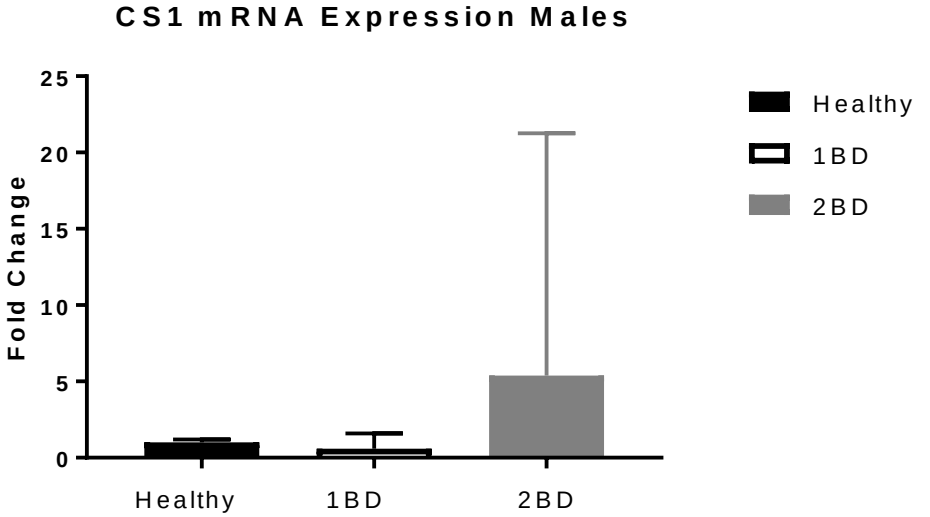

Figure 22: Comparison of CS1 mRNA expression in total PBMCs between male healthy subjects and male ALL subjects. Healthy n=11, ALL subjects samples from 1st blood draw n=18, ALL subjects samples from 2nd blood draw n=9. Bars represent the median, and the range was calculated using median absolute deviation.

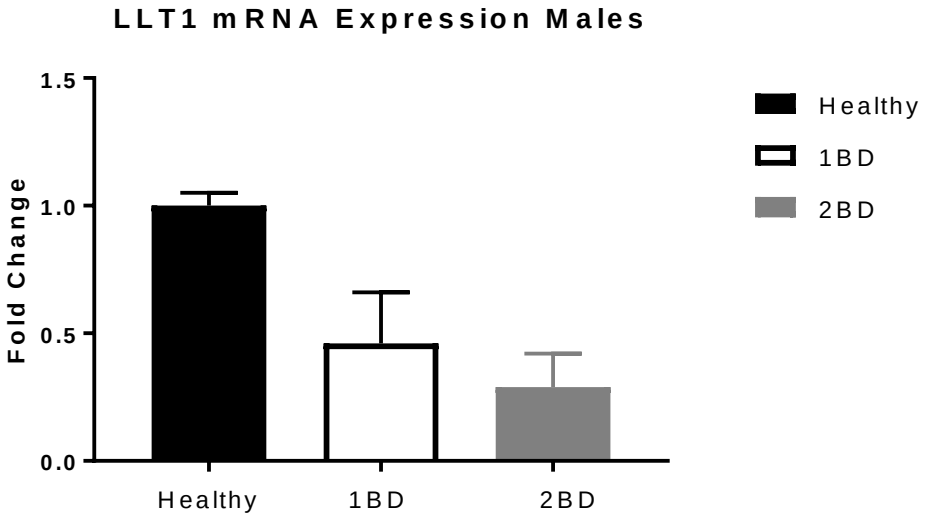

Figure 23: Comparison of LLT1 mRNA expression in total PBMCs between male healthy subjects and male ALL subjects. Healthy n=11, ALL subjects samples from 1st blood draw n=18, ALL subjects samples from 2nd blood draw n=9. Bars represent the median, and the range was calculated using median absolute deviation.

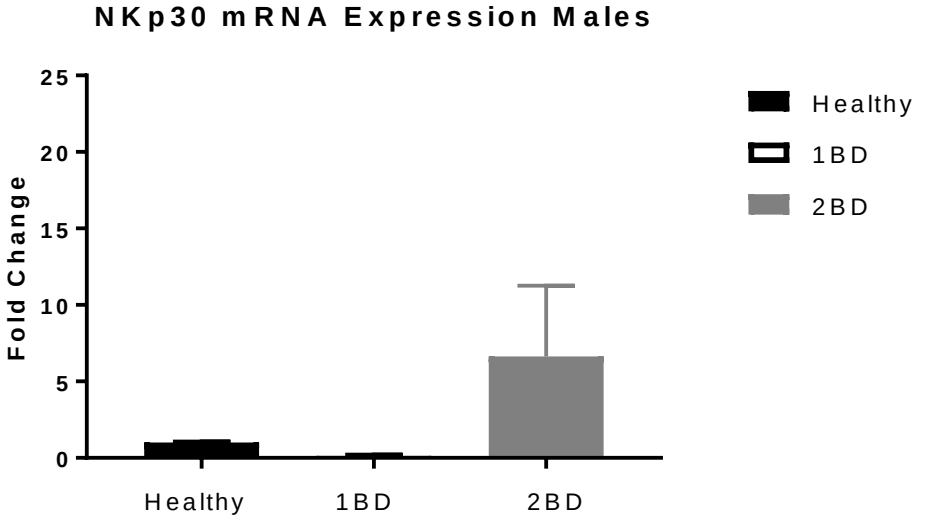

Figure 24: Comparison of NKp30 mRNA expression in total PBMCs between male healthy subjects and male ALL subjects. Healthy n=11, ALL subjects samples from 1st blood draw n=18, ALL subjects samples from 2nd blood draw n=9. Bars represent the median, and the range was calculated using median absolute deviation.

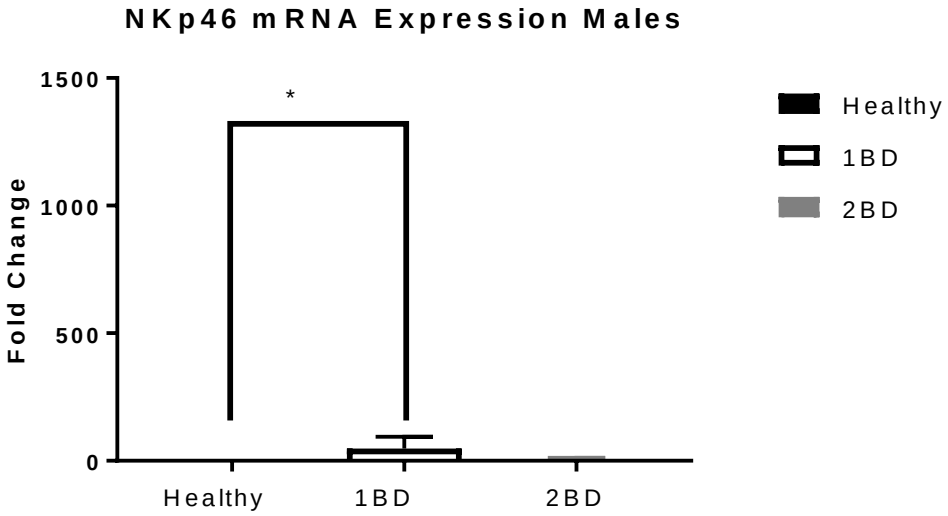

Figure 25: Comparison of NKp46 mRNA expression in total PBMCs between male healthy subjects and male ALL subjects. Healthy n=11, ALL subjects samples from 1st blood draw n=18, ALL subjects samples from 2nd blood draw n=9. * indicates a p value of less than 0.05. Bars represent the median, and the range was calculated using median absolute deviation.

Chapter IV

Surface Protein Expression of Immune Receptors in Healthy Subjects and Subjects with Pediatric Acute Lymphoblastic Leukemia

Purpose of Aim

The purpose of this aim was to determine the surface protein expression of immune receptors of healthy subjects and subjects with pediatric ALL. Cell surface expression was determined by flow cytometry on isolated PBMCs of healthy subjects and ALL subjects. For the ALL subjects blood samples were collected at two time points, the first blood draw (1BD) was at the time of diagnosis, and the second blood draw (2BD) was 29 days after initial chemotherapy treatment. If immune receptor expression of ALL subjects is found to be different from healthy subjects, this could indicate that the function of these receptors play a role in the onset or progression of this disease.

4.1 Percent Expression of Receptors

Although the results from the qRT-PCR are interesting, I cannot assume that mRNA expression automatically translates to protein expression. Likewise, the protein may get expressed, but it may remain intracellular. Immune cells rely on cell to cell contact and receptor-ligand interaction regulate cell function. To determine the cell surface expression, I employed flow cytometry.

The purpose of flow cytometry was to evaluate the cell surface expression of the receptors of interest. A Beckman Coulter FC500 was used and the population of immune cells were gated by forward and side scatter to separate the lymphocytes and monocytes from other cells by size and granularity. Various fluorochrome markers were used to differentiate: CD3 for T cells, CD19 for B cells, CD56 for NK cells, and CD14 for monocytes. These fluorochromes are used to further gate the acquired cells and differentiate the cell types. This is one huge advantage over the PCR analysis as those results show total PBMCs, while flow cytometry is able to analyze each cell type separately. Each immune receptor of interest was labeled with PE. Samples were stained with anti-2B4, anti-CS1, anti LLT1, anti-NKp30 and anti-NKp46 mAb to determine percentage and quantitative cell surface expression of these receptors on each immune cell type. In this section, I also examined CD161, the ligand for LLT1, and CD48, the ligand for 2B4. This is because a change in expression of the ligands for 2B4 and LLT1 could potentially affect the function of these receptors.

I analyzed the flow cytometry data in two ways: by percentage of receptor expression and by mean fluorescence intensity (MFI). MFI will be discussed in section 4.2. Percent receptor expression gives insight as to what percentage of cells collected express the receptor of interest. Percent expression does not differentiate by how much each cell expresses, only whether there is expression or there is not expression. The data is represented on a dot plot and the color code for the first blood draw and second blood draw correlate with the same patient. Black data points have no counterpart. This was done to analyze trends between the data sets.

Figures 31 and 33 show that CS1 is significantly overexpressed on T cells and monocytes of ALL subjects first blood draw compared to healthy subjects. This is interesting considering the recent findings of overexpressed CS1 in multiple myeloma discussed above. Figure 36 shows that LLT1 is overexpressed on NK cells in the first blood draw subjects compared to the healthy subjects. Interestingly, figures 38,39 and 41 show that NKp30 is overexpressed on B cells, T cells and monocytes but not NK cells of the first blood draw compared to healthy subjects. NKp30 is a known activating receptor of NK cells that has been studied in other leukemias. NKp46 was also shown to be overexpressed on T cells and monocytes (figures 43 and 45 respectively), but not NK cells. CD161, the ligand for LLT1, was greatly increased on B cells, T cells, and monocytes in the ALL subject first blood draw compared with healthy subjects as seen in figures 46, 47 and 49. When CD161 ligates with LLT1 on a NK cell, the NK cell gets activated to secrete interferon-gamma. However, the overexpression of CD161 on ancillary cells that are not the cancer targets may be overstimulating the NK cells causing an anergic effect as is known to happen when an activating receptor is overstimulated.[44] CD48, the ligand of 2B4, was overexpressed on B cells of ALL subjects second blood draw after treatment. This is interesting because it is the only significant change that occurs after ALL subjects have undergone

treatment. It is yet to be seen if this is a beneficial change or not. Intuitively, it would appear beneficial because more CD48 would ligate more 2B4 on NK cells, causing more activation. However, as stated above this may also produce an anergic effect.[44] Finally, in figure 51, CD48 is overexpressed on T cells in ALL subjects compared to healthy subjects.

4.2 Mean Fluorescence Intensity (MFI) of Cell Surface

Mean fluorescence intensity (MFI), was used to calculate immune receptor expression at the surface level. MFI measures the surface density of the receptor in question. As stated above, percentage expression identifies whether the receptor is expressed on the cell or not. MFI is a way to look at how much of the receptor is expressed. A low MFI means only a few of the receptors are expressed on a cell, while a high MFI means a large amount is expressed. Because NK cells specifically need more activating receptors to ligate than inhibiting receptors in order to produce an immune response, the amount of receptors expressed on a cell surface becomes very important. One drawback however is that MFI is very susceptible to error. Only a few of the MFI graphs that follow show statistical significance, however it is still very useful to look at trends that may show significance if a larger sample size is obtained.

Figure 56 and 64 show a trend that NK cells express less surface density of 2B4 and LLT1 respectively. This is an important trend as it could indicate why NK cells are not achieving the proper activation signals in ALL. Figure 78 also shows a slight increase in CD48 on B cells. Since ALL is a cancer of precursor B cells, this overexpression of CD48 on the cancer cells could potentially be very enlightening if the sample size is increased.

When MFI was broken down into the age groups 0-11 and 12-21, I found the same trends taking place, except for a few exceptions that are worth mentioning. In figures 81,82, and 83, CD48 is increased significantly in the first blood draw of ALL subjects ages 0-11.

The flow cytometry analysis confirms that there is altered expression of immune receptors between healthy subjects and ALL subjects. The next step is to confirm if this change in expression translates into a change of function.

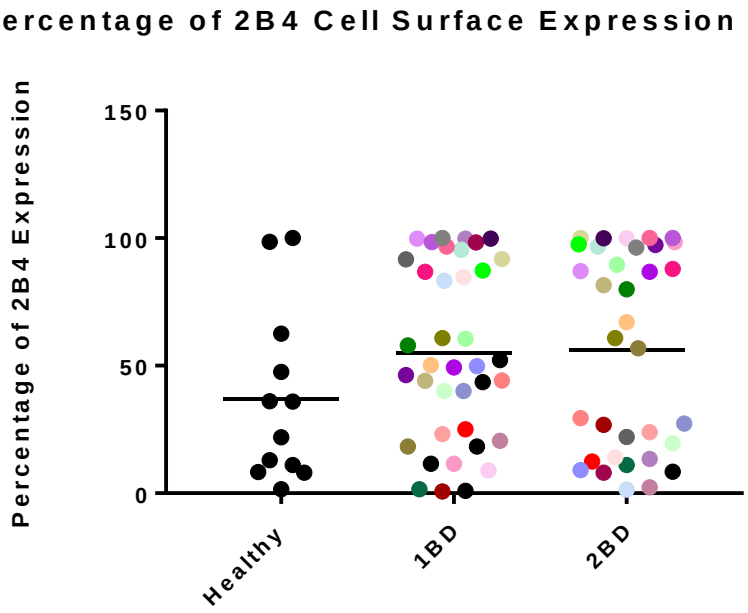

Figure 26: Comparison of percentage of 2B4 cell surface expression on B cells between healthy subjects n=12, ALL subjects sample first blood draw n=38, and second blood draw n=34. Lines represent the mean.

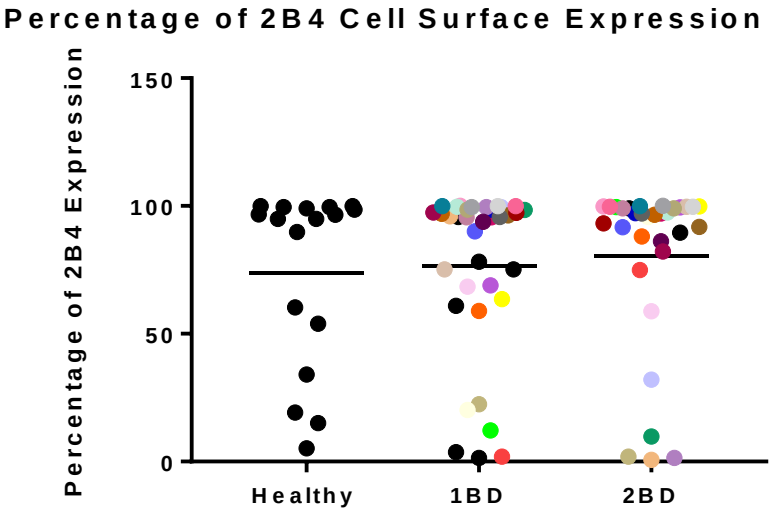

Figure 27: Comparison of percentage of 2B4 cell surface expression on T cells between healthy subjects n=17, ALL subjects sample first blood draw n=36, and second blood draw n=32. Lines represent the mean.

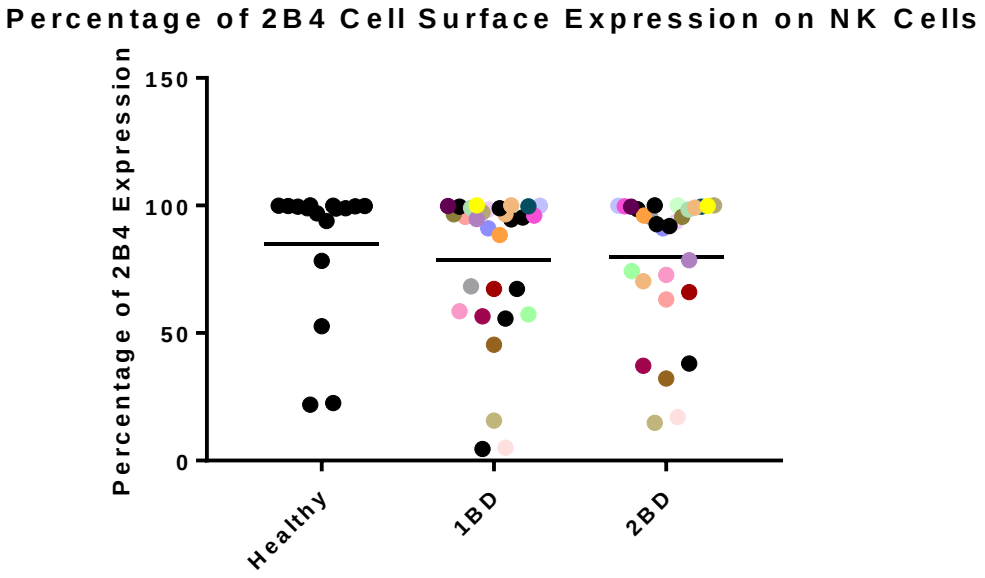

Figure 28: Comparison of percentage of 2B4 cell surface expression on NK cells between healthy subjects n=16, ALL subjects sample first blood draw n=31, and second blood draw n=29. Lines represent the mean.

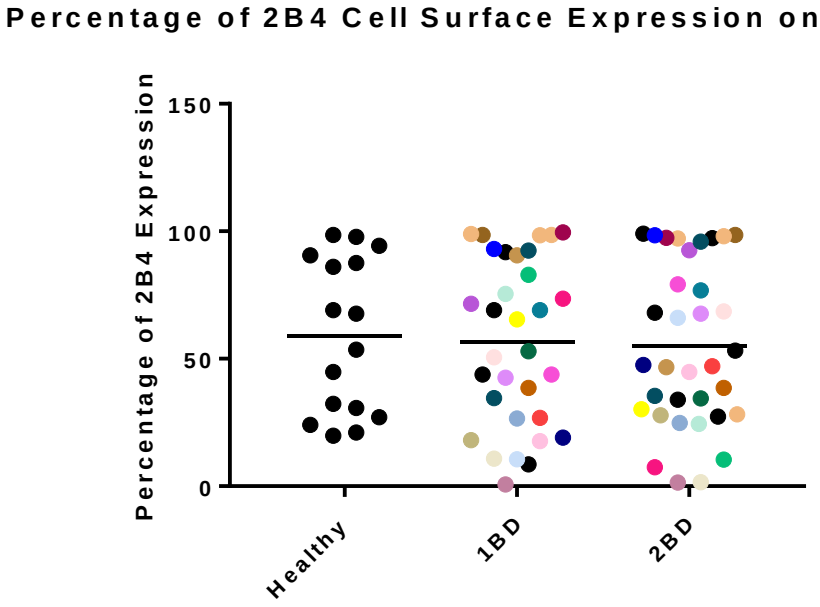

Figure 29: Comparison of percentage of 2B4 cell surface expression on monocytes between healthy subjects n=16, ALL subjects sample first blood draw n=32, and second blood draw n=34. Lines represent the mean.

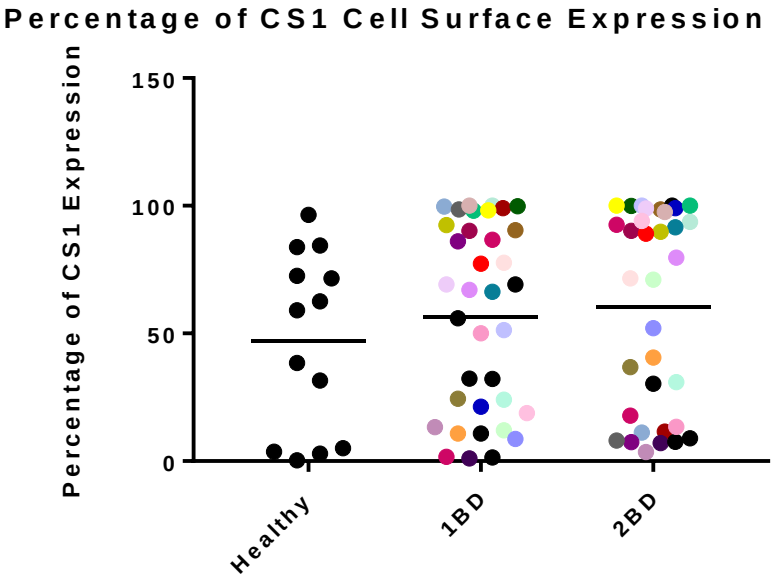

Figure 30: Comparison of percentage of CS1 cell surface expression on B cells between healthy subjects n=13, ALL subjects sample first blood draw n=36, and second blood draw n=34. Lines represent the mean.

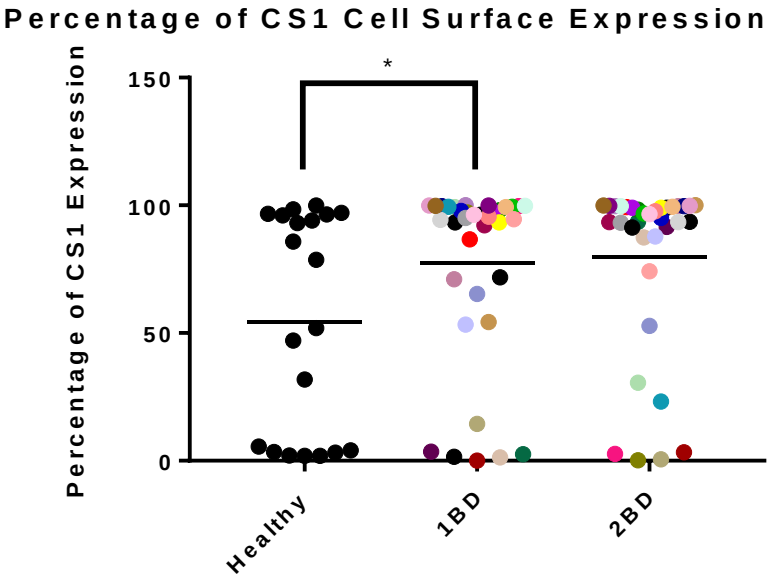

Figure 31: Comparison of percentage of CS1 cell surface expression on T cells between healthy subjects n=20, ALL subjects sample first blood draw n=37, and second blood draw n=35. * indicates a p value < 0.05. Lines represent the mean.

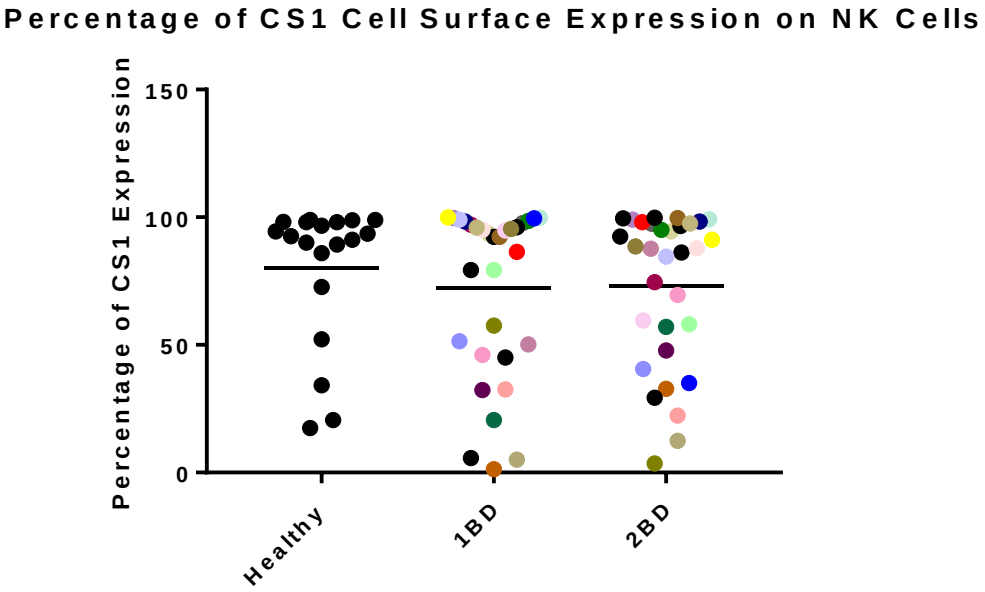

Figure 32: Comparison of percentage of CS1 cell surface expression on NK cells between healthy subjects n=19, ALL subjects sample first blood draw n=31, and second blood draw n=32. Lines represent the mean.

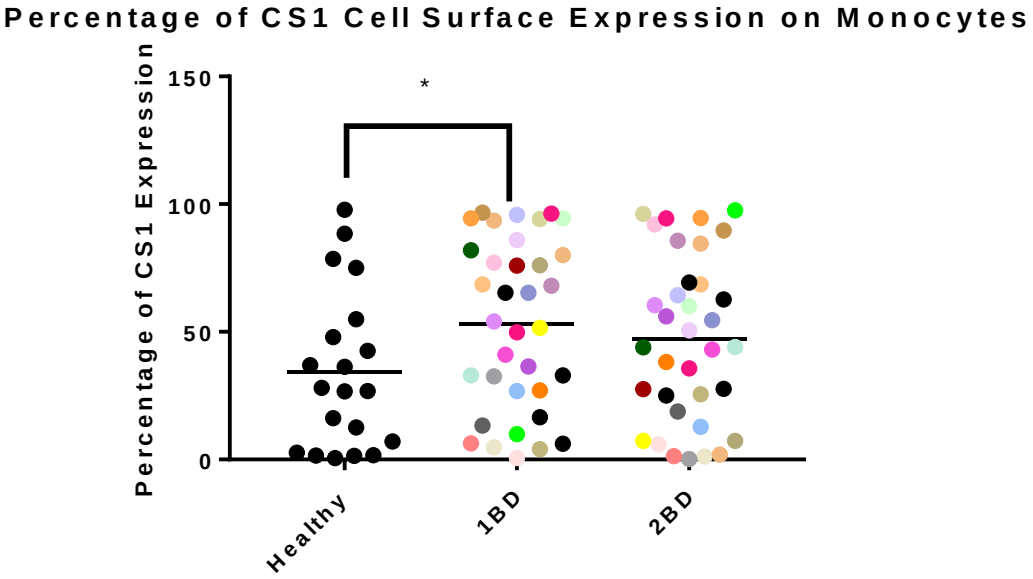

Figure 33: Comparison of percentage of CS1 cell surface expression on monocytes between healthy subjects n=20, ALL subjects sample first blood draw n=35, and second blood draw n=35. * indicates a p value < 0.05. Lines represent the mean.

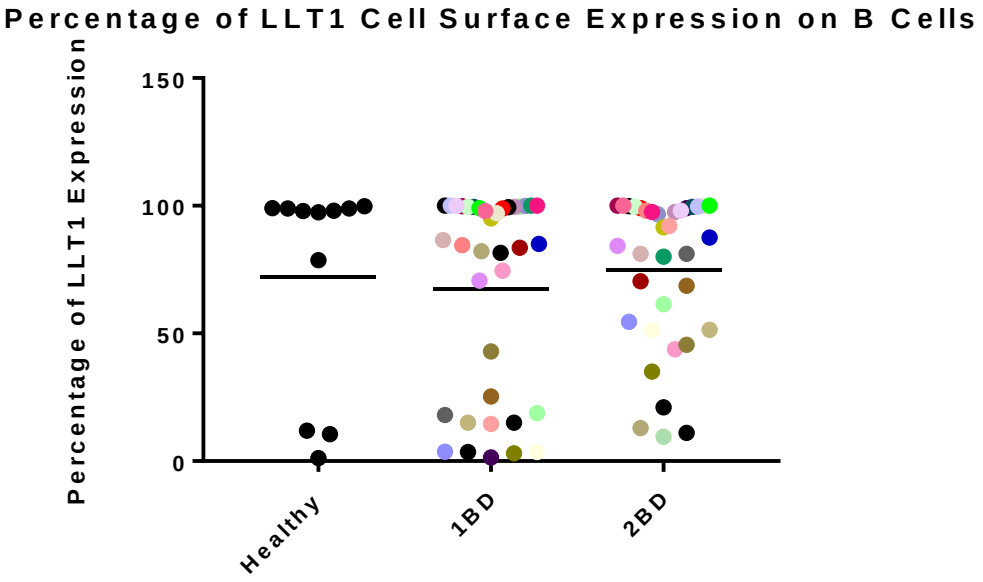

Figure 34: Comparison of percentage of LLT1 cell surface expression on B cells between healthy subjects n=11, ALL subjects sample first blood draw n=37, and second blood draw n=35. Lines represent the mean.

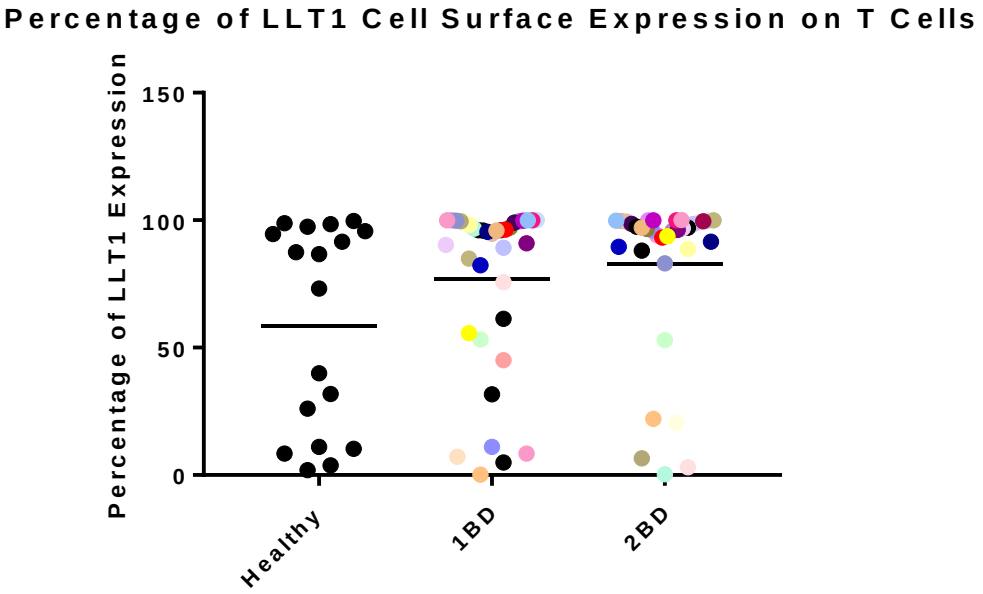

Figure 35: Comparison of percentage of LLT1 cell surface expression on T cells between healthy subjects n=18, ALL subjects sample first blood draw n=37, and second blood draw n=35. Lines represent the mean.

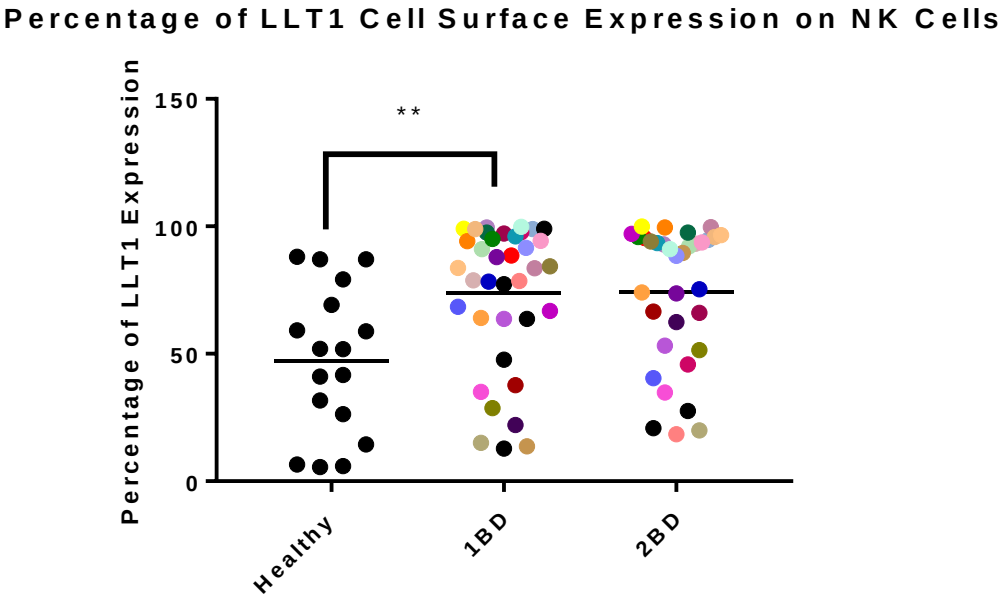

Figure 36: Comparison of percentage of LLT1 cell surface expression on NK cells between healthy subjects n=17, ALL subjects sample first blood draw n=37, and second blood draw n=34. ** indicates a p value of < 0.005. Lines represent the mean.

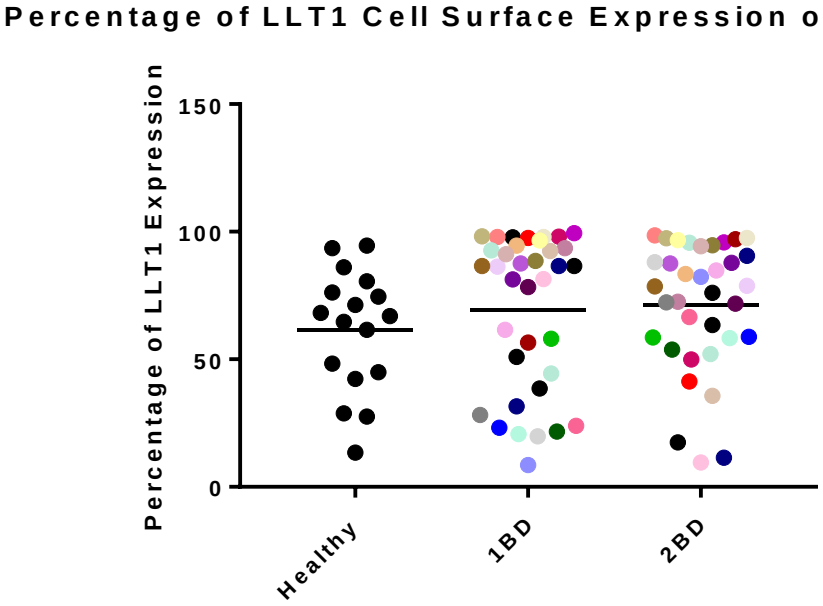

Figure 37: Comparison of percentage of LLT1 cell surface expression on monocytes between healthy subjects n=17, ALL subjects sample first blood draw n=36, and second blood draw n=35. Lines represent the mean.

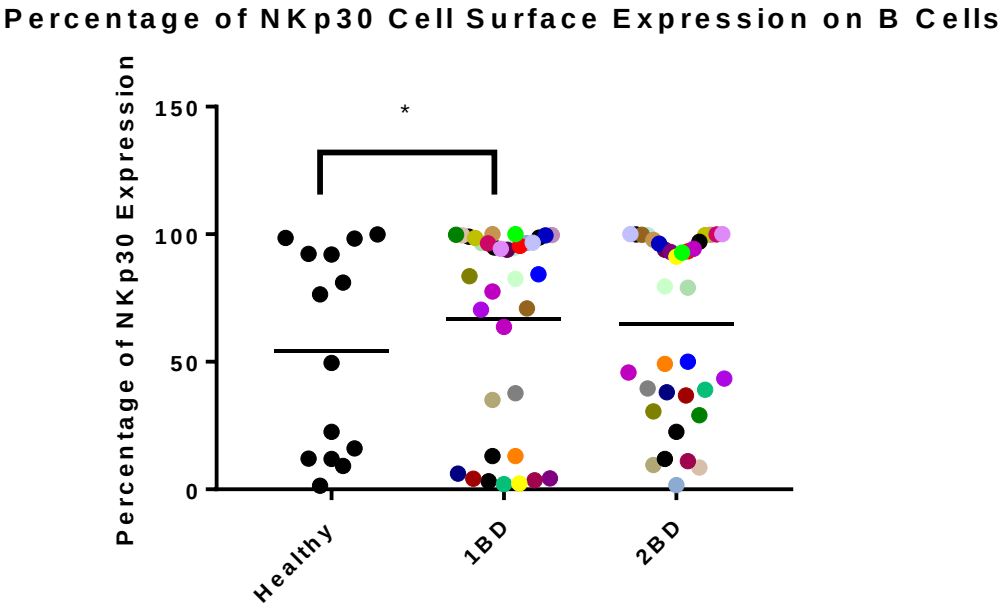

Figure 38: Comparison of percentage of NKp30 cell surface expression on B cells between healthy subjects n=14, ALL subjects sample first blood draw n=36, and second blood draw n=35. * indicates a p value < 0.05. Lines represent the mean.

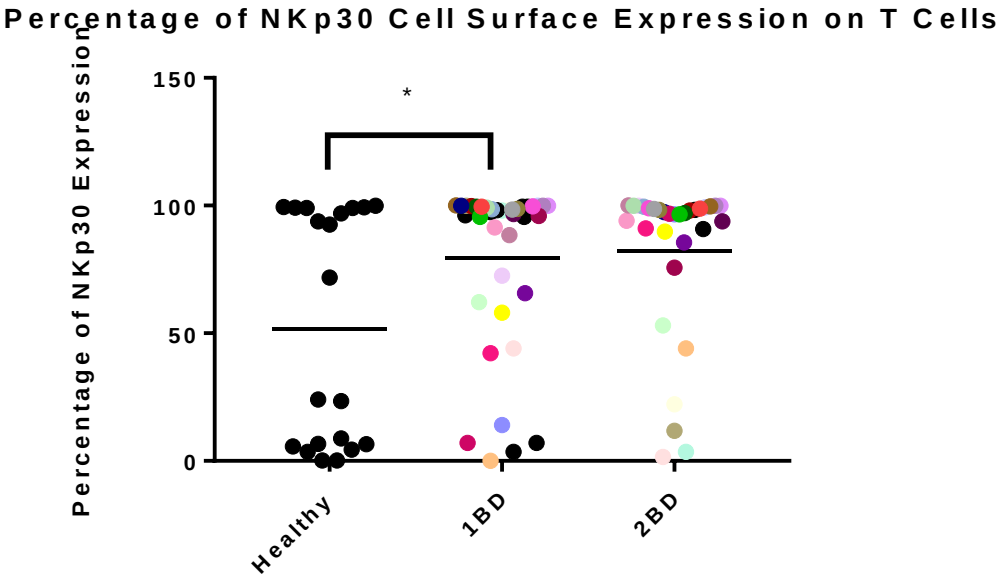

Figure 39: Comparison of percentage of NKp30 cell surface expression on T cells between healthy subjects n=20, ALL subjects sample first blood draw n=38, and second blood draw n=32. * indicates a p value < 0.05. Lines represent the mean.

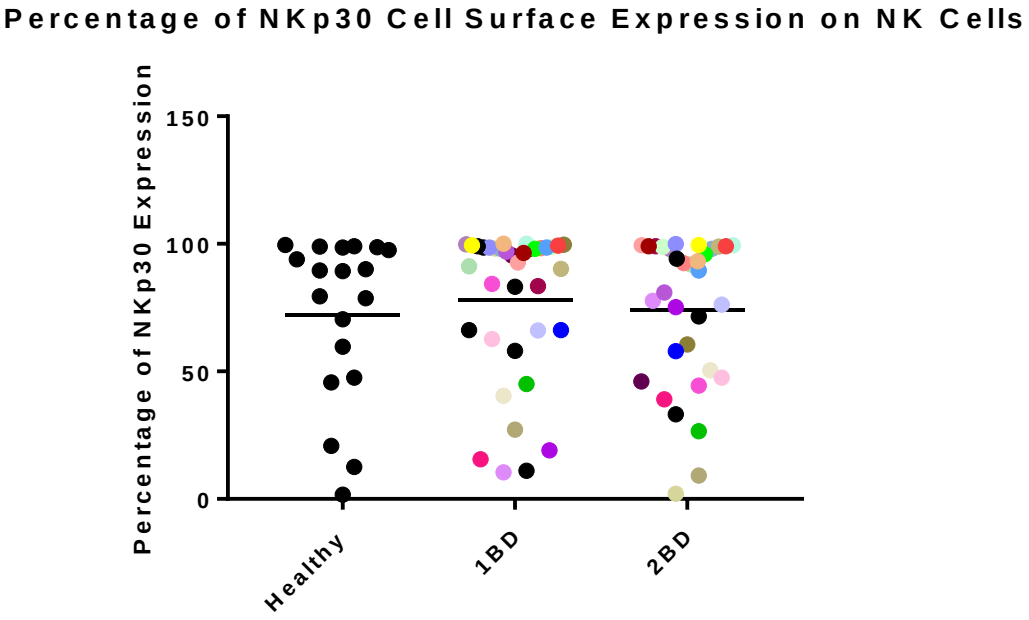

Figure 40: Comparison of percentage of NKp30 cell surface expression on NK cells between healthy subjects n=19, ALL subjects sample first blood draw n=37, and second blood draw n=33. Lines represent the mean.

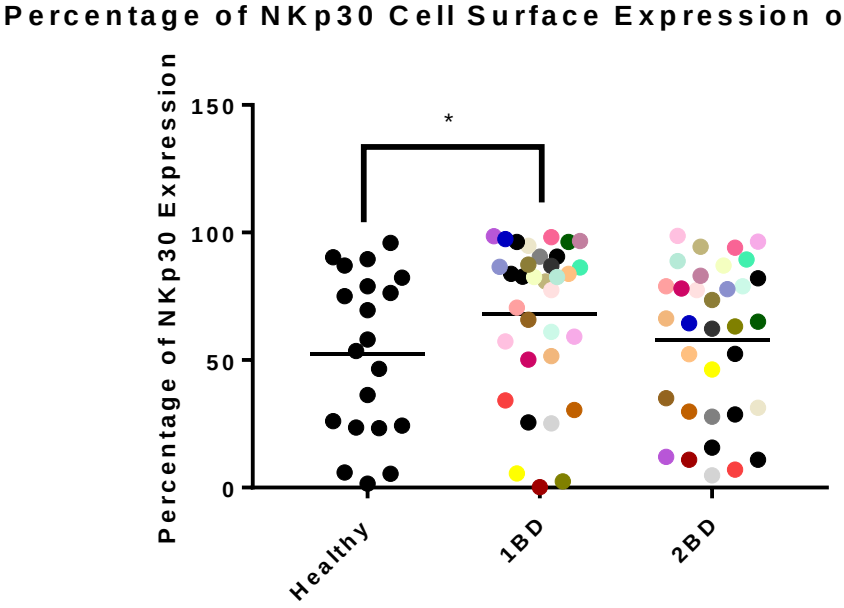

Figure 41: Comparison of percentage of NKp30 cell surface expression on monocytes between healthy subjects n=19, ALL subjects sample first blood draw n=34, and second blood draw n=34. * indicates a p value < 0.05. Lines represent the mean.

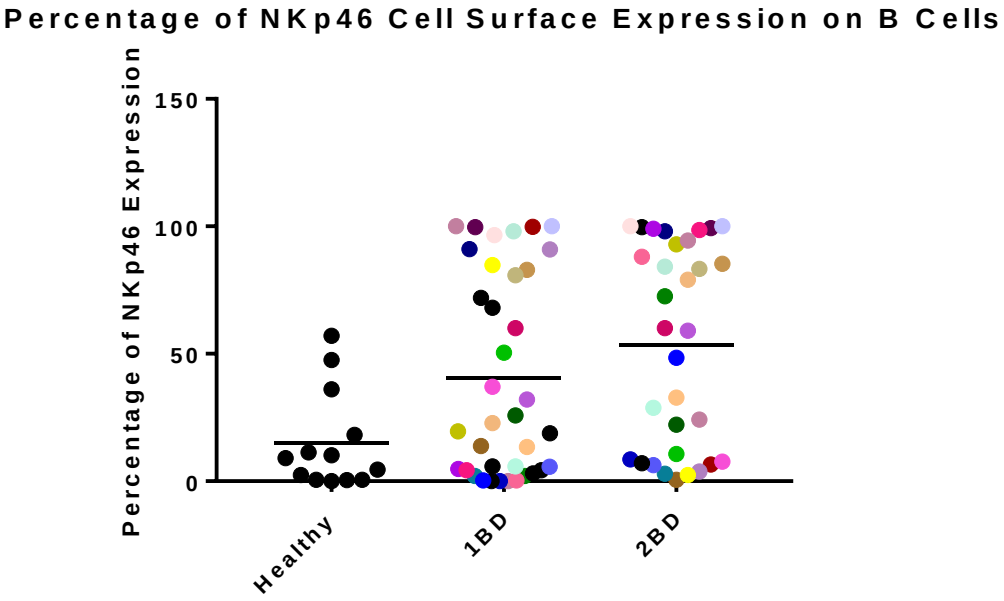

Figure 42: Comparison of percentage of NKp46 cell surface expression on B cells between healthy subjects n=13, ALL subjects sample first blood draw n=37, and second blood draw n=32. Lines represent the mean.

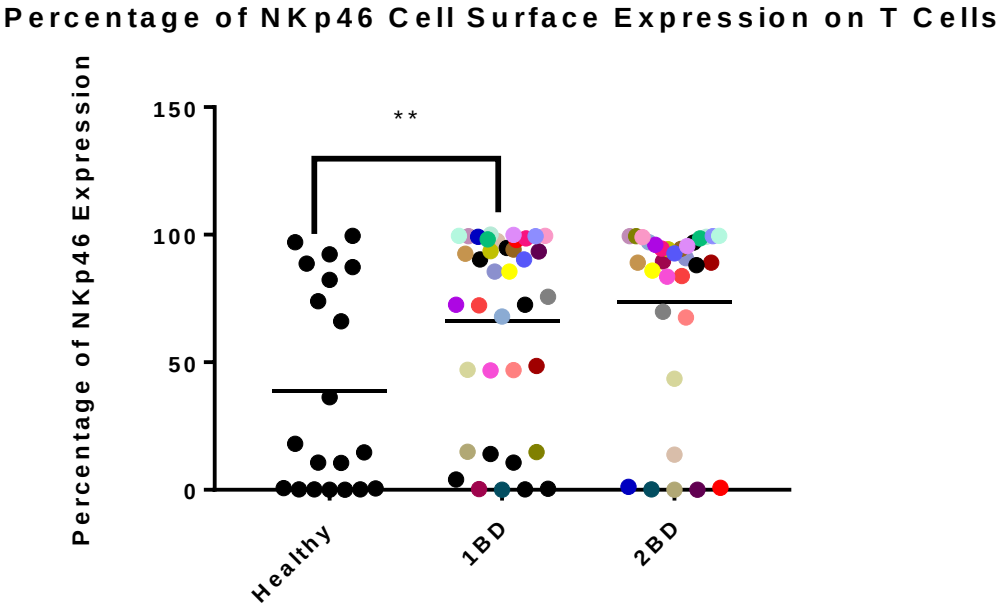

Figure 43: Comparison of percentage of NKp46 cell surface expression on T cells between healthy subjects n=20, ALL subjects sample first blood draw n=38, and second blood draw n=32. ** indicates a p value < 0.005. Lines represent the mean.

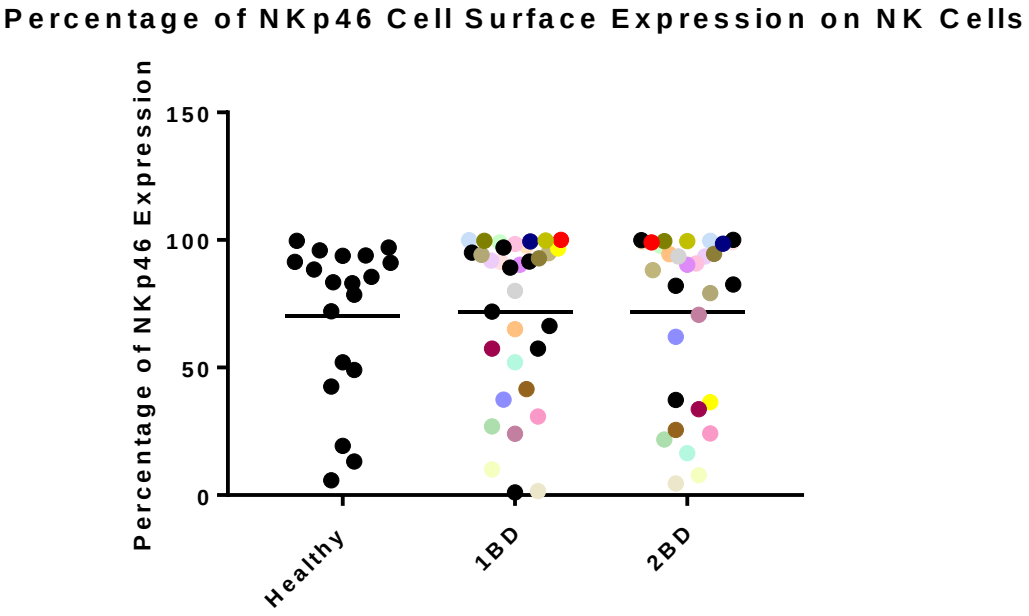

Figure 44: Comparison of percentage of NKp46 cell surface expression on NK cells between healthy subjects n=19, ALL subjects sample first blood draw n=34, and second blood draw n=31. Lines represent the mean.

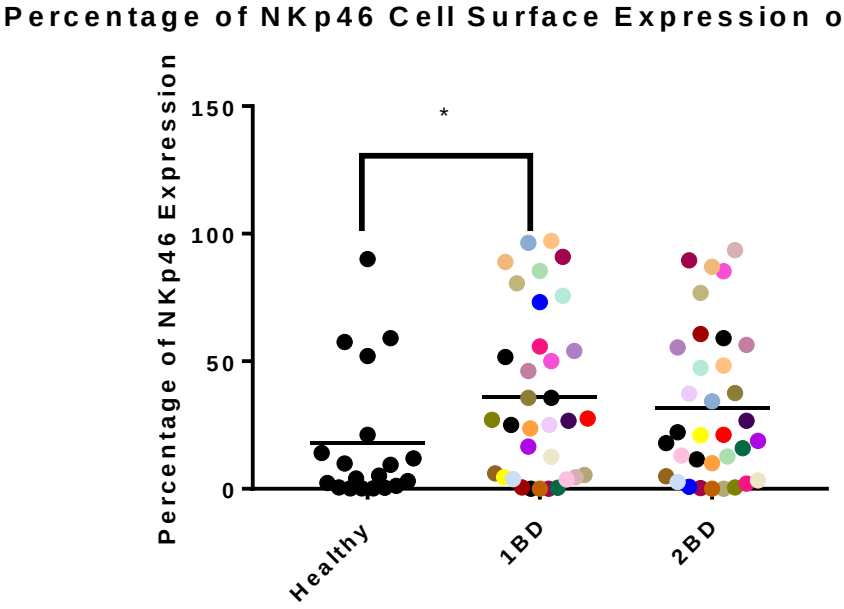

Figure 45: Comparison of percentage of NKp46 cell surface expression on monocytes between healthy subjects n=19, ALL subjects sample first blood draw n=34, and second blood draw n=34. * indicates a p value < 0.05. Lines represent the mean.

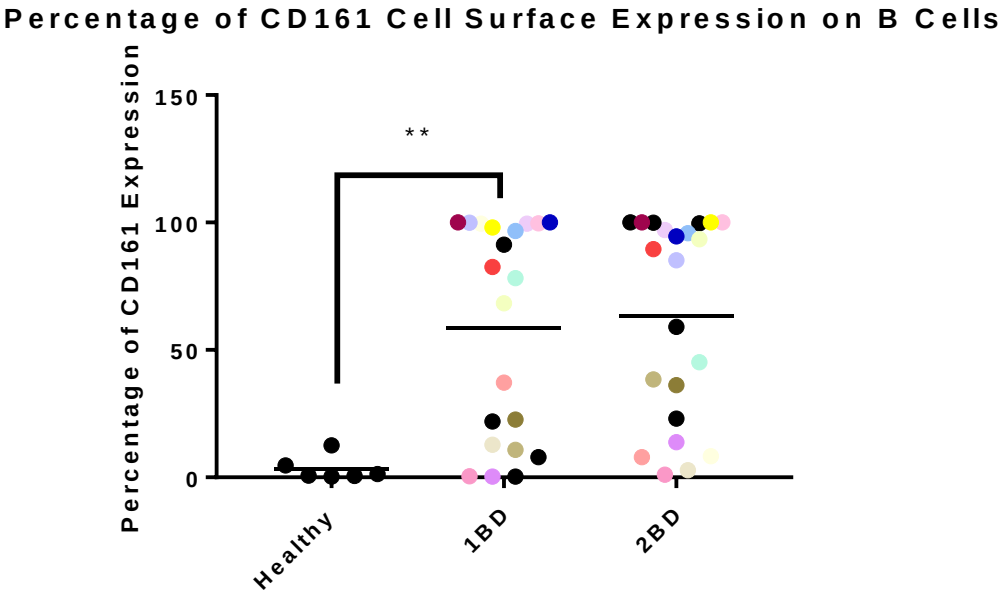

Figure 46: Comparison of percentage of CD161 cell surface expression on B cells between healthy subjects n=6, ALL subjects sample first blood draw n=21, and second blood draw n=22. ** indicates a p value < 0.005. Lines represent the mean.

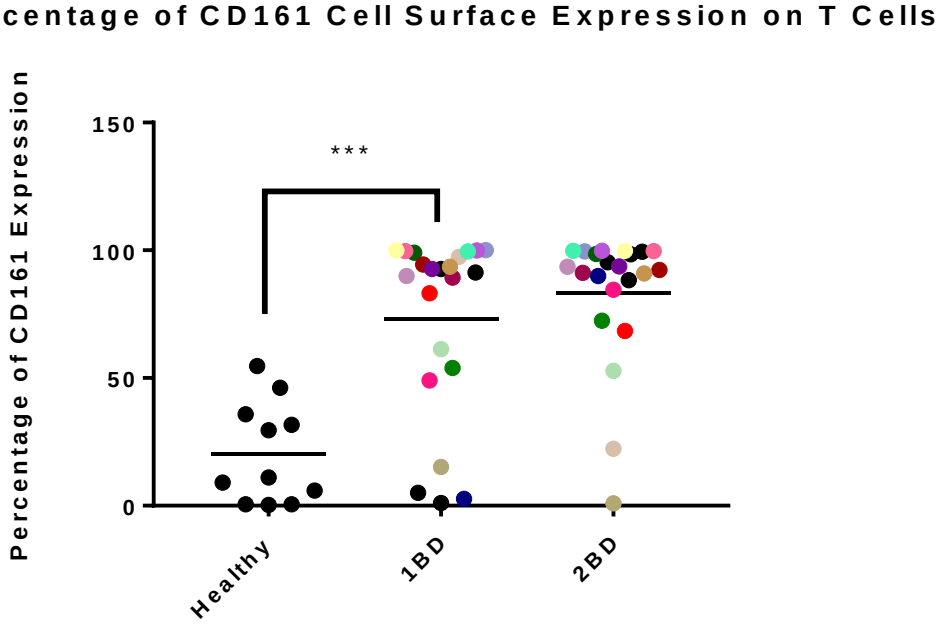

Figure 47: Comparison of percentage of CD161 cell surface expression on T cells between healthy subjects n=11, ALL subjects sample first blood draw n=22, and second blood draw n=22. *** indicates a p value < 0.0005. Lines represent the mean.

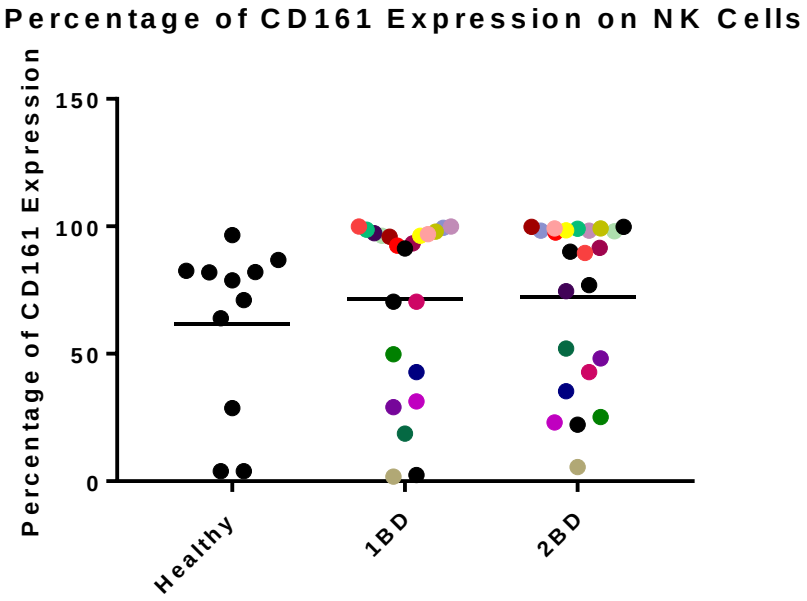

Figure 48: Comparison of percentage of CD161 cell surface expression on NK cells between healthy subjects n=11, ALL subjects sample first blood draw n=22, and second blood draw n=23. Lines represent the mean.

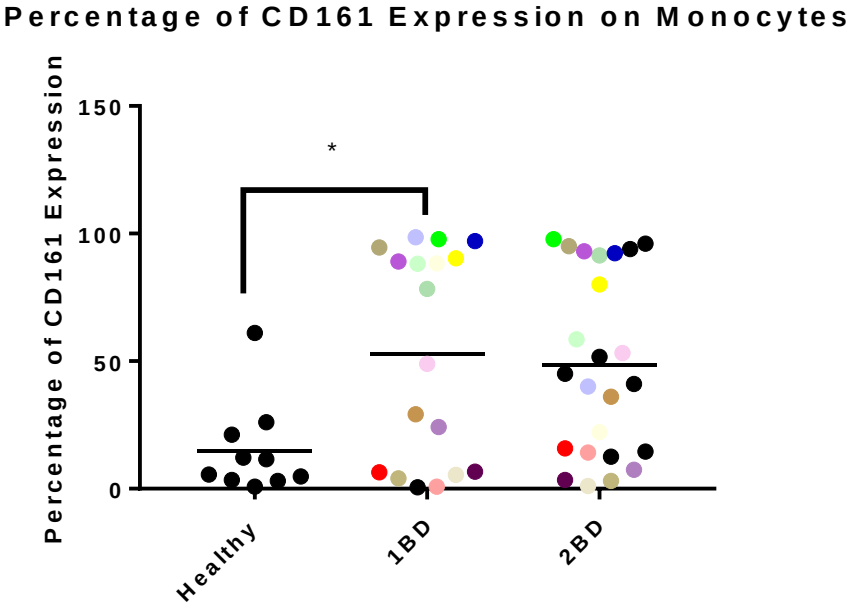

Figure 49: Comparison of percentage of CD161 cell surface expression on monocytes between healthy subjects n=10, ALL subjects sample first blood draw n=18, and second blood draw n=24. Lines represent the mean.

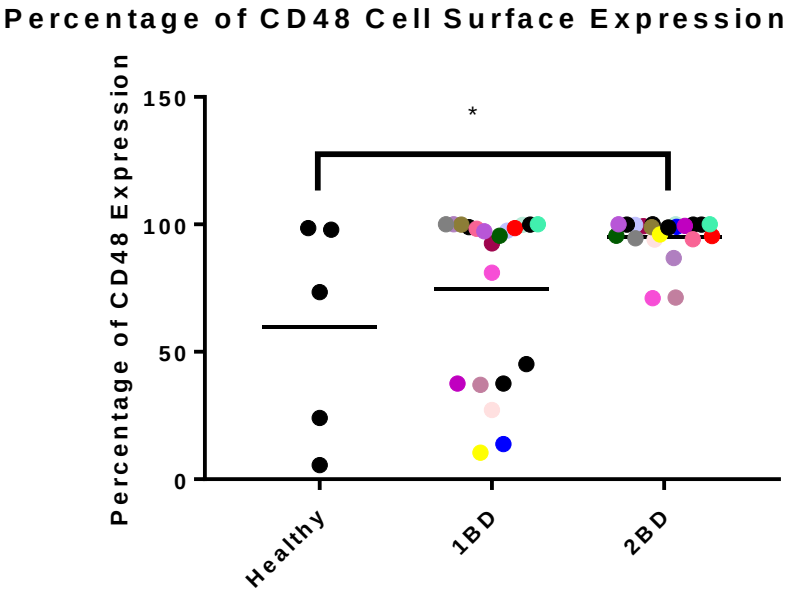

Figure 50: Comparison of percentage of CD48 cell surface expression on B cells between healthy subjects n=5, ALL subjects sample first blood draw n=21, and second blood draw n=22. * indicates a p value of < 0.05. Lines represent the mean.

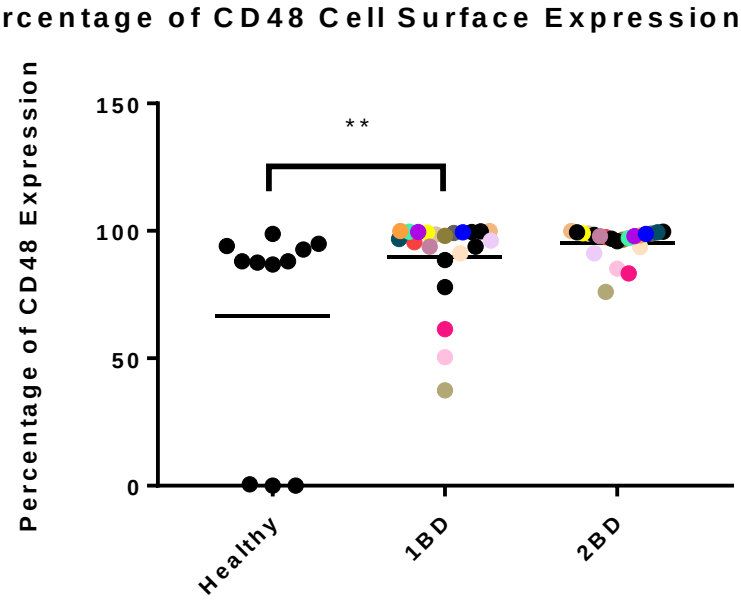

Figure 51: Comparison of percentage of CD48 cell surface expression on T cells between healthy subjects n=11, ALL subjects sample first blood draw n=22, and second blood draw n=22. ** indicates a p value of < 0.05. Lines represent the mean.

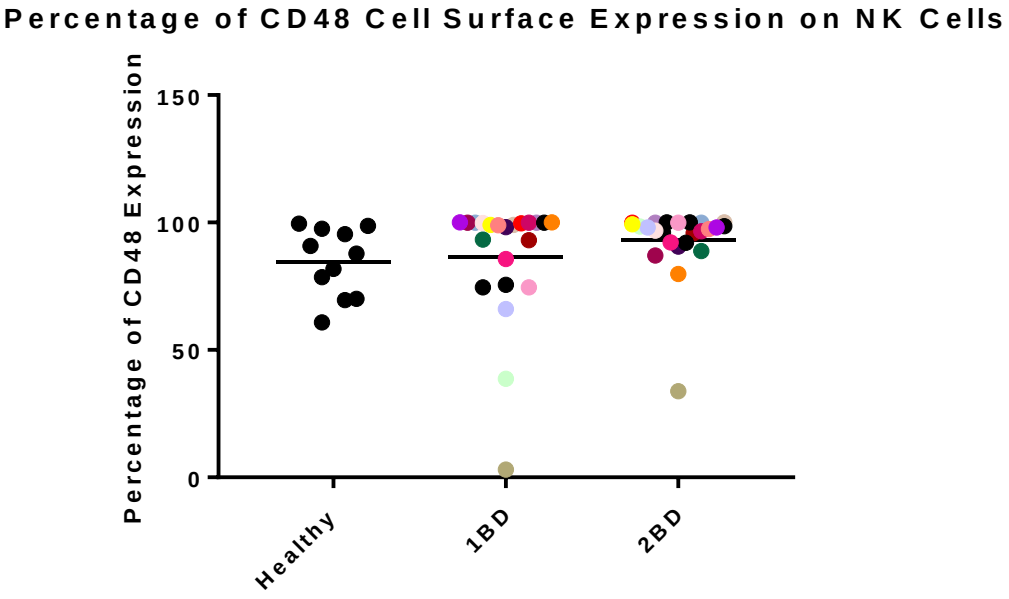

Figure 52: Comparison of percentage of CD48 cell surface expression on NK cells between healthy subjects n=11, ALL subjects sample first blood draw n=22, and second blood draw n=24. Lines represent the mean.

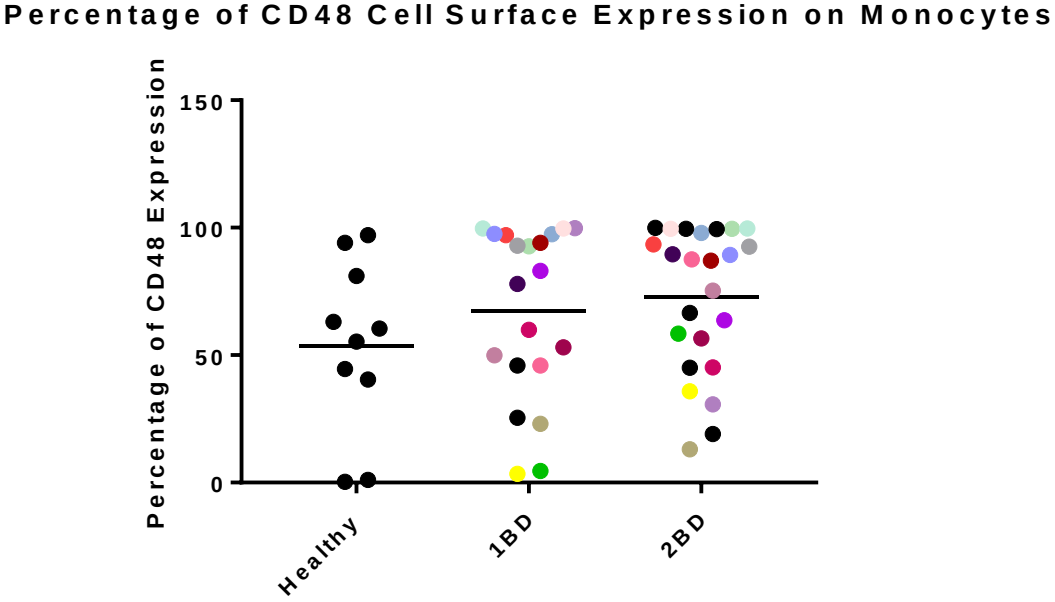

Figure 53: Comparison of percentage of CD48 cell surface expression on monocytes between healthy subjects n=10, ALL subjects sample first blood draw n=20, and second blood draw n=24. Lines represent the mean.

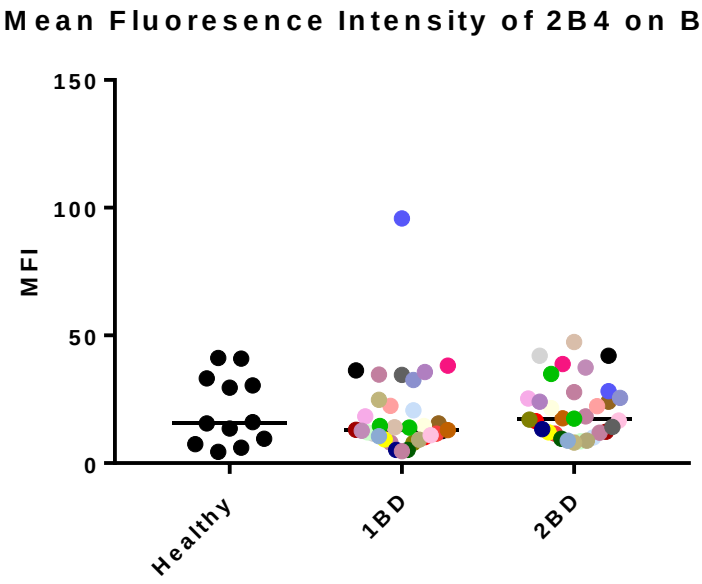

Figure 54: Comparison of mean fluorescence intensity (MFI) of 2B4 on B cells in healthy subjects n=12, patient subjects samples from first blood draw n=33, and second blood draw n=32. Line represents the median.

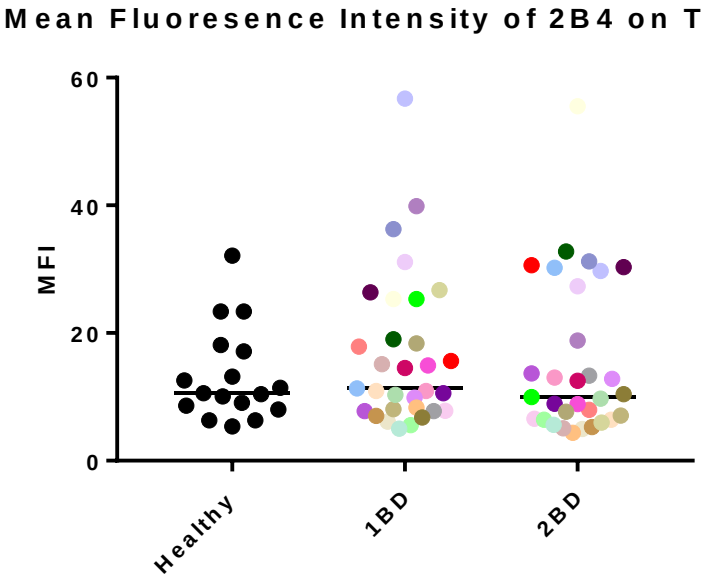

Figure 55: Comparison of mean fluorescence intensity (MFI) of 2B4 on T cells in healthy subjects n=17, patient subjects samples from first blood draw n=31, and second blood draw n=31. Line represents the median.

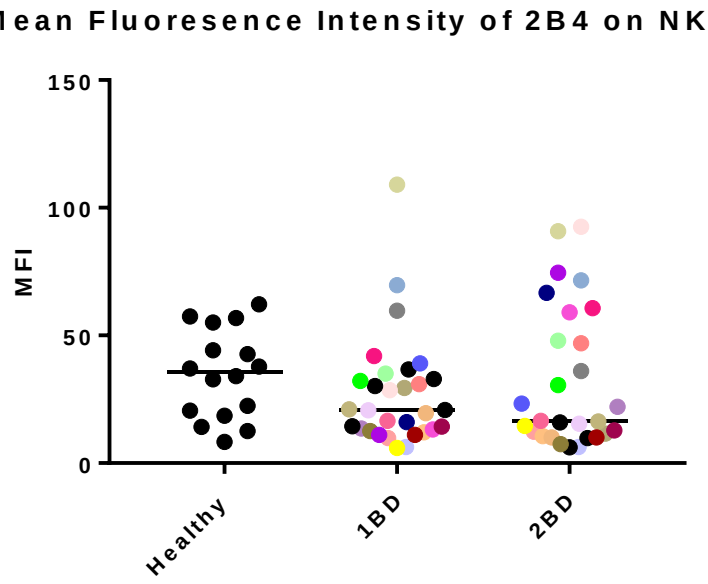

Figure 56: Comparison of mean fluorescence intensity (MFI) of 2B4 on NK cells in healthy subjects n=16, patient subjects samples from first blood draw n=30, and second blood draw n=28. Line represents the median.

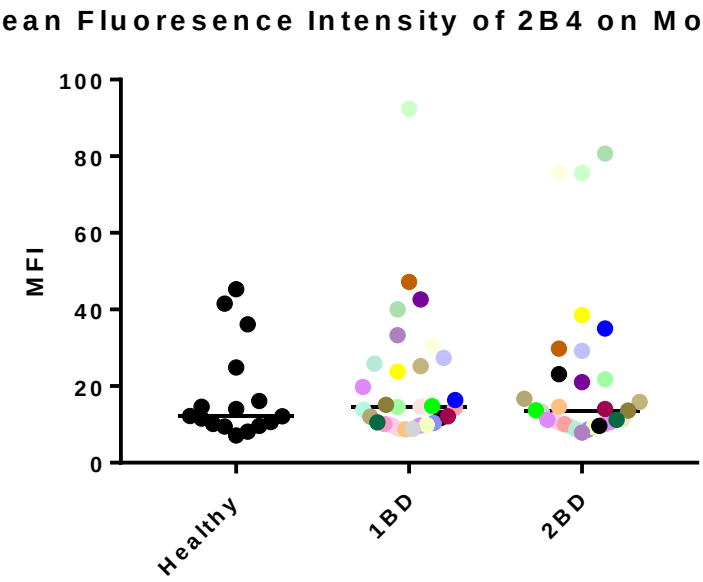

Figure 57: Comparison of mean fluorescence intensity (MFI) of 2B4 on monocytes in healthy subjects n=16, patient subjects samples from first blood draw n=30, and second blood draw n=31. Line represents the median.

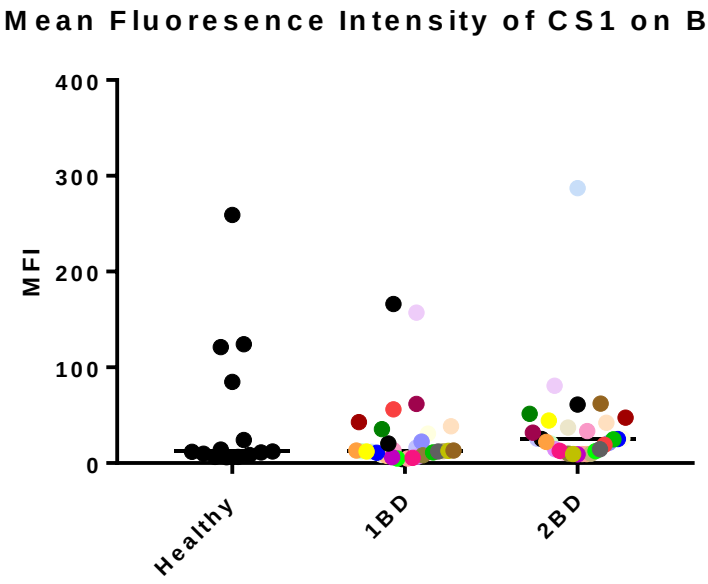

Figure 58: Comparison of mean fluorescence intensity (MFI) of CS1 on B cell in healthy subjects n=14, patient subjects samples from first blood draw n=32, and second blood draw n=31. Line represents the median.

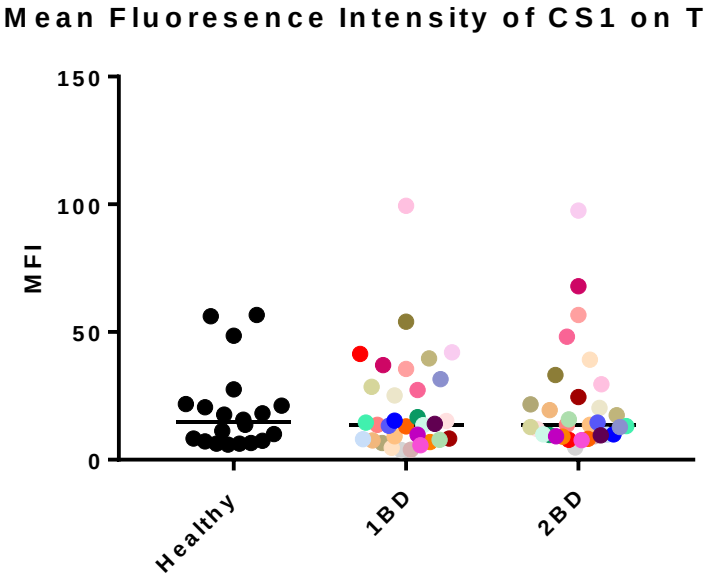

Figure 59: Comparison of mean fluorescence intensity (MFI) of CS1 on T cells in healthy subjects n=20, patient subjects samples from first blood draw n=32, and second blood draw n=32. Line represents the median.

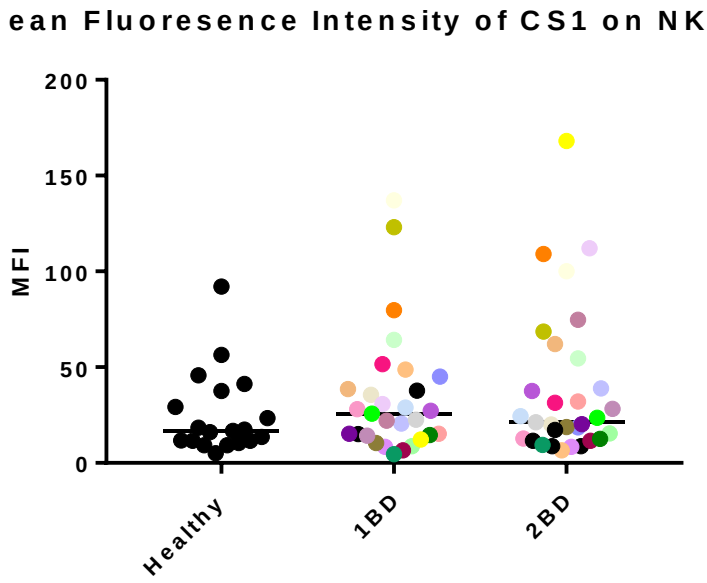

Figure 60: Comparison of mean fluorescence intensity (MFI) of CS1 on NK cells in healthy subjects n=19, patient subjects samples from first blood draw n=29, and second blood draw n=31. Line represents the median.

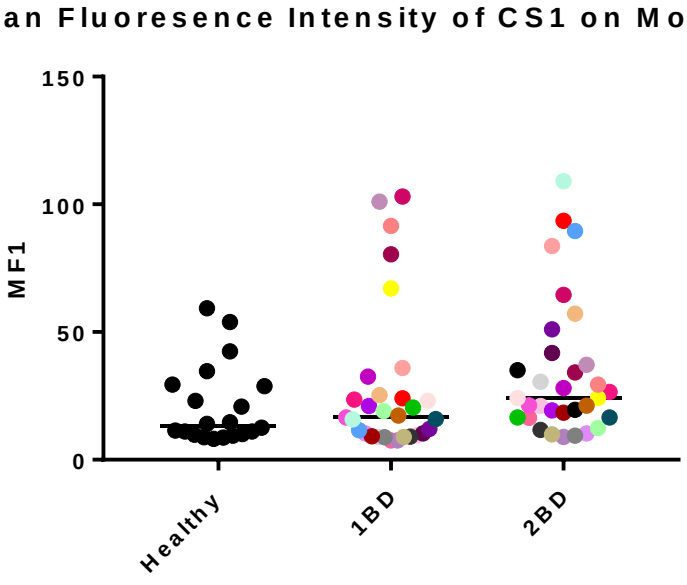

Figure 61: Comparison of mean fluorescence intensity (MFI) of CS1 on monocytes in healthy subjects n=20, patient subjects samples from first blood draw n=30, and second blood draw n=32. Line represents the median.

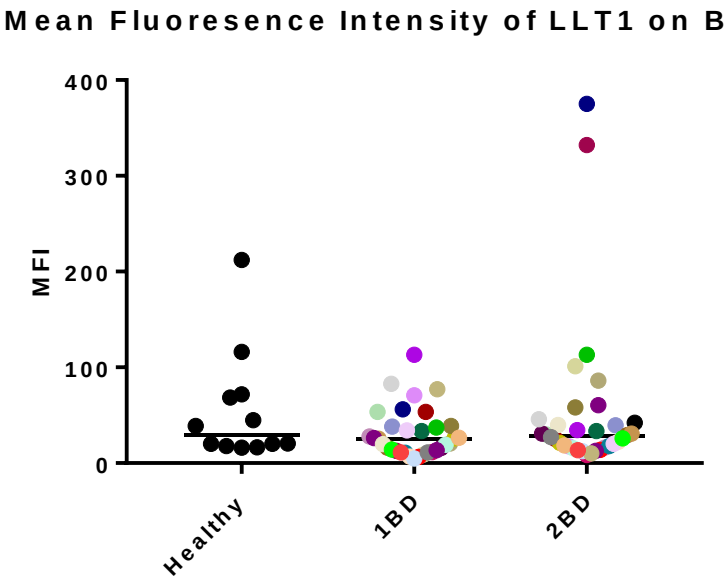

Figure 62: Comparison of mean fluorescence intensity (MFI) of LLT1 on B cells in healthy subjects n=12, patient subjects samples from first blood draw n=33, and second blood draw n=34. Line represents the median.

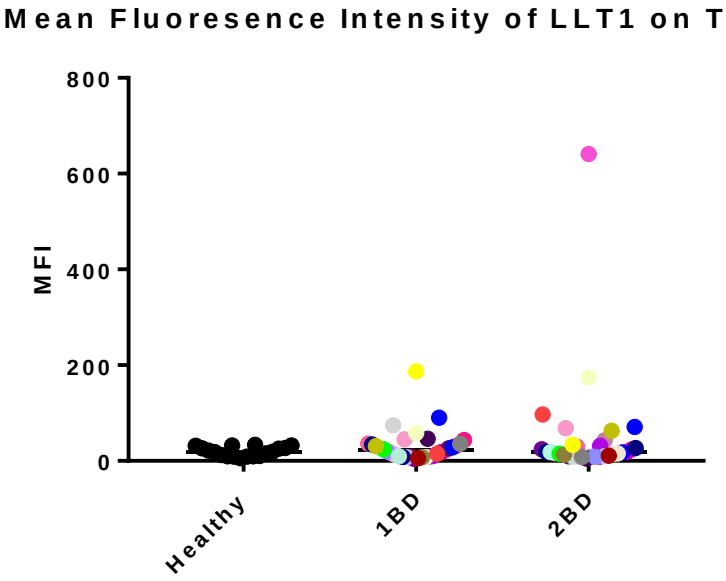

Figure 63: Comparison of mean fluorescence intensity (MFI) of LLT1 on T cells in healthy subjects n=18, patient subjects samples from first blood draw n=32, and second blood draw n=32. Line represents the median.

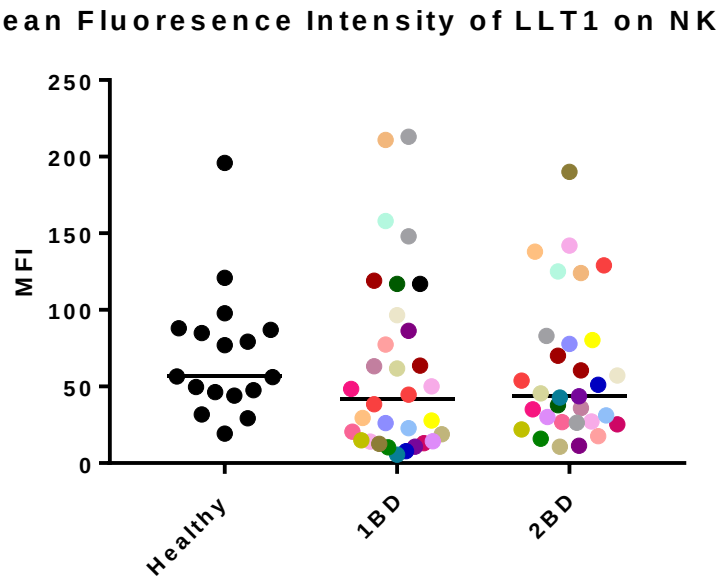

Figure 64: Comparison of mean fluorescence intensity (MFI) of LLT1 on NK cells in healthy subjects n=17, patient subjects samples from first blood draw n=32, and second blood draw n=31. Line represents the median.

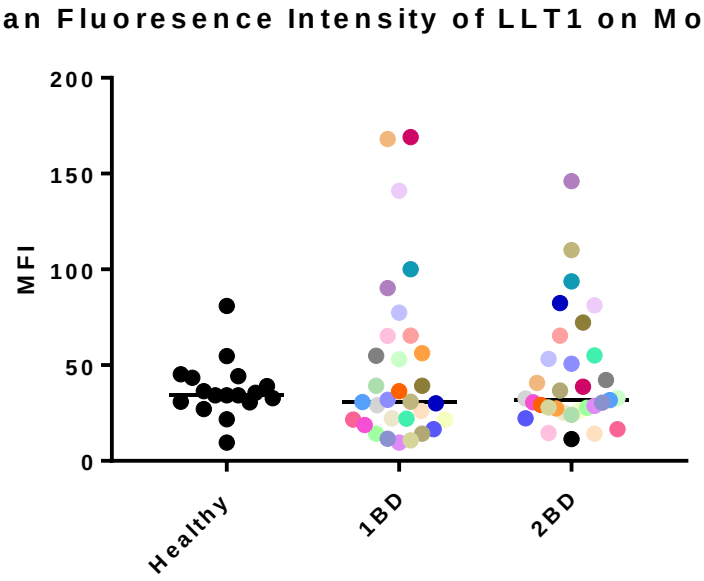

Figure 65: Comparison of mean fluorescence intensity (MFI) of LLT1 on monocytes in healthy subjects n=17, patient subjects samples from first blood draw n=31, and second blood draw n=32. Line represents the median.

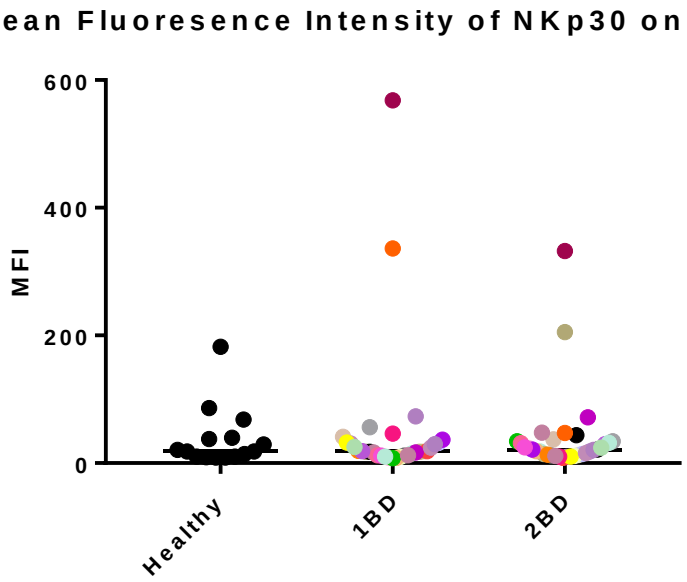

Figure 66: Comparison of mean fluorescence intensity (MFI) of NKp30 on B cells in healthy subjects n=15, patient subjects samples from first blood draw n=32, and second blood draw n=33. Line represents the median.

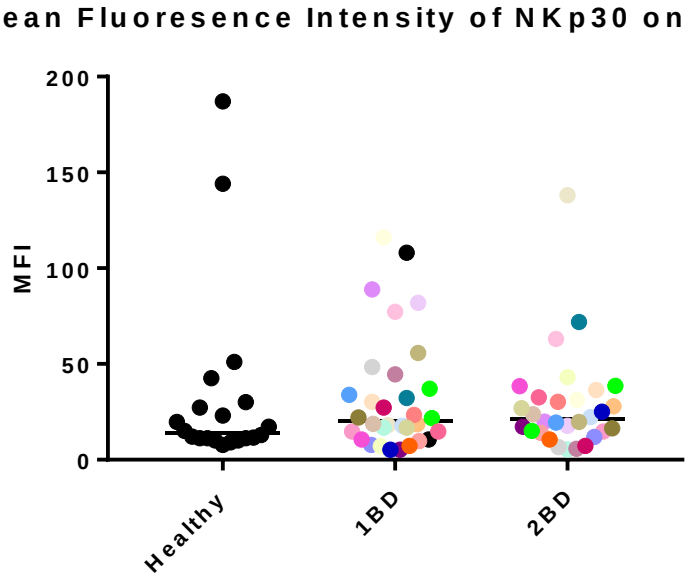

Figure 66: Comparison of mean fluorescence intensity (MFI) of NKp30 on T cells in healthy subjects n=20, patient subjects samples from first blood draw n=32, and second blood draw n=30. Line represents the median.

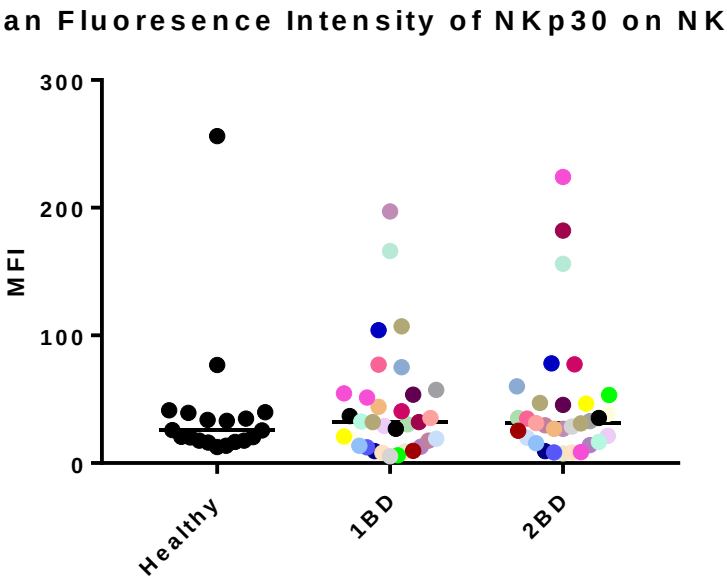

Figure 67: Comparison of mean fluorescence intensity (MFI) of NKp30 on NK cells in healthy subjects n=19, patient subjects samples from first blood draw n=33, and second blood draw n=32. Line represents the median.

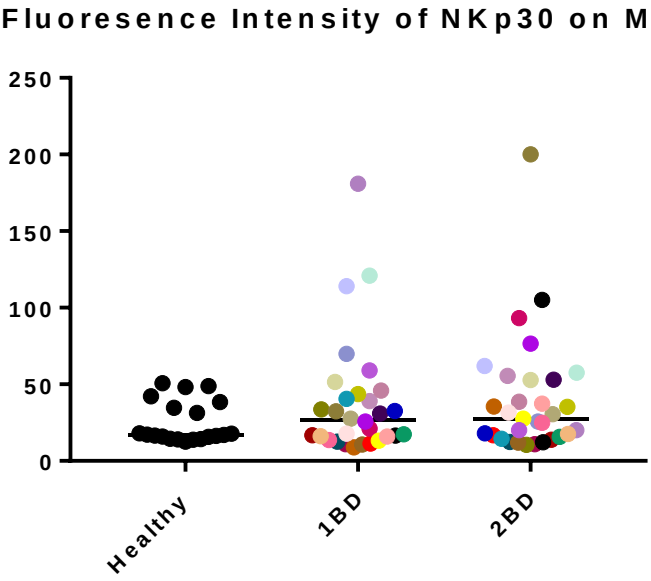

Figure 68: Comparison of mean fluorescence intensity (MFI) of NKp30 on monocytes in healthy subjects n=20, patient subjects samples from first blood draw n=30, and second blood draw n=31. Line represents the median.

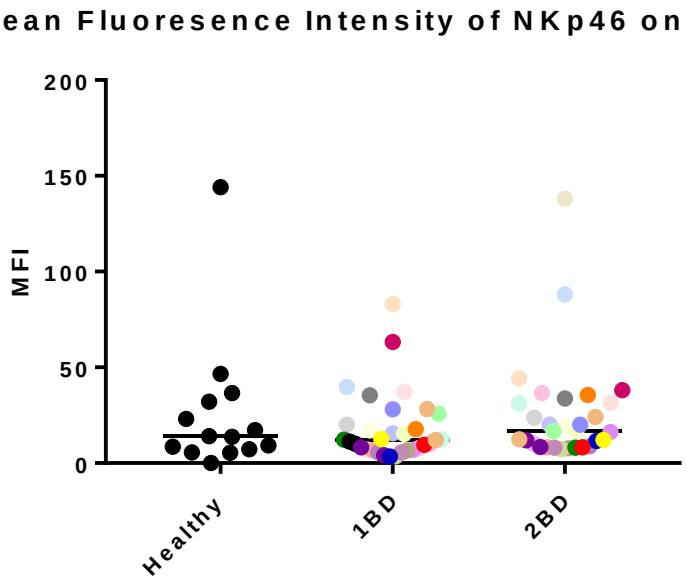

Figure 69: Comparison of mean fluorescence intensity (MFI) of NKp46 on B cells in healthy subjects n=14, patient subjects samples from first blood draw n=32, and second blood draw n=30. Line represents the median.

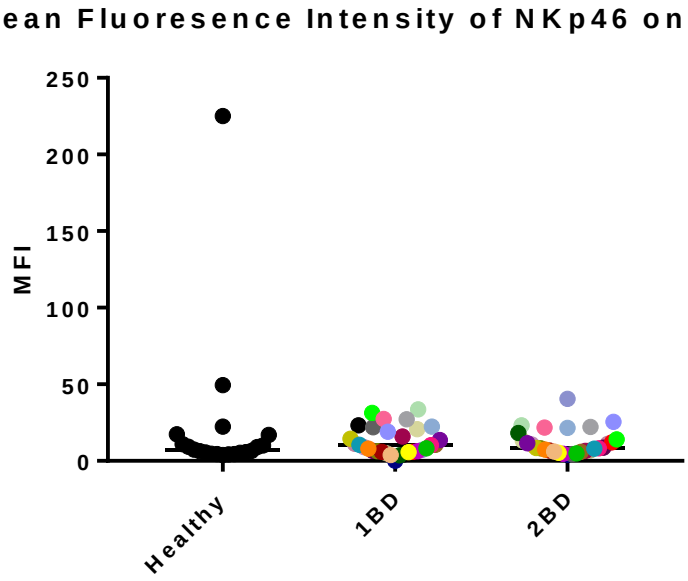

Figure 70: Comparison of mean fluorescence intensity (MFI) of NKp46 on T cells in healthy subjects n=20, patient subjects samples from first blood draw n=31, and second blood draw n=29. Line represents the median.

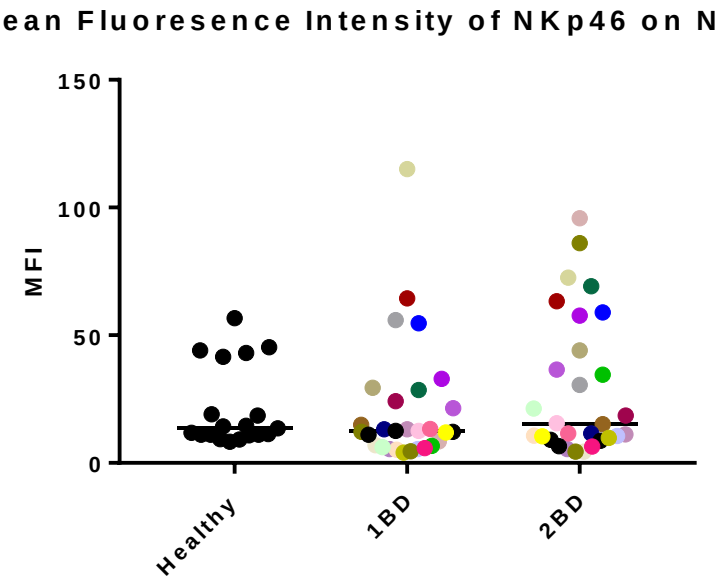

Figure 71: Comparison of mean fluorescence intensity (MFI) of NKp46 on NK cells in healthy subjects n=19, patient subjects samples from first blood draw n=29, and second blood draw n=29. Line represents the median.

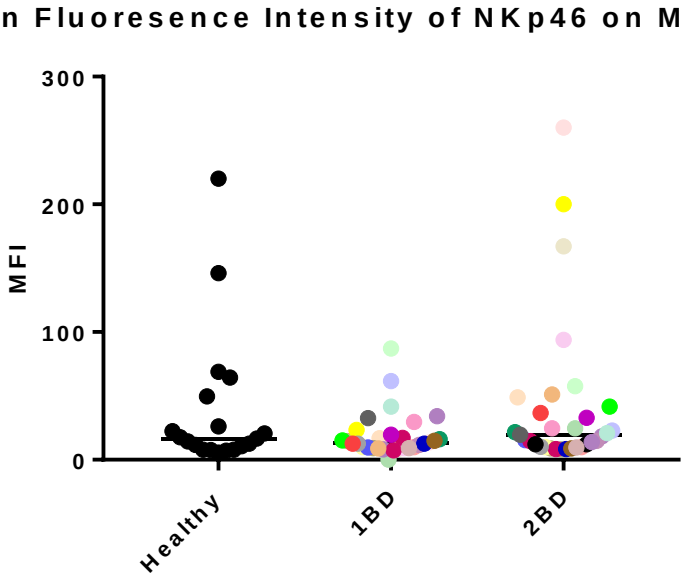

Figure 72: Comparison of mean fluorescence intensity (MFI) of NKp46 on monocytes in healthy subjects n=19, patient subjects samples from first blood draw n=30, and second blood draw n=32. Line represents the median.

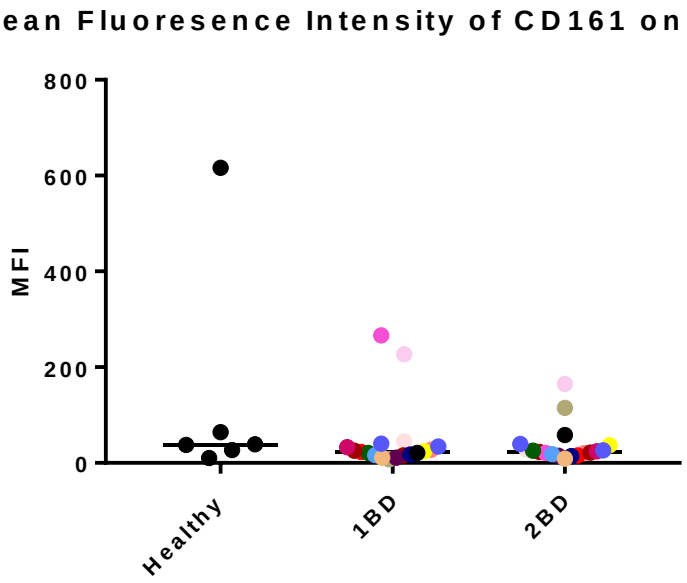

Figure 73: Comparison of mean fluorescence intensity (MFI) of CD161 on B cells in healthy subjects n=6, patient subjects samples from first blood draw n=18, and second blood draw n=18. Line represents the median.

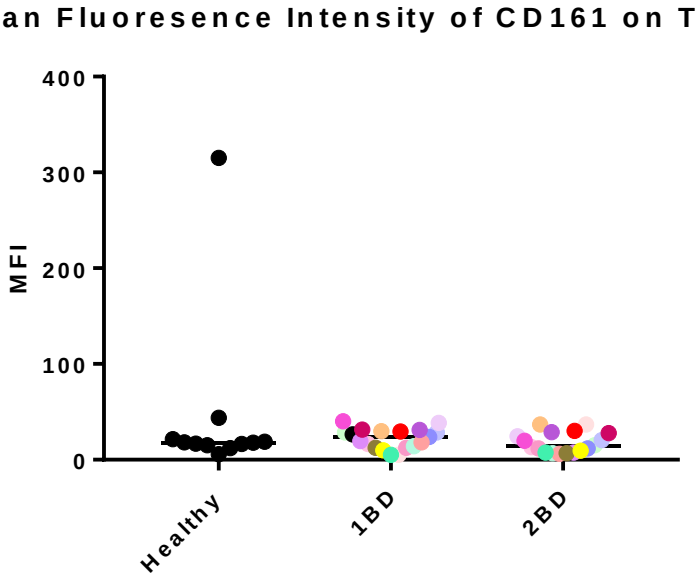

Figure 74: Comparison of mean fluorescence intensity (MFI) of CD161 on T cells in healthy subjects n=11, patient subjects samples from first blood draw n=19, and second blood draw n=18. Line represents the median.

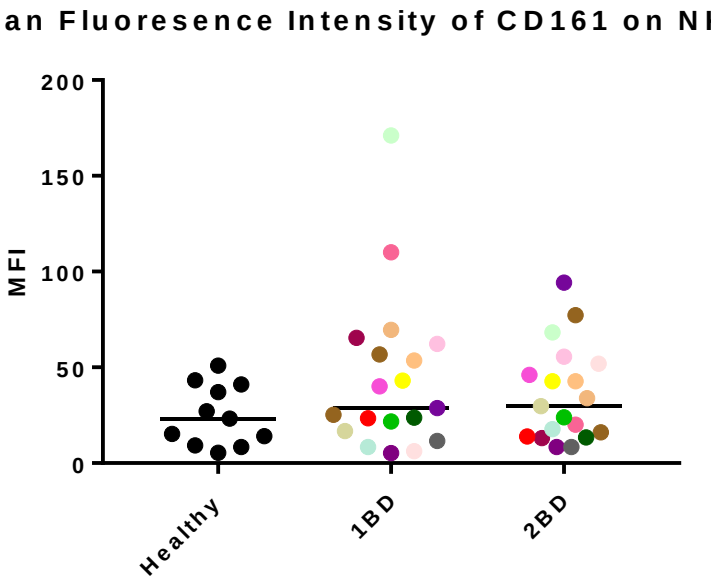

Figure 75: Comparison of mean fluorescence intensity (MFI) of CD161 on NK cells in healthy subjects n=11, patient subjects samples from first blood draw n=19, and second blood draw n=19. Line represents the median.

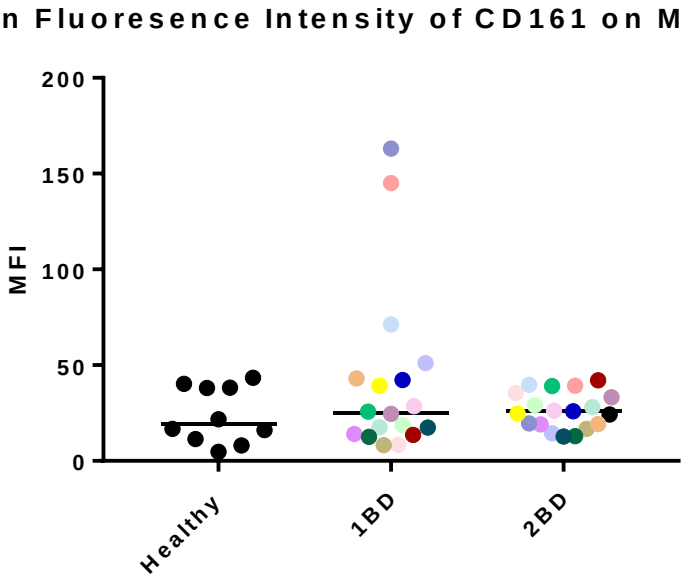

Figure 76: Comparison of mean fluorescence intensity (MFI) of CD161 on monocytes in healthy subjects n=10, patient subjects samples from first blood draw n=18, and second blood draw n=19. Line represents the median.

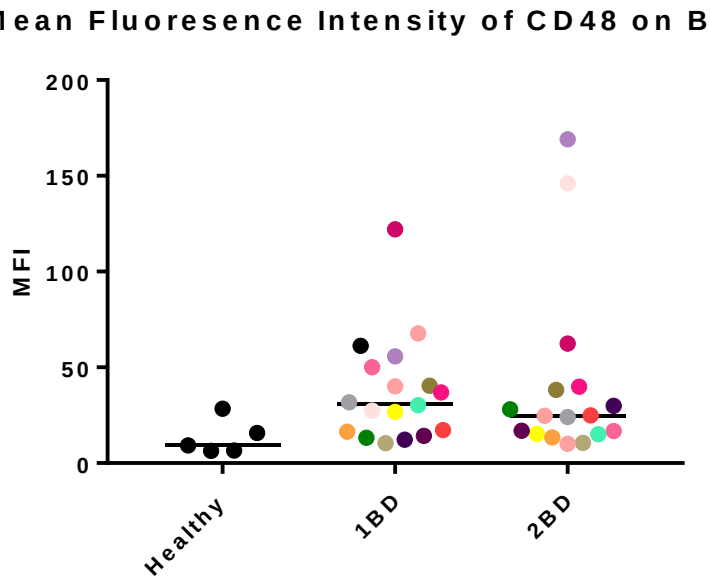

Figure 77: Comparison of mean fluorescence intensity (MFI) of CD48 on B cells in healthy subjects n=5, patient subjects samples from first blood draw n=18, and second blood draw n=17. Line represents the median.

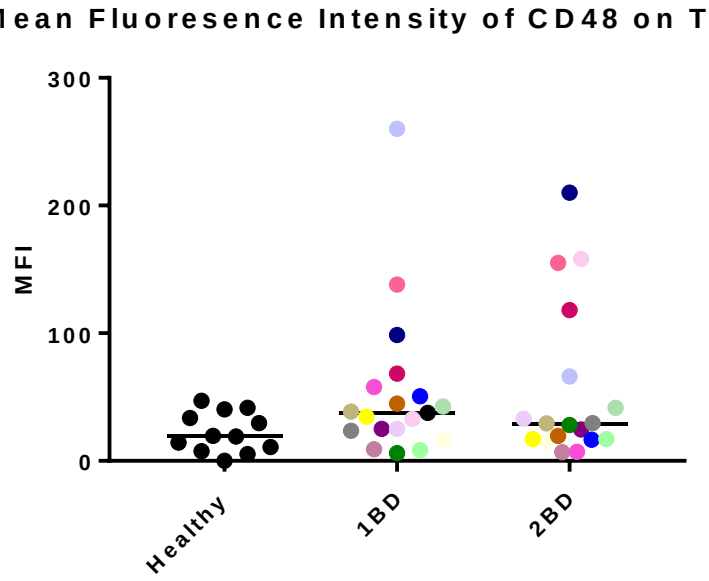

Figure 78: Comparison of mean fluorescence intensity (MFI) of CD48 on T cells in healthy subjects n=12, patient subjects samples from first blood draw n=19, and second blood draw n=18. Line represents the median.

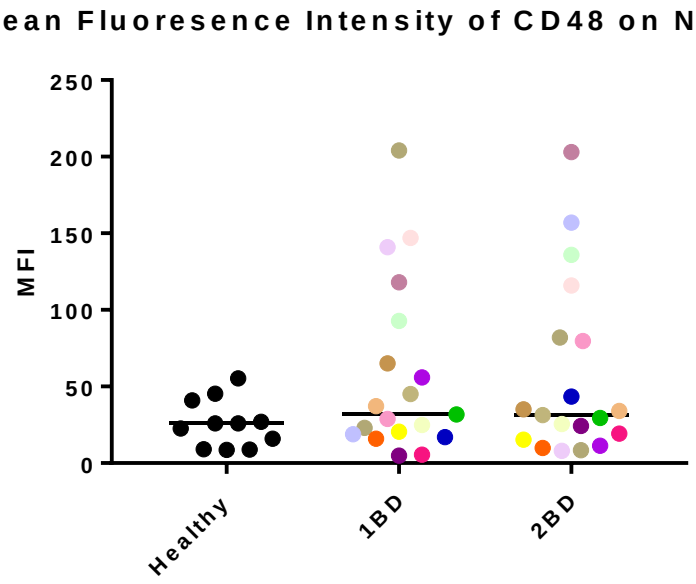

Figure 79: Comparison of mean fluorescence intensity (MFI) of CD48 on NK cells in healthy subjects n=11, patient subjects samples from first blood draw n=19, and second blood draw n=19. Line represents the median.

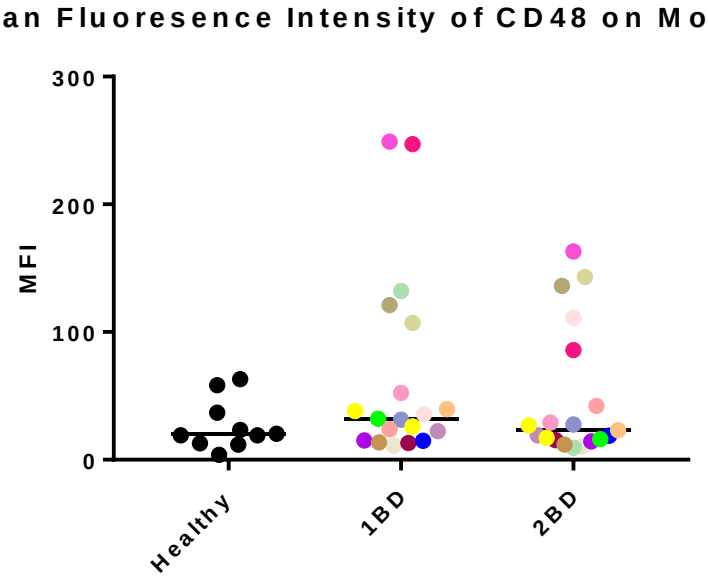

Figure 80: Comparison of mean fluorescence intensity (MFI) of CD48 on monocytes in healthy subjects n=10, patient subjects samples from first blood draw n=19, and second blood draw n=19. Line represents the median.

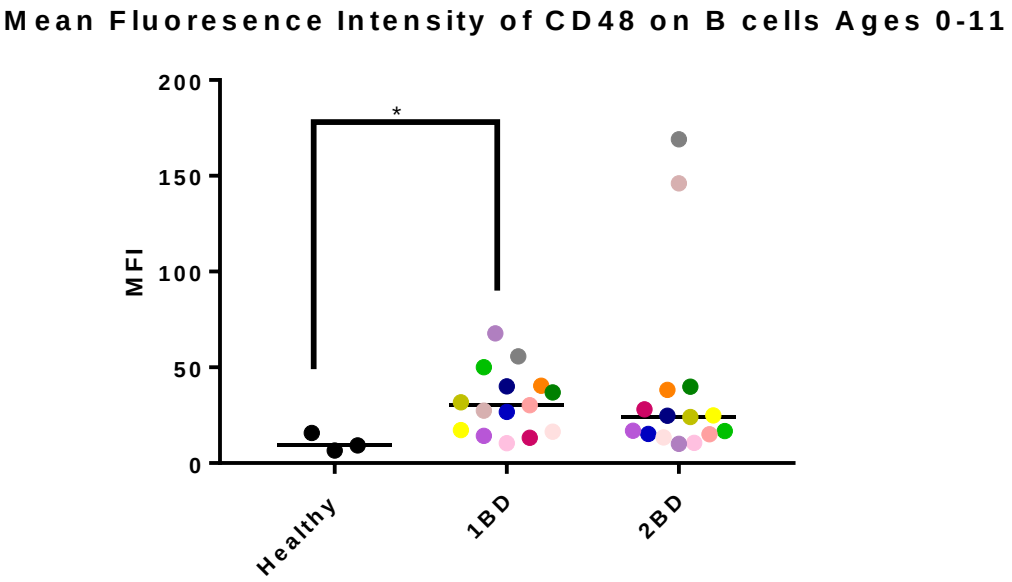

Figure 81: Comparison of mean fluorescence intensity (MFI) of CD48 on B cells in healthy subjects n=3, patient subjects samples from first blood draw n=15, and second blood draw n=15. All subjects are aged 0-11. Line represents the median. * indicates a p value less than 0.05.

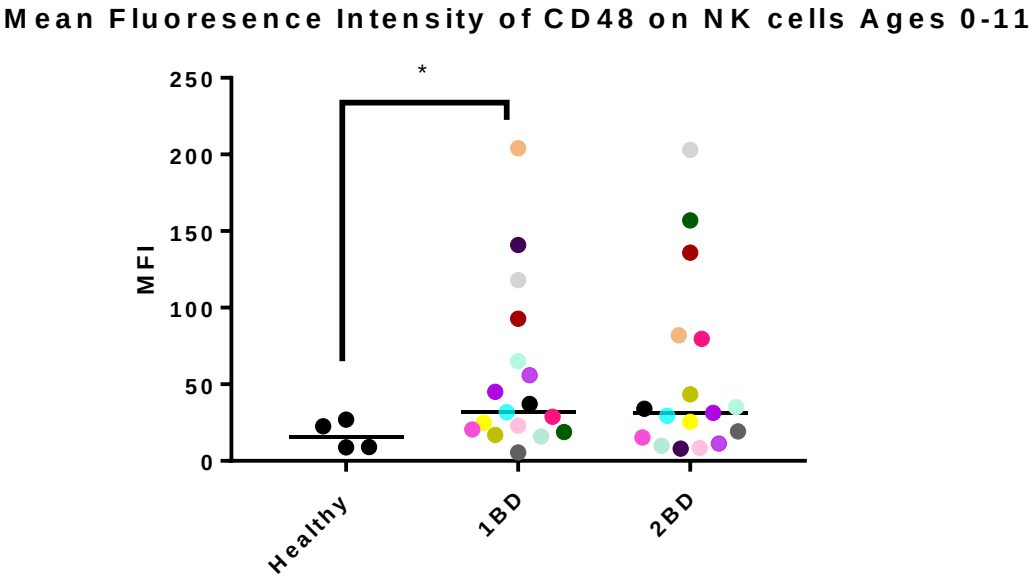

Figure 82: Comparison of mean fluorescence intensity (MFI) of CD48 on NK cells in healthy subjects n=4, patient subjects samples from first blood draw n=17, and second blood draw n=17. All subjects are aged 0-11. Line represents the median. * indicates a p value less than 0.05.

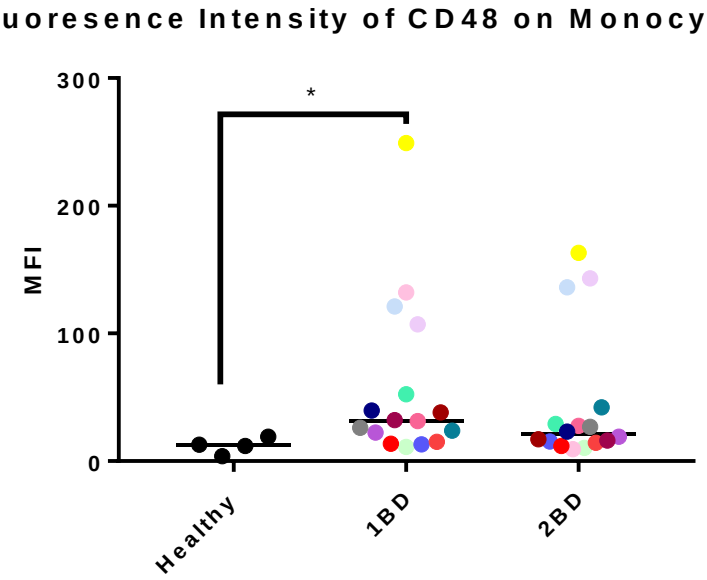

Figure 83: Comparison of mean fluorescence intensity (MFI) of CD48 on monocytes in healthy subjects n=4, patient subjects samples from first blood draw n=16, and second blood draw n=16. All subjects are aged 0-11. Line represents the median. * indicates a p value less than 0.05.

Chapter V

Detection of soluble CS1, CD48 and BAT3 in Sera of Healthy Subjects and Subjects with Pediatric Acute Lymphoblastic Leukemia

Purpose of this Aim

The purpose of this aim was to determine if the concentration of soluble receptors in serum differs between healthy subjects and ALL subjects, and if concentration differs between the same patient before and after treatment. As mentioned previously, CS1 soluble receptor was shown to be upregulated in patients with multiple myeloma.[28] This is because the receptor is upregulated on the cell surface and sheds off into the patient serum. This could potentially become a marker for those who have a high risk for multiple myeloma and preventative measures could potentially be taken.

Soluble CD48, the ligand for 2B4, as well as soluble BAT3, a ligand for NKp30, have been shown to be overexpressed in a few diseases and other leukemias, causing NK cell dysfunction.[40] For this reason, these three soluble receptors; CS1, CD48, and BAT3; were quantified by indirect ELISA to determine the concentration of these receptors in healthy and patient plasma.

Figure 84 shows results of CS1 concentration of total subjects. The figure shows there is no significance between the groups. Figures 85 and 86 separate the subjects into two age groups: 0-11 and 12-21 years old respectively. For the age group 0-11, there is a significant increase of soluble CS1 in the first blood draw of patients with ALL compared to healthy subjects. For ages 12-21, there is no statistical significance, however interestingly, when comparing the same patient over the first and second blood draw, all the patients have an increase of soluble CS1 after receiving treatment, except the only white male, UNTALL-11. This interesting trend led to a separation of ethnicities. Figure 87 shows the concentration of soluble CS1 in subjects with African descent. Because ALL has the least frequency in the African American population, this study only had serum for first and second blood draw for one ALL patient of African descent. This patient had a lower CS1 concentration than the healthy group and a marked increase in CS1 after treatment. To draw any definitive conclusions, more patients of African descent must be obtained and evaluated. However, the observation does show an interesting potential for future research.

Figure 88 shows a highly significant increase in soluble CS1 in patients of white/Hispanic descent ages 0-11 and figure 89 shows the same trend and significance in male patients of ALL ages 0-11 when compared to healthy subjects. The high concentration of

soluble CS1 in these groups as opposed to the African group may shed some light on why this particular cancer is more prevalent in Caucasian and Hispanic males.

Figure 90 shows the concentration of soluble CD48 in all of the subjects. There is significant increase in CD48 concentration from first blood draw to second blood draw. The next figure, figure 91, shows the concentration of soluble CD48 in subjects aged 0-11 years old. The significant increase after treatment for the second blood draw is more pronounced. One outlier in the healthy subject group was the only subject of African descent in the 0-11 age range, so for figure 92, all the subjects of African descent were analyzed. The healthy subjects form a tight cluster, and the one patient sample in this group shows a dramatic decrease in soluble CD48 at the first blood draw, and then seemingly returns to the healthy control concentration at the second blood draw.

The next figure, figure 93, shows the age group 12-21. Once again there is no statistical significance, however the same white male is the only subject going against the trend of an increase in soluble CD48 after treatment is received. Figure 94 shows white/Hispanics aged 0-11, with a significant increase in soluble CD48 at first blood draw.

Figures 95, 96, and 98 show BAT-3 significantly overexpressed in first blood draw patients, with the most noticeable increase in the white/Hispanic population ages 0-11.

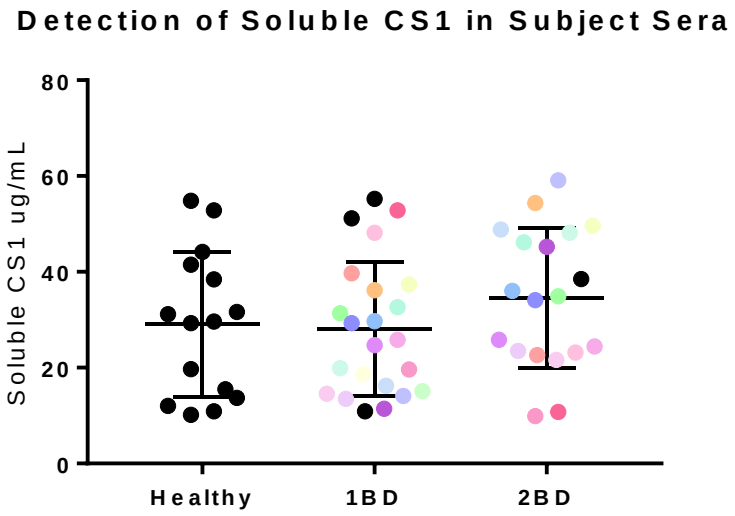

Figure 84: Comparison of total soluble CS1 ug/mL detected in healthy subjects (n=15), first blood draw (1BD) of ALL subjects (n=23) and second blood draw (2BD) of ALL subjects (n=21). No significance found between medians of groups. Lines mark mean and standard deviation.

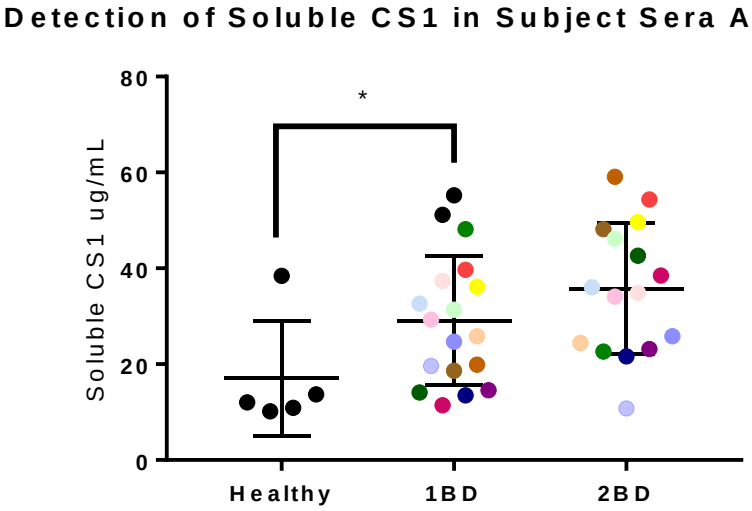

Figure 85: Comparison of soluble CS1 ug/mL detected in healthy subjects (n=5), first blood draw (1BD) of ALL subjects (n=18) and second blood draw (2BD) of ALL subjects (n=16). All samples are between ages 0-11 years old. * indicates significance of a p value< 0.05. Lines mark mean and standard deviation.

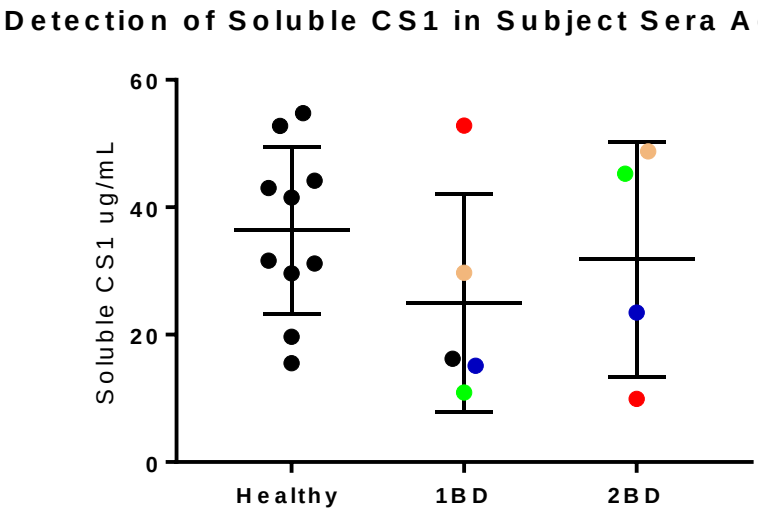

Figure 86: Comparison of soluble CS1 ug/mL detected in healthy subjects (n=10), first blood draw (1BD) of ALL subjects (n=5) and second blood draw (2BD) of ALL subjects (n=4). All samples are between ages 12-21 years old. No significance found between medians of groups. **The patient marked in red is the only white male in patient data set, UNTALL-11.** Lines mark mean and standard deviation.

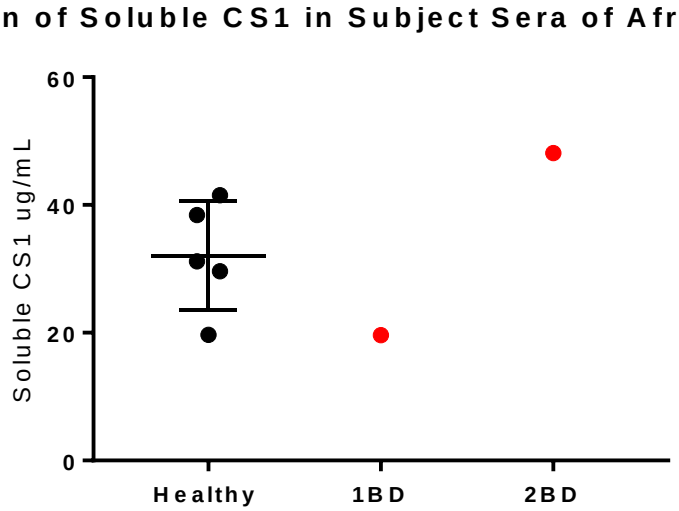

Figure 87: Comparison of soluble CS1 ug/mL detected in healthy subjects (n=5), and the 1BD and 2BD of the same patient. All subjects are of African descent. Lines mark mean and standard deviation.

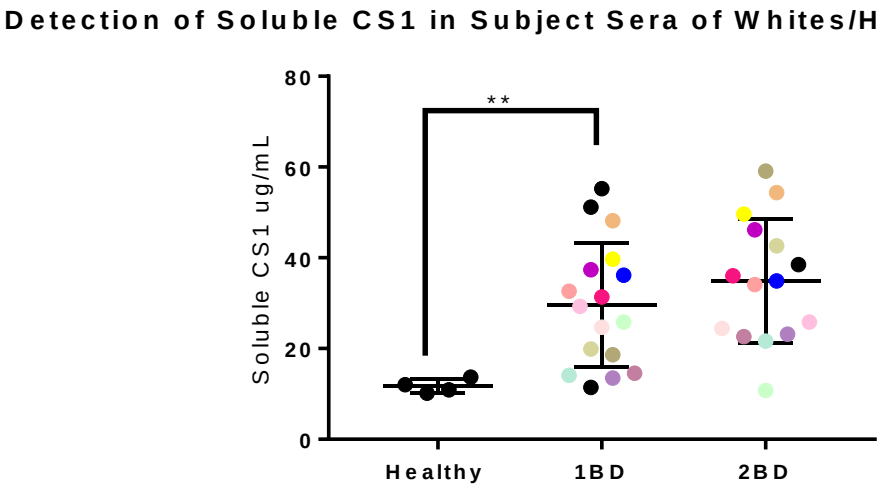

Figure 88: Comparison of soluble CS1 ug/mL detected in healthy subjects (n=4), first blood draw (1BD) patients (n=17) and second blood draw (2BD) patients (n=15). All samples are between ages 0-11 years old and of white or Hispanic descent. ** indicates significance of a p value< 0.005. Lines mark mean and standard deviation.

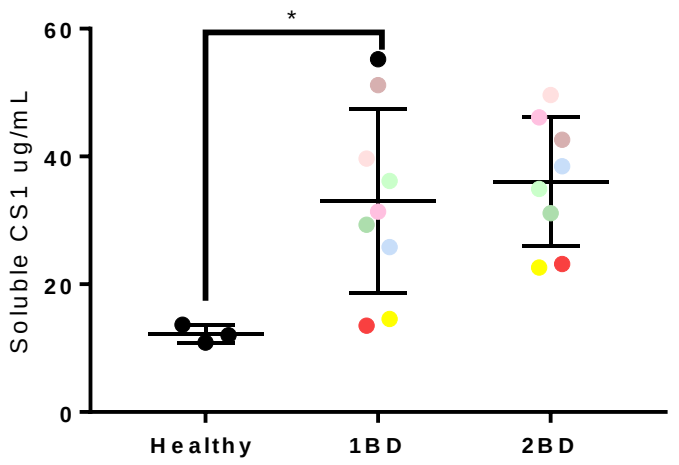

Figure 89: Comparison of soluble CS1 ug/mL detected in healthy subjects (n=3), first blood draw (1BD) of ALL subjects (n=9) and second blood draw (2BD) of ALL subjects (n=8). All samples are male between ages 0-11 years old. * indicates significance of a p value< 0.05. Lines mark mean and standard deviation.

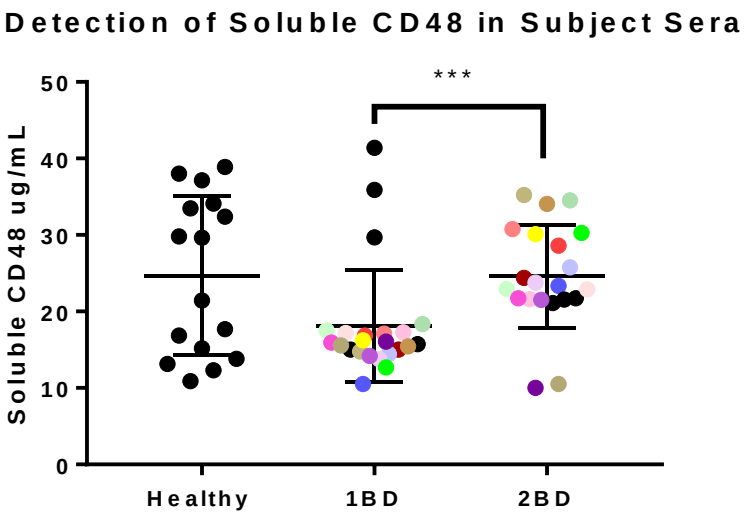

Figure 90: Comparison of soluble CD48 ug/mL detected in healthy subjects (n=16), first blood draw (1BD) of ALL subjects (n=23) and second blood draw (2BD) of ALL subjects (n=21). *** indicates significance of a p value< 0.0005. Lines mark mean and standard deviation.

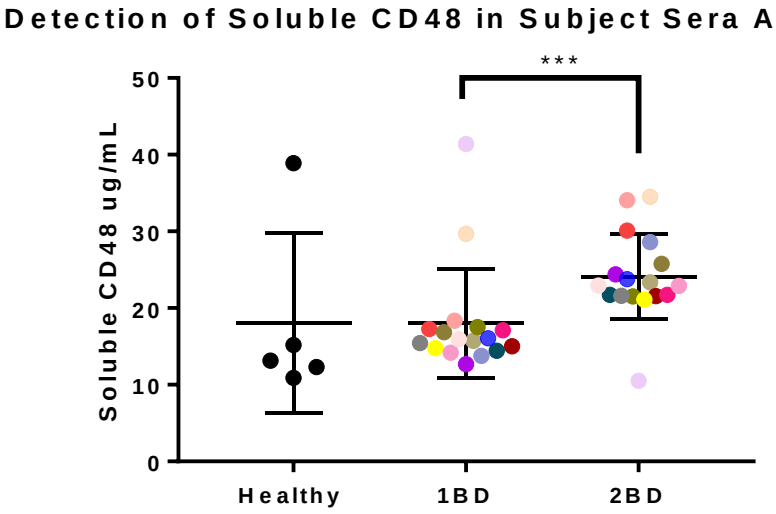

Figure 91: Comparison of soluble CD48 ug/mL detected in healthy subjects (n=5), first blood draw (1BD) of ALL subjects (n=17) and second blood draw (2BD) of ALL subjects (n=17). *** indicates significance of a p value< 0.0005. Lines mark mean and standard deviation.

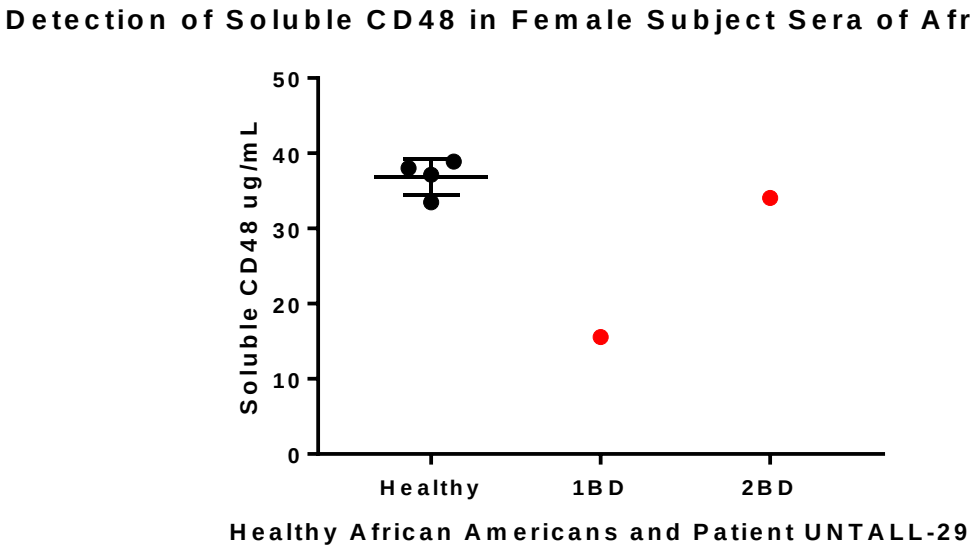

Figure 92: Comparison of soluble CD48 ug/mL detected in healthy subjects (n=4), and the 1BD and 2BD of the same patient. All subjects are females of African descent. Lines mark mean and standard deviation.

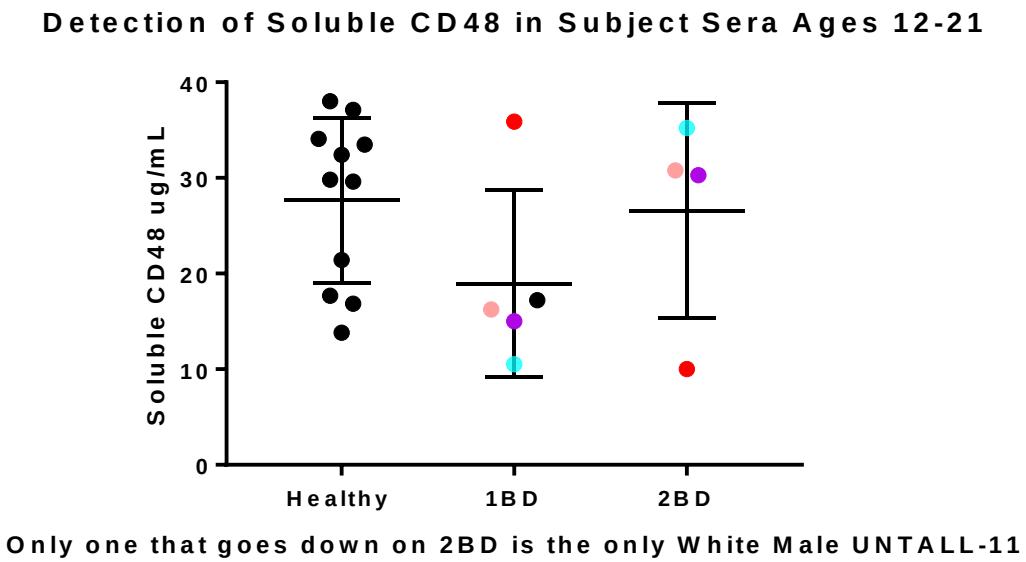

Figure 93: Comparison of soluble CD48 ug/mL detected in healthy subjects (n=11), first blood draw (1BD) of ALL subjects (n=5) and second blood draw (2BD) of ALL subjects (n=4). All samples are between ages 12-21 years old. No significance found between medians of groups. **The patient marked in red is the only white male in patient data set UNTALL-11.** Lines mark mean and standard deviation.

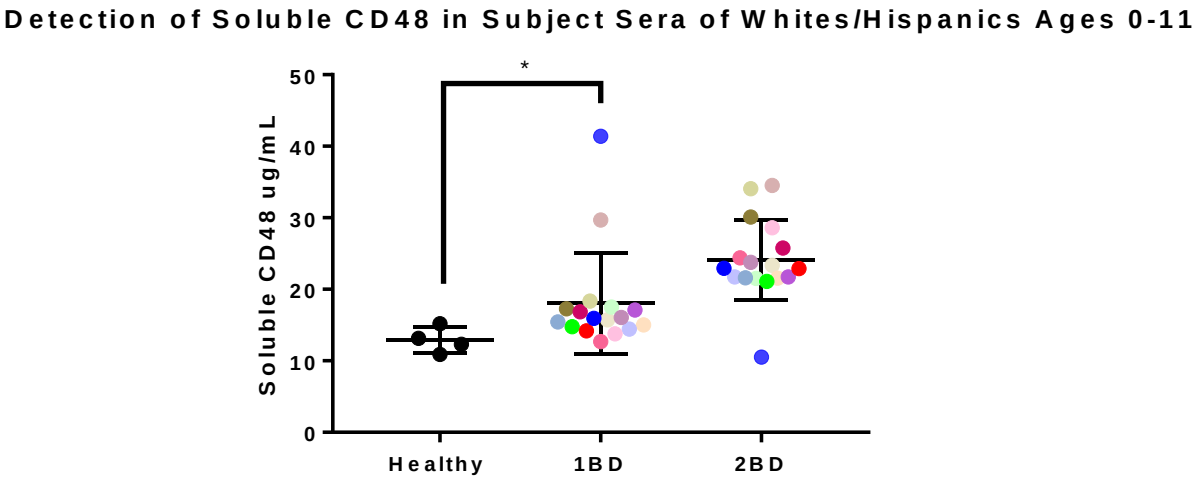

Figure 94: Comparison of soluble CD48 ug/mL detected in healthy subjects (n=4), first blood draw (1BD) of ALL subjects (n=17) and second blood draw (2BD) of ALL subjects (n=17). All samples are between ages 0-11 years old and of white or Hispanic descent. * indicates significance of a p value< 0.05. Lines mark mean and standard deviation.

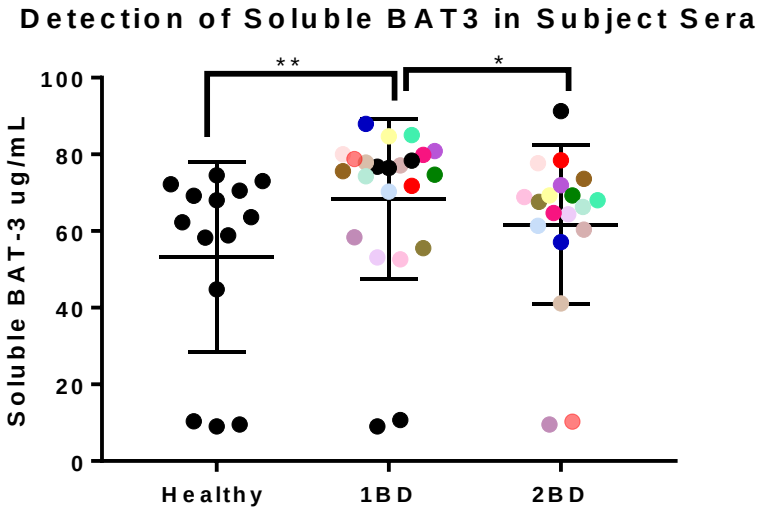

Figure 95: Comparison of soluble BAT-3 ug/mL detected in healthy subjects (n=14), first blood draw (1BD) of ALL subjects (n=23) and second blood draw (2BD) of ALL subjects (n=19). * indicates significance of a p value< 0.05. ** indicates significance of a p value < 0.005. Lines mark mean and standard deviation.

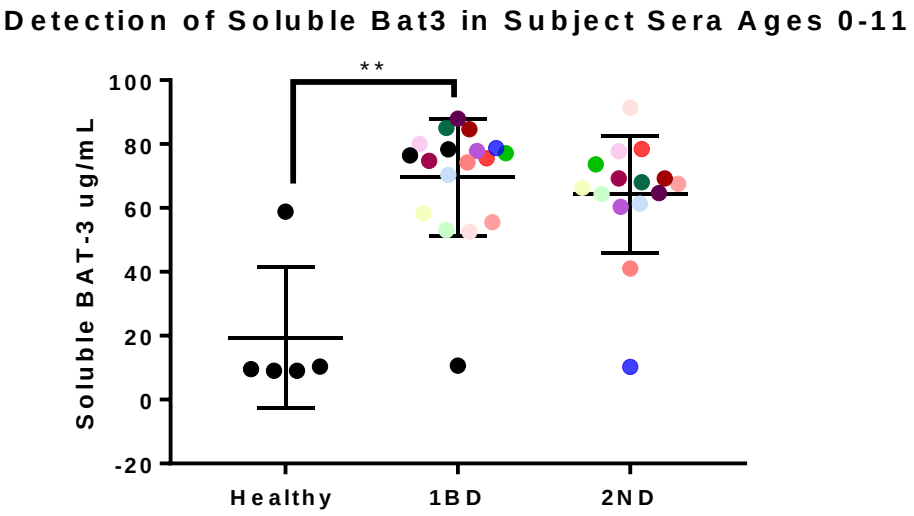

Figure 96: Detection of soluble BAT-3 ug/mL detected in healthy subjects (n=5), first blood draw (1BD) of ALL subjects (n=18) and second blood draw (2BD) of ALL subjects (n=15). ** indicates significance of a p value< 0.005. Lines mark mean and standard deviation.

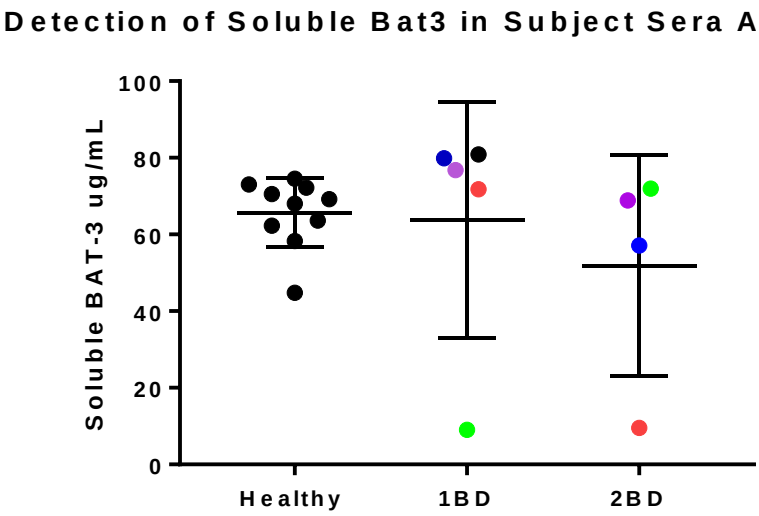

Figure 97: Comparison of soluble BAT-3 ug/mL detected in healthy subjects (n=10), first blood draw (1BD) of ALL subjects (n=5) and second blood draw (2BD) of ALL subjects (n=4). Lines mark mean and standard deviation.

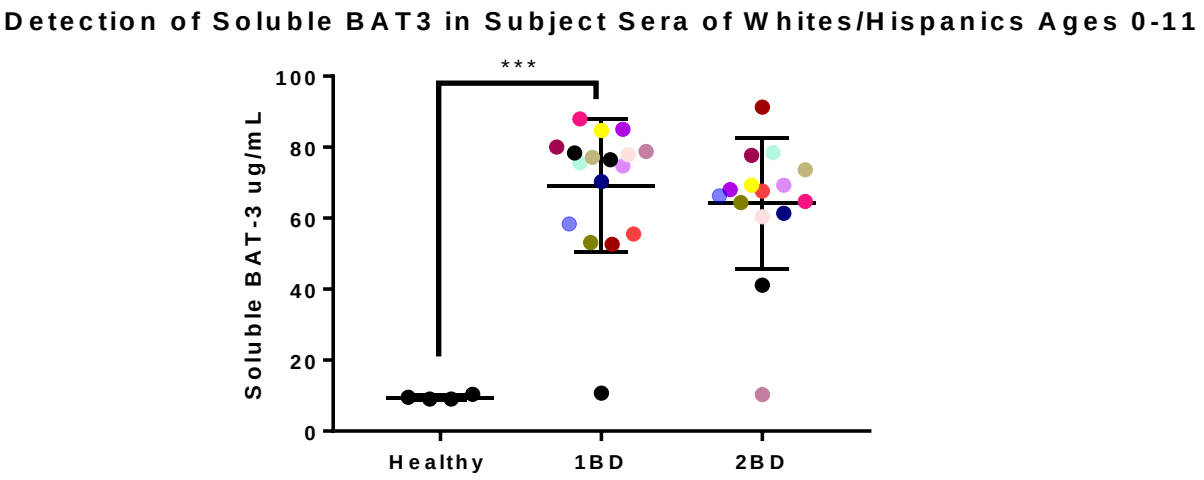

Figure 98: Comparison of soluble BAT-3 ug/mL detected in healthy subjects (n=4), first blood draw (1BD) of ALL subjects (n=17) and second blood draw (2BD) of ALL subjects (n=15). *** indicates significance of a p value< 0.0005. Lines mark mean and standard deviation.

Chapter VI

Functional Analysis of Immune Receptors by NK Cell Cytotoxic Killing Assay

Flow-based Natural Killer Cell Cytotoxicity Assay

To analyze functional changes in NK killing, I performed a cytotoxicity assay to determine if a change in receptor availability improved or inhibited receptor function. The target cell line I used for this assay was Jurkat, a well-established T cell leukemia line and also known for having resistance to NK cell killing.

Figure 100 shows the results of a flow cytometry experiment to determine whether or not Jurkat cells expressed any of our receptors of interest. I examined 2B4, CS1, CD48, NKp30 and NKp46. It was already established in the literature that Jurkat does not express LLT1.[37] The results of the flow cytometry show that 2B4, NKp30, and NKp46 are not expressed. CS1 is

expressed at a very low percentage. CD48, however, had a very high level of expression on Jurkat cancer cells.

Figure 101 shows the killing of Jurkat cells by a leukemic NK cell line NK92-MI, measured by annexin V fluorescence.[43] Jurkat cells were incubated for three hours with a control antibody or with an anti-CD48 antibody. Three ratios of effector to target cells were measured and done in triplicates: 20:1, 5:1 and 1:1. The results show that when Jurkat cells were incubated with anti-CD48, the NK cells had reduced killing, and significantly reduced killing at the 5:1 ratio. With CD48 blocked on Jurkat cells, they were not able to ligate with the NK cell 2B4. As mentioned previously, 2B4 is an important activating receptor as well as a co-activator, enhancing the activation of other receptors. The results show that when 2B4 is not ligated, NK cells are not able to achieve activation and thus have reduced killing.

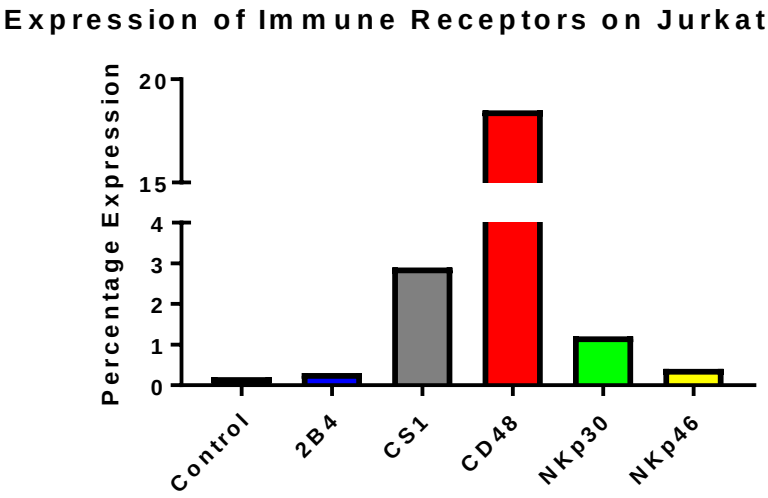

Figure 99: Shows expression of immune receptors on Jurkat cell line. CD48 was expressed the most at 18.5%.

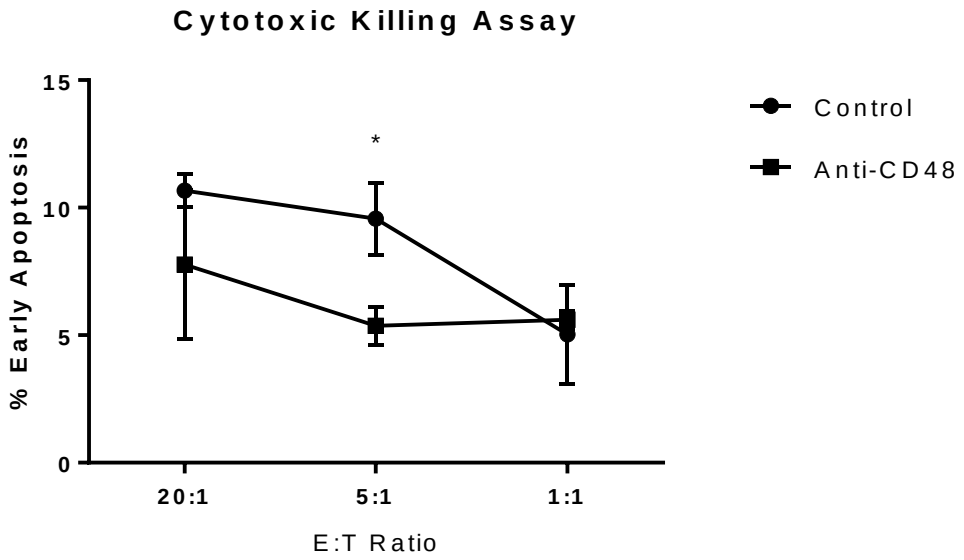

Figure 100: Comparison of early apoptosis caused by NK cell killing Jurkat cells between the control antibody and anti-CD48 antibody. * indicates a p value < 0.05.

Chapter VII

Discussion

The immune receptors 2B4, CS1, LLT1, NKp30 and NKp46 have been shown to play a role in the pathogenesis and progression of various diseases, including leukemias. The lack of sufficient 2B4 receptor on NK cells has been shown to play a role in decreased activation of NK cells. CS1 expression is upregulated in multiple myeloma and lupus. LLT1 is shown to have an increased expression in various cancers. A decreased expression of the NCRs, NKp30 and NKp46, have been shown to correlate with poor prognosis in other leukemias. The goal of this project was to determine if the expression and function of these immune receptors are altered in ALL compared with healthy subjects.

PBMCs from 43 ALL subjects and 21 healthy subjects were isolated from whole blood. Two blood samples were collected from ALL subjects: one blood draw (1BD) at the time of diagnosis and a second blood draw (2BD) 29 days later after receiving the initial chemotherapy treatment. Only one blood sample was collected from healthy subjects.

The qRT-PCR experiments to determine fold change of mRNA expression of the immune receptors revealed that there is in fact a change in mRNA expression. 2B4, LLT1, and NKp30 mRNA expression was all reduced in the first blood draw of ALL subjects compared to the healthy subjects (Figures 1,3, and 4). NKp46 was found to be reduced in the first blood draw in the age group 12-21 (Figure 15). This may be an indication as to why the NK cells in ALL do not appear to achieve the proper activating signals as two important activating ligands, 2B4 and LLT1, are seen to be reduced. Also, lowered expression of NKp30 has already been shown to be an indicator of poor prognosis in other leukemias.[13] CS1 was the only immune receptor that had an increase of fold change in mRNA expression in the second blood draw, after treatment was given to the ALL subjects. Further studies will need to be done to determine if this change is beneficial or not. The increase in CS1 may indicate the presence of more activated B cells and perhaps a healthier cellular environment.

Flow cytometric analysis was conducted to determine the surface protein expression of these receptors on the specific immune cell types: B cells, T cells, NK cells and monocytes. The flow percentage expression, which determines at what percentage a receptor is expressed, revealed that CS1 was increased on the ALL subjects first blood draw in T cells and monocytes (figures 31 and 33). CS1 on monocytes was shown in the literature to have an inhibitory role in immune responses.[45] Therefore, an increase in CS1 on monocytes as well as T cells could be inhibiting the immune response of NK cells against ALL cells. LLT1 was increased on NK cells for the first blood draw of ALL subjects (figure 36). However, LLT1's ligand, CD161, was increased on all cell types except NK cells (figure 46, 47, 49). Even though there appears to be an increase of an activating receptor on NK cells, there also might be interference created by an increase of LLT1 ligand on many cells, not just the cancer cells. CD48 was also increased on B

cells after treatment (Figure 50). Studies have shown that when NK cells are overstimulated through their activating receptors, what results is an anergic effect.[27,44]

When I look at MFI, which displays surface density of a receptor, figure 56 shows that 2B4 surface density appears to be decreased on the NK cells in first blood draw ALL subjects when compared to healthy subjects. This is important because NK cells need a majority of activating receptors to be ligated in order to produce an immune response. With fewer activating receptors available on the cell surface, the less likely the NK cell will achieve its function. When I further looked at the age group 0-11, the age group most susceptible to ALL, I found that CD48 is significantly increased on B cells, NK cells and monocytes in first blood draw subjects compared to healthy subjects.

Next I used ELISA testing to look at soluble receptors CS1, CD48 and BAT3 in sera. CS1 was shown in figure 85 to be significantly increased in first blood draw of ALL subjects ages 0-11. In the age group 12-21, I observed a trend in figure 87, where after treatment, all the patient subjects have an increase in CS1 in the sera except the only white male who has a dramatic decrease. I do not have data on the current state for the white male, UNTALL-11, however a follow-up on his and other patients' remission and relapse rates may be insightful.

In the age group of 0-11 only including those of white or Hispanic descent, there is a significant increase in soluble CS1 in the subject sera for first blood draw compared to healthy subjects. Because this was the same outcome seen in patients with multiple myeloma, ALL subjects may also benefit from the current Empliciti (anti-CS1 mAB) treatment for multiple myeloma.[31]

Soluble CD48 was also seen to be significantly increased in the first blood draw in ALL subjects in the age group 0-11 of white or Hispanic descent. When I look at total subjects, there

was also a significant increase from first blood draw to second blood draw, after treatment. As stated above, increased CD48 in the sera of patients, has produced an anergic effect on NK cells in other diseases.[27]

Soluble BAT3 was also shown to have a highly significant increaase in the first blood draw of ALL subjects ages 0-11 in Caucasian or Hispanic descent. These findings may shed some light as to why ALL is much more prevalent in young children of Caucasian or Hispanic descent.

The final experiment I conducted was a killing assay to investigate the function of these immune receptors. CD48 has shown a trend throughout this project of being a receptor of interest in ALL, as well as its ligand 2B4 in other diseases. After first determining that CD48 was expressed on the Jurkat T cell ALL cell line (figure 99), I conducted a killing assay to determine its role in NK cell killing. I incubated the Jurkat cells with an anti-CD48 antibody. I also blocked the NK cells with an FC blocker to ensure they could not kill via ADCC. This meant that any killing I witnessed had to be through the NK cell's other activating receptors. Figure 100 shows that, compared to our control, the NK cell killing was significantly reduced when 2B4 was not able to ligate CD48 on the target cell. This could suggest that either the 2B4 on NK cells of ALL subjects is expressed less and therefore, not activating the NK cell, or that the soluble CD48 is over-activating the NK cell causing anergy, or that the 2B4 on NK cells of ALL subjects is mutated or an isoform that functions slightly differently.[46,47]

Conclusion

The immune receptors 2B4, CS1, LLT1, NKp30 and NKp46 showed altered expression in mRNA and protein surface expression on immune cells of ALL subjects compared to healthy subjects. I also found that blocking CD48, the ligand for 2B4, negatively affected the killing ability of NK cells. Based on the findings in this project, as well as corresponding literature, it appears that ALL cells are able to escape NK cell killings through a variety of ways of inhibiting NK cell activation.

Future Studies

Our goal with the project is that the results here will aid further studies toward developing an immunotherapy drug that could contribute to the treatment of ALL. For future research, mRNA expression should be measured for each individual immune cell. This way I could determine if expression of a receptor is truly altered at the protein or mRNA. More patient subjects need to be recruited, as well as healthy subjects with more emphasis on age and ethnicity to look at disparities in these groups. Also, for flow cytometric analysis, it would be of interest to distinguish the normal B cells from the cancerous B cells to give us a clearer picture as to what specifically is happening with the cancer cells. Finally, a killing assay should be conducted using actual patient cells if possible. Since the ultimate goal is to create a treatment option much like the CS1 monoclonal antibody, a killing assay should be done using CD48 as the target to determine if a monoclonal antibody could increase NK cell ADCC killing of target cells. With continuing research, an immunotherapy treatment could be created for ALL patients to reduce relapse rate and improve quality of life.

REFERENCES

1. Cooper, S. L., et al. (2015). "Treatment of pediatric acute lymphoblastic leukemia." Pediatr Clin North Am **62**(1): 61-73.
2. Pui, C. H., et al. (2013). "A 50-year journey to cure childhood acute lymphoblastic leukemia." Semin Hematol **50**(3): 185-196.
3. Hunger, S. P., et al. (2012). "Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group." J Clin Oncol **30**(14): 1663-1669.
4. Bhojwani, D., et al. (2015). "Biology of childhood acute lymphoblastic leukemia." Pediatr Clin North Am **62**(1): 47-60.
5. Frishman-Levy, L., et al. (2015). "Central nervous system acute lymphoblastic leukemia: role of natural killer cells." Blood **125**(22): 3420-3431.
6. Lelaidier, M., et al. (2015). "TRAIL-mediated killing of acute lymphoblastic leukemia by plasmacytoid dendritic cell-activated natural killer cells." Oncotarget **6**(30): 29440-29455.
7. Sun, J. C., et al. (2011). "NK cells and immune "memory"." J Immunol **186**(4): 1891-1897.
8. Boles, K. S., et al. (1999). "Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4." Tissue Antigens **54**(1): 27-34.
9. Boles, K. S., et al. (2001). "Molecular cloning of CS1, a novel human natural killer cell receptor belonging to the CD2 subset of the immunoglobulin superfamily." Immunogenetics **52**(3-4): 302-307.

10. Boles, K. S., et al. (1999). "Cloning of a new lectin-like receptor expressed on human NK cells." Immunogenetics **50**(1-2): 1-7.
11. Kim, J. R., et al. (2010). "Altered expression of signalling lymphocyte activation molecule (SLAM) family receptors CS1 (CD319) and 2B4 (CD244) in patients with systemic lupus erythematosus." Clin Exp Immunol **160**(3): 348-358.
12. Parolini, S., et al. (2000). "X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells." J Exp Med **192**(3): 337-346.
13. Costello, R. T., et al. (2002). "Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia." Blood **99**(10): 3661-3667.
14. Davila, M. L., et al. (2014). "Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia." Sci Transl Med **6**(224): 224ra225.
15. Maude, S. L., et al. (2015). "CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia." Blood **125**(26): 4017-4023.
16. Pegram, H. J., et al. (2011). "Activating and inhibitory receptors of natural killer cells." Immunol Cell Biol **89**(2): 216-224.
17. Moretta, L., et al. (2001). "Human natural killer cell function and receptors." Curr Opin Pharmacol **1**(4): 387-391.
18. Anfossi, N., et al. (2006). "Human NK cell education by inhibitory receptors for MHC class I." Immunity **25**(2): 331-342.
19. Elliott, J. M., et al. (2011). "Unifying concepts of MHC-dependent natural killer cell education." Trends Immunol **32**(8): 364-372.

20. Brown, M. H., et al. (1998). "2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48." J Exp Med **188**(11): 2083-2090.

21. Latchman, Y., et al. (1998). "Identification of the 2B4 molecule as a counter-receptor for CD48." J Immunol **161**(11): 5809-5812.

22. Vaidya, S. V., et al. (2006). "Of mice and men: different functions of the murine and human 2B4 (CD244) receptor on NK cells." Immunol Lett **105**(2): 180-184.

23. Mathew, S. O., et al. (2009). "Functional role of human NK cell receptor 2B4 (CD244) isoforms." Eur J Immunol **39**(6): 1632-1641.

24. Assarsson, E., et al. (2005). "2B4/CD48-mediated regulation of lymphocyte activation and function." J Immunol **175**(4): 2045-2049.

25. Chlewicki, L. K., et al. (2008). "Molecular basis of the dual functions of 2B4 (CD244)." J Immunol **180**(12): 8159-8167.

26. Wu, Y., et al. (2013). "Monocyte/macrophage-elicited natural killer cell dysfunction in hepatocellular carcinoma is mediated by CD48/2B4 interactions." Hepatology **57**(3): 1107-1116.

27. Smith, G. M., et al. (1997). "Detection of a soluble form of the leukocyte surface antigen CD48 in plasma and its elevation in patients with lymphoid leukemias and arthritis." J Clin Immunol **17**(6): 502-509.

28. Tai, Y. T., et al. (2008). "Anti-CS1 humanized monoclonal antibody HuLuc63 inhibits myeloma cell adhesion and induces antibody-dependent cellular cytotoxicity in the bone marrow milieu." Blood **112**(4): 1329-1337.

29. Hsi, E. D., et al. (2008). "CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma." Clin Cancer Res **14**(9): 2775-2784.

30. Lee, J. K., et al. (2007). "CS1 (CRACC, CD319) induces proliferation and autocrine cytokine expression on human B lymphocytes." J Immunol **179**(7): 4672-4678.
31. Balasa, B., et al. (2015). "Elotuzumab enhances natural killer cell activation and myeloma cell killing through interleukin-2 and TNF-alpha pathways." Cancer Immunol Immunother **64**(1): 61-73.
32. Einsele, H., et al. (2016). "Treatment of multiple myeloma with the immunostimulatory SLAMF7 antibody elotuzumab." Ther Adv Hematol **7**(5): 288-301.
33. http://szpiczak.org/lang/aktualnosci/kongresy/rok_2014/pdf/0905_EHA%202014_MM_Richardson.pdf
34. Mathew, P. A., et al. (2004). "The LLT1 receptor induces IFN-gamma production by human natural killer cells." Mol Immunol **40**(16): 1157-1163.
35. Rosen, D. B., et al. (2005). "Cutting edge: lectin-like transcript-1 is a ligand for the inhibitory human NKR-P1A receptor." J Immunol **175**(12): 7796-7799.
36. Roth, P., et al. (2007). "Malignant glioma cells counteract antitumor immune responses through expression of lectin-like transcript-1." Cancer Res **67**(8): 3540-3544.
37. Mathew, S. O., et al. (2016). "Overexpression of LLT1 (OCIL, CLEC2D) on prostate cancer cells inhibits NK cell-mediated killing through LLT1-NKRP1A (CD161) interaction." Oncotarget.
38. Hecht, M. L., et al. (2009). "Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences." J Proteome Res **8**(2): 712-720.
39. Delahaye, N. F., et al. (2011). "Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors." Nat Med **17**(6): 700-707.

40. Reiners, K. S., et al. (2013). "Soluble ligands for NK cell receptors promote evasion of chronic lymphocytic leukemia cells from NK cell anti-tumor activity." Blood **121**(18): 3658-3665.

41. Hecht, M. L., et al. (2009). "Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences." J Proteome Res **8**(2): 712-720.

42. Goni, R., et al. (2009). "The qPCR data statistical analysis." Integromics White Paper September 1-9.

43. Hingorani, R. (2011) Detection of Apoptosis Using the BD Annexin V FITC Assay on the BD FACSVerse™ System. BD Biosciences.

44. Orr, M. T., et al. (2010). "Natural killer cell education and tolerance." Cell **142**(6): 847-856.

45. Kim, J. R., et al. (2013). "CS1 (SLAMF7) inhibits production of proinflammatory cytokines by activated monocytes." Inflamm Res **62**(8): 765-772.

46. Mathew, S. O., et al. (2009). "Functional role of human NK cell receptor 2B4 (CD244) isoforms." Eur J Immunol **39**(6): 1632-1641.

47. Mathew, S. O., et al. (2005). "Mutational analysis of the human 2B4 (CD244)/CD48 interaction: Lys68 and Glu70 in the V domain of 2B4 are critical for CD48 binding and functional activation of NK cells." J Immunol **175**(2): 1005-1013.