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Innate immunity genes as determinants of resistance/susceptibility to human disease: studies in leukemia patients.

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Ce mémoire intitulé :

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Summary

Investigating genetic determinants that play a role in conferring susceptibility/resistance to the development of acute B cell leukemia (B-ALL) in children is highly desirable. We hypothesized that activating Killer-cell Immunoglobulin-like Receptor (KIR) genes, which are implicated in NK cell activation, may represent one of these determinants. To test this hypothesis, we conducted a case-control study in French-Canadian children in which we used genomic DNA from 100 B-ALL patients and 245 healthy controls. The presence or absence of each KIR gene was detected by PCR using sequence-specific primers. We found that the frequencies of these genes are significantly reduced in B-ALL cases when compared with their healthy counterparts. Furthermore, we found that these genes had an additive effect in reducing risk for developing the cancer. The results may be useful in early identification of children at risk for developing this cancer. Moreover, KIR-based therapies may prove to be useful in treating this cancer.

Résumé

La leucémie lymphoblastique aiguë des cellules Pré-B (B-ALL) reste le type de cancer le plus souvent diagnostiqué chez les enfants. Des études ont montré que des déterminants génétiques jouent un rôle important dans la susceptibilité/résistance au développement de ce cancer. À cet égard, les gènes *Killer-cell Immunoglobulin-like Receptor (KIR) s*ont d'une importance particulière. Ces gènes sont fortement polymorphiques et codent pour des récepteurs qui contrôlent l'activité fonctionnelle des cellules *Natural Killer* (NK). Notre hypothèse est que les gènes activateurs des KIR s'associent avec la résistance innée pour développer la B-ALL. Afin d'évaluer cette hypothèse, nous avons entrepris une étude de cas-contrôles chez des enfants canadiens-français dans laquelle nous avons utilisé l'ADN génomique de 100 patients atteints de B-ALL ainsi que l'ADN de 245 individus sains. La présence ou l'absence de chaque gène KIR a été détectée par PCR en utilisant des amorces de séquences spécifiques. Nous avons trouvé que la présence des gènes KIR activateurs est significativement diminuée chez les enfants leucémiques par rapport aux témoins. En outre, le nombre de ces gènes a aussi montré une association significative linéaire avec la résistance au développement d'une B-ALL. Cela suggère des effets additifs de ces gènes permettant de conférer une protection contre ce cancer. Ces résultats pourraient être utiles afin de déceler de façon précoce les enfants ayant un risque de développer cette leucémie. Enfin, des stratégies thérapeutiques basées sur les récepteurs KIR pourraient être envisagées et s'avérer utiles concernant le traitement de ce cancer chez les enfants.

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Abbreviations

ALL:	Acute Lymphoblastic Leukemia
AML:	Acute Myeloblastic Leukemia
CD:	Cluster of Differentiation
cCD3:	Cytoplasmic CD3
CALLA:	Common c-ALL Antigen
CLL:	Chronic Lymphoblastic Leukemia
CML:	Chronic Myeloid Leukemia
CTL:	Cytotoxic T Cell
CEACAM-1:	Carcinoembryonic Antigen-related Cell Adhesion Molecule 1
DAP:	Dynax Activation Protein
DNA:	Deoxyribonucleic Acid
DNAM-1:	DNAX Accessory Molecule 1
FAB:	French American British
FCRL:	FcR-like protein
GM-CSF:	Granulocyte-macrophage colony stimulating factor
GPI:	Glycosylphosphatidyl inositol
GVHD:	Graft Versus Host Disease
HCMV:	Human Cytomegalovirus
HLA:	Human Leukocyte Antigen
IFN-γ:	Interferon-gamma
IL-10:	Interleukin-10
ILT:	Immunoglobulin-Like Transcript
ITAM:	Immunotyrosine-based Activating Motifs
ITIM:	Immunoreceptor Tyrosine-based Inhibitory Motifs

KIR:	Killer-cell Immunoglobulin-like Receptor
KLRF1:	Killer-cell Lectin-like Receptor, subfamily F member 1
LAIR:	Leukocyte Associated Ig-like Receptor
LFA-1:	Lymphocyte Function-Associated Antigen 1
mAb:	Monoclonal Antibody
MAdCAM:	Mucosal Adressin Cell Adhesion Molecule
MHC:	Major Histocompatibility Complex
MICA:	MHC-class I Chain-related protein A
MICB:	MHC-class I Chain-related protein B
MIP-1α:	Macrophage Inflammatory Protein1α
MIP-1β:	Macrophage Inflammatory Protein 1β
MIR:	Macrophage Ig-like Receptors
N-CAM:	Neural Cell Adhesion Molecule
NHL:	Non Hodgkin Lymphoma
NK cells:	Natural Killer cells
NKG2:	Natural Killer Group 2
NKp44:	NK cell protein
NKR:	Natural Killer cell Receptors
NO:	Nitric Oxide
RAG:	Recombination Activating Gene
Rantes:	Regulated on Activation Normal T cell Expressed and Secreted
sIg:	Surface Immunoglobulin
SHP-1:	SH2-domain containing phosphatase-1
SAP:	SLAM-Associated Protein
SIGLEC-7:	Sialic acid binding Ig-like lectin 7
SLAM:	Signalling Lymphocyte Activation Molecule
SRRs:	SLAM-Related Receptors
TCR:	T-Cell Receptor
TdT:	Terminal deoxynucleotidyl Transferase
TGFβ:	Transforming Growth Factor beta

TH1:	T helper cell
TNF:	Tumor Necrosis Factor
ULBPs:	UL-16 Binding Proteins
VCAM:	Vascular Cell Adhesion Molecule
VLA-4:	Very Late Antigen-4
WBC:	White Blood Cell
WHO:	World Health Organization

CHAPTER 1

INTRODUCTION

Approximately 15000 children are diagnosed with cancer every year in North America, a rate that is increasing especially among Caucasian children (1). Among the diagnosed childhood cancers, the most common is acute lymphoblastic leukemia (ALL) (2). Studies show that genetic determinants play an important role in conferring susceptibility/resistance to the development of this cancer in children. For example, mutations in several genes involved in the regulation of cell cycle, DNA repair and metabolism of toxic substances in the body have been associated with susceptibility/resistance to this cancer in children (3). It is worth noting that Natural Killer (NK) cells spontaneously kill tumor cells, particularly the leukemic ones (4, 5). Therefore, these cells are likely to play a role in controlling leukemia. The functional activities of NK cells are mainly regulated by Killer-cell Immunoglobulin-like Receptors (KIR), which are encoded by the KIR family of genes (6). Interestingly, these genes are highly polymorphic (7).

KIRs are expressed on the surface of NK and other cells and act as receptors to recognize specific molecular structures on the surface of target cells. By activating or inhibiting NK cells, they control the functional activities of the cells. These receptors (especially their inhibitory versions) bind to different subsets of MHC class I antigens. The KIR genes encode two different kinds of receptors: activating and inhibitory, which activate and inhibit NK cell functions, respectively (8). The functions include killing of the target cells and secretion of cytokines and chemokines. The KIR gene polymorphism has been associated with resistance/susceptibility to infectious agents, malignancy and

autoimmune diseases (9, 10). However, little is known about the association of these genes with a child's innate susceptibility or resistance to ALL. This issue is addressed in this study. In the following sections, scientific literature on leukemia, KIR genes and their ligands is briefly reviewed.

1.1- Leukemia

1.1.1- Definition

Leukemia is a malignant proliferation of white blood cell precursors (blasts) in bone marrow or lymphoid tissue and their accumulation in peripheral blood, bone marrow, and body tissues. The word leukemia is literally a Greek word that means "white blood", referring to the color of the blood that it acquires due to accumulation of white blood cells.

1.1.2- Classification

Leukemia in children can be classified according to time (duration of its occurrence) as either acute or chronic. Acute leukemia is rapidly growing and can overwhelm the body within a few weeks or months. On the other hand, chronic leukemia is slow growing and worsens progressively over years. It is worth noting that chronic leukemia is rare in children. Leukemia can also be classified according to the cell line that becomes malignant and proliferates without control. The proliferative defect can occur in the lymphoid or myeloid lineage, resulting in acute lymphoblastic leukemia or acute myeloid leukemia (AML), respectively. Eighty percent of all childhood acute leukemia is ALL (60 to 80% is of B origin or B-ALL, 15-20% is of T origin or T-ALL and the rest are of uncertain origin (11, 12).

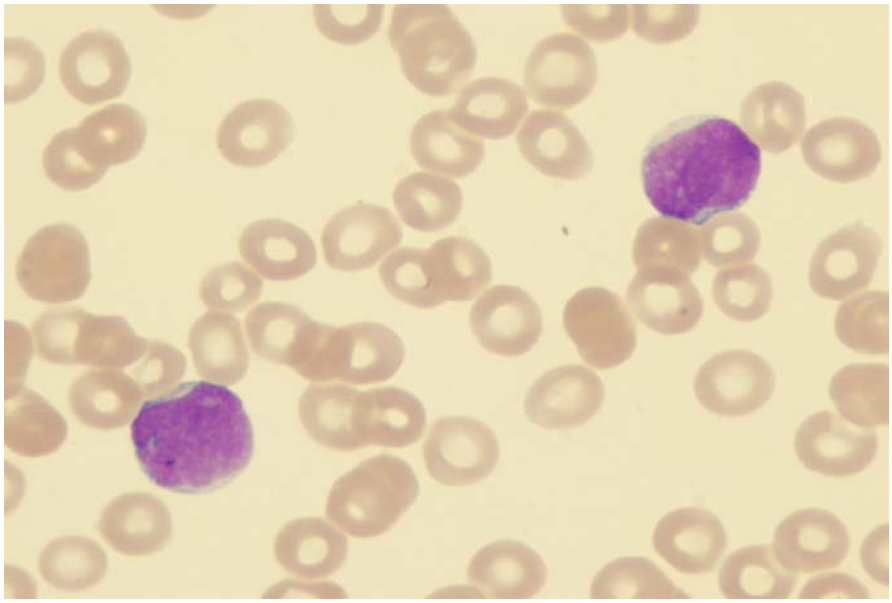
ALL cells can be examined under a light microscope and can be classified morphologically according to the criteria established by a group of French, American and British (FAB**)** hematologists (13-15).These criteria form the basis of the so-called FAB classification. The FAB classification distinguishes three variants of ALL:

ALL-L1: Most common, small uniform cells.

ALL-L2: Less common, large varied cells.

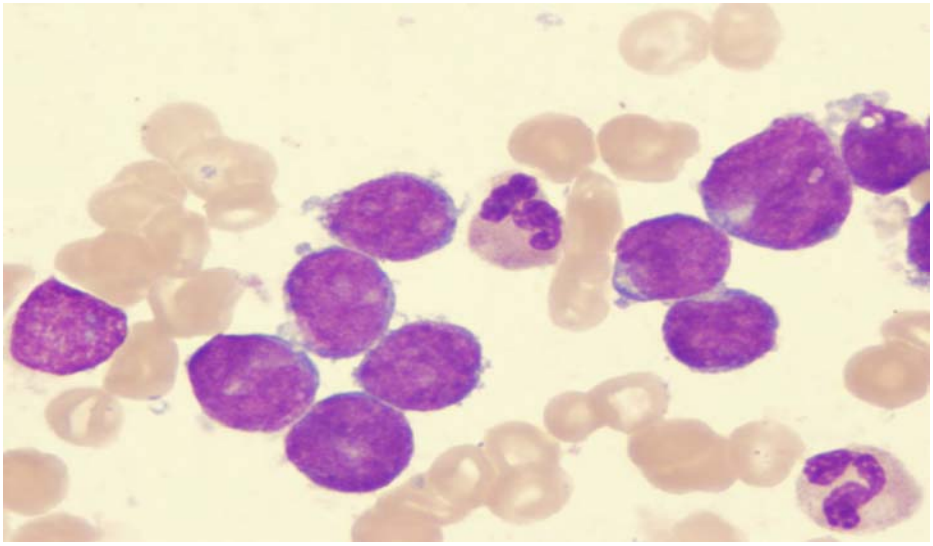
ALL-L3: Very rare, large varied cells with vacuoles (bubble-like features). The three types of ALL cells are shown in Figures 1 A, B and C.

Figure 1A. L-1 stage ALL



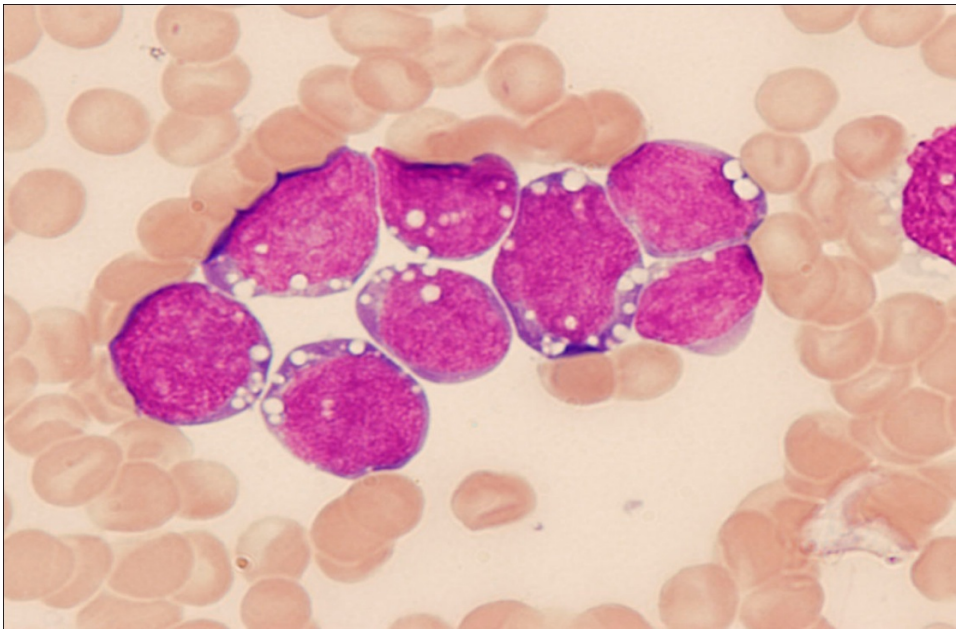
The two purple colored cells represent L-1 ALL cells. The other cells in the Figure are RBCs. Reproduced from Hess CE and Krstic L, 2009 (reference 14) after permission.

Figure 1B. L-2 stage ALL



The purple colored cells represent L-2 ALL cells. These cells have more abundant cytoplasm than L1. Reproduced from the Website (reference 14).

Figure 1C. L-3 stage ALL



Burkitt's Leukemia cells are uniform in size. The cytoplasm is very basophilic (blue) and contains a variable number of lipid-laden vacuoles appearing as white spots. Reproduced from Hess CE and Krstic L, 2009 (reference 14) after permission.

The WHO classification simply classifies ALL cases as precursor-B and precursor-T without additional categorization. The most common B-lineage ALL is the precursor-B phenotype with B-cell markers (CD19, CD22), TdT, cytoplasmic CD79A, CD34, CD10. This type has variably been called common precursor B ALL, early precursor-B ALL or simply B-ALL. The less common type, termed pro-B ALL, has a worse prognosis. Pre-B ALL is more mature than the common precursor B (16).

T lineage ALL accounts for only 15–20% of cases and can also be separated into phenotypic groups which may correspond to different stages of thymic T cell development (12). As in B-lineage ALL, the intermediate differentiated type is the most common. This common thymocyte type shows expression of the pan T-cell markers, CD2, cytoplasmic CD3 (cCD3), CD7, CD5 and characteristically shows coexpression of CD4 and CD8, and expression of CD1a. A more primitive type is called prothymocyte or immature thymocyte. Finally, a more mature phenotype than the common thymocyte type is called mature thymocyte. Again, because of a lack of conformity and variability in marker expression, the WHO classification recognizes only the precursor-T and -B groups without further immunophenotypic categorization (16). B and T lineage ALL phenotypes are listed in Table 1.

Table 1. Commonly used markers for immunophenotyping leukemia

Phenotype	Marker
General	CD34, HLA-DR, TdT, CD45
B-cell markers	CD10, CD19, cCD22, CD20, cCD79A, CD24, sIg
T-cell markers	CD1a, CD2, cCD3, CD4, CD8, CD5, CD7
Myeloid	cMPO, CD117, CD13, CD33, CD11c, CD14,CD15
B-lineage ALL phenotypes:	
Pro-B	TdT+, CD19/22/79A+, CD10–, c_–, sIg–
Common precursor-B	TdT+, CD19/22/79A+, CD10+,c_–, sIg–
Pre-B	TdT+, CD19/22/79A+, CD10+, c_+, sIg–
Burkitt	TdT–, CD19/22/79A+, CD10+, sIg+
T-lineage ALL phenotypes:	
Pro/immature Thymocyte	TdT+, cCD3+, CD2/5/7+/–
Common thymocyte	TdT+, cCD3+, CD2/5/7+,CD4+/CD8+, CD1a+
Mature thymocyte	TdT+/-, CD3+, CD2/5/7+, CD4+ or CD8+, CD1a–

TdT: Terminal deoxynucleotidyl transferase, cMPO: Cytoplasmic myeloperoxidase; Modified from Larson RA and Anastasi J, 2007 (reference 16).

1.1.3- Signs and symptoms of ALL

As shown in Figure 2, healthy bone marrow contains stem cells, which proliferate to produce progeny cells that develop and differentiate into three types of cells found in blood:

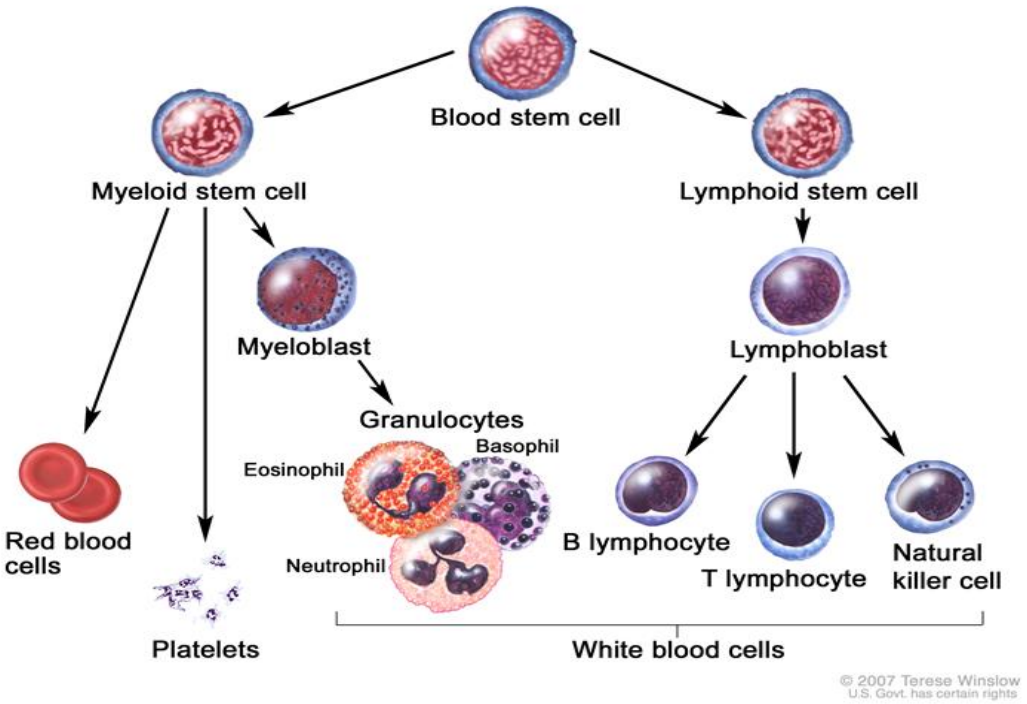
1. Red blood cells, which oxygenate the body in the healthy state.
2. White blood cells, which are responsible for controlling infections and abnormal cells.
3. Platelets, which assist in clotting to prevent blood loss.

In acute leukemia, cancerous cells multiply very quickly and replace normal cells. They takeover normal cells, as shown in Figure 3, and become permanent cells of the bone marrow, resulting in bone marrow failure. A person with ALL is more likely to bleed and have repeated infections because of fewer platelets and abnormal WBC. Other symptoms of acute leukemia include frequent or unexplained fever, bone and joint pain (as a result of the spread of lymphoblasts to the surface of the bone or into the joint from the marrow cavity), generalized weakness and fatigue, night- sweating, shortness of breath, excessive and unexplained bruising, and skin changes, which include lumps, rashes, and paleness, etc.

1.1.4- Incidence and risk factors

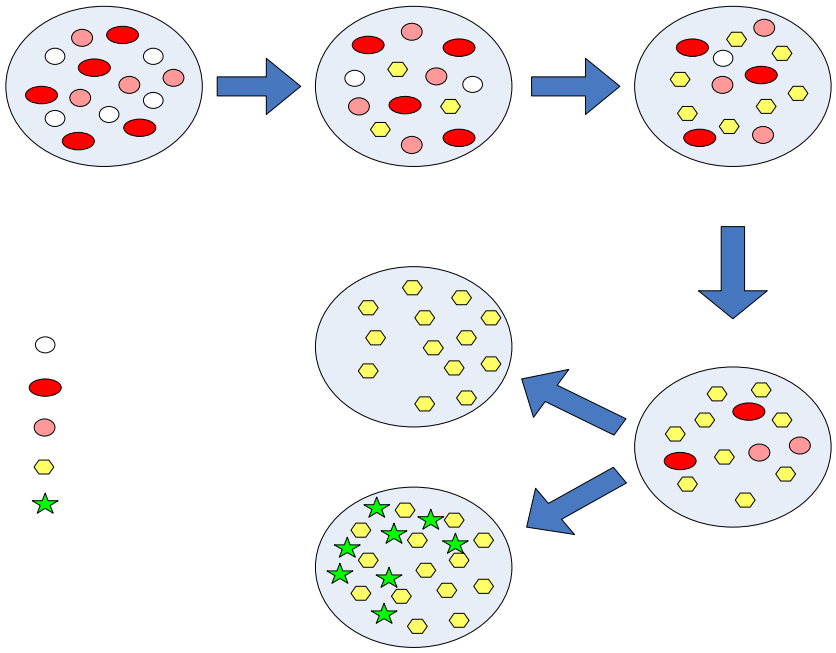
With the exception of leukemia, cancer in children and adolescents is rare. In North America, about 15000 new cases of cancer are diagnosed in Caucasian children (1). Pre-B ALL is the most common form of leukemia and represents about 95% of acute leukemia cases among children younger than 12 years of age. Most cases occur in children aged 3 to 7 years.

Figure 2. Normal hematopoietic stem cell proliferation



Three types of cells are produced by hematopoietic stem cells. The cells differentiate progressively as shown by the arrows. Reproduced from Winslow T, 2008 (reference 17) after permission.

Figure 3. Disturbances in the relative numbers of blood cell populations occurring in leukemia.



Cells are not drawn to actual scale. Reproduced from the Website (reference 18).

Normal Blood Cells

Appearance
WBC

WBC

RBC

Furthermore, it occurs slightly more often in boys than in girls, and the prognosis is better in girls (19). One reason for the worse prognosis in boys is the occurrence of testicular relapses among boys. The boys also appear to be at increased risk of bone marrow and CNS relapses for reasons that are not well understood (19). In contrast, acute myelogenous leukemia (AML) is more common in females. In addition, ALL occurs in Caucasian and Hispanic children more often than in black children. It may also occur in adults, but is not very common (20). Untreated acute leukemia is a fatal condition, usually because of complications that result from the infiltration of leukemic cells into bone marrow or vital organs. With treatment, the prognosis varies.

The factors that predispose children to leukemia are not fully understood. They seem to be complex and are likely to result from interplay between genetic make-up and environmental stimuli (21, 22). The risk of childhood leukemia is higher if a sibling has been diagnosed with the disease (3). Despite extensive studies on different molecules, proteomics and gene expression profiles, the exact cause of leukemia is still unknown. However, the clinicopathologic and molecular characterizations of different cytogenetic subgroups of B-ALL have been well established (23; reviewed in 24, 25). They are summarized in Table 2.

Other risk factors include exposure to very high levels of radiation, as people exposed to radioactive iodine radiation are much more likely to develop leukemia than those who have not been exposed. This is supported by the fact that the incidence of leukemia was increased in Japan and Chernobyl as a result of atomic bomb explosions and nuclear accidents, respectively (26). Internationally, the highest incidences of acute lymphoblastic leukemia (ALL) occur in Italy, the United States, Switzerland, and Costa Rica (27).

Table 2.Cytogenetic subgroups of pre B-ALL and their clinicopathologic features

Cytogenetic Subgroup	Frequency (%)	Cytogenetic Abnormality	Fusion Gene	Unique Immunophenotypic Features	Additional Molecular Abnormalities	Pharmacologic Features
Hyperdiploid ALL	27–29 (P) 6–8 (A)	51-65 chromosomes (+4, +14,+21, +X)	NA	NA	uncommon BCDG mutations (13%), FLT3 mutations (21%–25%)	Higher sensitivity to MTX, MP
ALL with t(12;21)	22–25 (P) 1–2 (A)	t(12;21) (p13;q22)	TEL/AML1 (ETVX/ RUNX1)	Early pre–B-ALL, My+	Monoallelic PAX5 deletions (28%)	Higher sensitivity to asparaginase
ALL with t(1;19)	3–6 (P) 5–7 (A)	t(1;19) (q23;p13)	E2A (TCF3)/ PBX1	Pre–B-ALL, CD34-/dim+, CD20-, CD9++	NA	NA
Philadelphia1 ALL	2–3 (P) 20–30 (A)	t(9;22) (q34;q11.2)	BCR/ABL (P190, P210)	NA	IKZF1 (Ikaros) deletions, BCDG mutations in 66%	NA
ALL with t(v;11q23); MLL rearranged	2–3 (P) 5–7 (A)	t(4;11) (q21;q23) t(19;11) (p13;q23)	AF4/MLL ENL/MLL	Early pre-B, CD10-, CD15+, sCD22-, CD65+, NG2+	FLT3 mutations (18%) Increased expression of HOX genes	Higher sensitivity to cytarabine
Hypodiploid ALL	5%–6% (P) NA (A)	<46 chromosomes (typically near haploid or low hypodiploid)	NA	NA	BCDG mutations in 100%	NA
ALL with eosinophilia	<1	t(5;14 q31;q32)	IL3/IGH	NA	NA	NA

Abbreviations: A and P in the Frequency column: % in adults and children, respectively, BCDG: B-cell development genes (eg, PAX5, EBF1, IKZF1, LEF1, TCF3, BLNK), FLT3: fms-related tyrosine kinase 3, MP: mercaptopurines, MTX: methotrexate, My: myeloid antigens, NA: not applicable or not known.
Redrawn from Onciu M, 2009 (reference 24).

Biological risk factors for ALL include genetic conditions such as Down's syndrome, neurofibromatosis type 1, Noonan syndrome, Fanconi anaemia and Poland syndrome. Additionally, some environmental risk factors include prenatal exposure to nicotine or alcohol, prenatal exposure to X-rays and previous chemotherapy treatment (2).

The prognosis for ALL depends on a patient's age, white blood cell count, the degree of metastasis to the central nervous system, and the initial response to chemotherapy. Bad prognostic risk factors include age less than one year or more than ten years at the time of diagnosis, high white blood cell counts, metastasis of the leukemic cells to the central nervous system, and absent or low response to initial chemotherapy (28).

1.1.5- Treatment

The most common treatment used in ALL is chemotherapy. It can be administrated by a variety of routes including oral, intramuscular, intravenous and intrathecal (injection into the fluid surrounding the brain and spinal cord). Chemotherapy for ALL uses a combination of several anti-cancer drugs given over a long period of time (usually about 2 years; reviewed in reference 29). The most commonly used drugs and their treatment protocols are listed in Table 3.Recently, monoclonal antibodies (mAB) have been used in the treatment of haematological malignancies, including ALL. In this regard, Rituximab, which is a mAb directed against the B cell lineage antigen CD20, has been used in combination with chemotherapy for treatment of adult lymphoma. The antibody shows improvement of disease-free survival in adults with non-Hodgkin's lymphoma (NHL) (30). It is unlikely that mAbs will have adequate activity to be effective for paediatric hematologic malignancies when used alone. However, rare cases of complete remissions in adults and children with ALL have been reported with mAbs targeting CD20, CD33, and CD52 (31, 32). Radiotherapy has also been used to kill tumour cells and prevent their growth.

The standard protocol for ALL treatment can be divided into three phases: remission-induction, consolidation, and maintenance. Of these, the maintenance phase is the most prolonged.

Remission-induction phase: The aim of this phase is to destroy leukemic cells, thereby placing the patient in temporary recovery, or a remission state, and restore normal hematopoiesis.

Consolidation phase: In this stage, the purpose is to eradicate any remaining inactive leukemic cells.

Maintenance and re-induction phase: Long-term maintenance therapy has been found to decrease the risk of relapses (recurrence of leukemia). It is important to prevent relapses of the disease because they are more difficult to treat. Secondary remissions, if they occur, are usually of short duration.

Bone marrow or cord-blood stem cell transplantation is commonly used to treat ALL that has not responded to chemotherapy (reviewed in 33). This transplantation consists of three steps: 1) collection of healthy stem cells from a donor without cancer or from the patient himself or herself, 2) administration of high doses of chemotherapy and possibly radiation therapy to kill any remaining leukemia cells, and 3) infusion of the healthy stem cells to produce normal, healthy blood-forming cells.

Table 3 Typical ALL-treatment protocols.

Week	Treatment	Schedule	Chemotherapeutic class
Continuation for high-risk patients (120 weeks)			
1	Etoposide; cyclophosphamide	Weekly	Topoisomerase II inhibitor; alkylating agent
2	Methotrexate; mercaptopurine	Weekly methotrexate; daily mercaptopurine	Antifolate; purineantimetabolite
3	Methotrexate; cytarabine	Weekly	Antifolate; cytidineantimetabolite
4	Dexamethasone; vincristine	Daily dexamethasone; weekly vincristine	Glucocorticoid; antimicrotubule agent
5	Etoposide; cyclophosphamide	Weekly	Topoisomerase II inhibitor; alkylating agent
6	High-dose methotrexate; mercaptopurine	Weekly methotrexate; daily mercaptopurine	Antifolate; purineantimetabolite
7	Etoposide; cytarabine	Weekly	Topoisomerase II inhibitor; cytidineantimetabolite
8	Dexamethasone; vincristine	Daily dexamethasone; weekly vincristine	Glucocorticoid; antimicrotubule agent
Continuation for low-risk patients (120 weeks)			
1	Methotrexate; mercaptopurine	Weekly methotrexate; daily mercaptopurine	Antifolate; purineantimetabolite
2	Methotrexate; mercaptopurine	Weekly methotrexate; daily mercaptopurine	Antifolate; purineantimetabolite
3	Methotrexate; mercaptopurine	Weekly methotrexate; daily mercaptopurine	Antifolate; purineantimetabolite
4	Dexamethasone; vincristine	Daily dexamethasone; weekly vincristine	Glucocorticoid; antimicrotubule agent
5	Methotrexate; mercaptopurine	Weekly methotrexate; daily mercaptopurine	Antifolate; purineantimetabolite
6	Methotrexate; mercaptopurine	Weekly methotrexate; daily mercaptopurine	Antifolate; purineantimetabolite
7	High-dose methotrexate; mercaptopurine	Weekly methotrexate; daily mercaptopurine	Antifolate; purineantimetabolite
8	Dexamethasone; vincristine	Daily dexamethasone; weekly vincristine	Glucocorticoid; antimicrotubule agent

Adapted from Cheok M, Evans W (reference 29).

1.2- Human Natural Killer (NK) Cells

NK cells are low-density large granular lymphocytes. They comprise approximately 10-15% of all circulating lymphocytes in the bloodstream and are also present in tissues and lymphoid organs. NK cells have the ability to kill virus-infected or tumor cells without prior sensitization and for this reason are called Natural Killer cells (34). NK cells respond to intracellular microbes by killing the infected cell and by producing the macrophage activating cytokine IFN-γ. Phenotypically, NK cells are CD3-, CD2+, CD16+, CD56+, CD14-, and CD19-. Human NK cells can be divided into two subsets based on their cell-surface density of CD56 and CD16. These two markers are usually expressed reciprocally on two subsets of NK cells. They are divided into $CD56^{bright}$ CD16- and $CD56^{dim}$ CD16+ subsets. The cells in the two subsets differ in their proliferative potential, homing characteristics, functional capabilities and responses to different cytokines (listed in Table 4). The $CD3^{-}CD56^{dim}$ $CD16^{+}$ subset is more cytotoxic, and expresses intermediate affinity receptors for IL-2 and higher levels of Killer-cell Ig-like Receptors (KIR). This subset comprises more than 90% of NK cells in blood. In contrast, the $CD56^{bright}$CD16- subset has greater capacity of cytokine production, but has lower ability for cytotoxicity and lacks perforin granules (35, 36).

Table 4. Characteristics of two major human NK cell subsets.

Characteristic	$CD56^{bright}CD16^{-}$	$CD56^{dim}CD16^{+}$
IL-2R	High affinity	Low affinity
Cytotoxicity	+	+++
Cytokine production[a]	+++	+
Perforin	Low	High
CD62L[c]	High	Low
CCR7[d]	High	Low
NKG2 Expression	High	Low
IL-7R	High	Low
ICAM-3	High	Low
KIR	Low	High
SHIP-1[e]	High	Low
c-Kit[g]	High	Low
LFA-1	Low	High
CD3ζ-chain[f]	Low	High
Lysozyme production	Yes	No
Main location	Lymph nodes[b]	Blood
ADCC Function	Inefficient	Efficient

Abbreviations: High and Low refer to levels of expression.
[a] Cytokines include IFN-γ, TNF-α, TNF-β, IL-5, IL-13, and GM-CSF.
[b] In T cell-rich areas of lymph nodes and other secondary lymphoid organs and in body tissues and organs such as liver.
[c,d] Lymph node homing receptors.
[e] Needed for IFN-γ production.
[f] Signaling partner for activating receptors such as CD16a.
[g] c-Kit receptor tyrosine kinase needed for IL-mediated proliferation. KIR: Killer-cell Ig-like Receptors, NKG2: NK cell group 2, CD62L: CD62 ligand.
Adapted from Iannello A et al., 2008 (reference 7).

Some immunologists also differentiate between $CD16^{high}$ and $CD16^{dim}$ subsets. They have described NK cells expressing both CD56 and CD16 (36a).The incubation of these cells with different cytokines may change their phenotypic appearance, as well as functional and homing characteristics. For instance, IL-2, IL-12 and IL-15 have the ability to convert both $CD56^{high}CD16^{-}$ and $CD56^{low}CD16^{+}$ NK cell subsets into $CD56^{bright}$CD16+ cells (7). On the other hand, TGF-β1 converts $CD16^{+}$ NK cells into $CD16^{-}$ NK cells (7, 36a). NK cells are important effectors and regulatory cells of the immune system. Their important functions are given below:

1- Defending the host against viral infections, intracellular pathogens and malignancy:

NK cells can recognize tumor cells and virus-infected cells and kill them without prior sensitization to their antigens. In addition to direct killing, they secrete anti-microbial peptides called α-defensins. Like activated T cells, NK cells can produce interferon-γ (IFN-γ), so named because of its ability to inhibit or interfere with viral replication. IFN-γ is a potent activator of macrophages, and leads to differentiation of naive CD4+ T cells into T helper type 1 (TH1) effector cells (7). In addition to IFN-γ, NK cells have also been shown to secrete TNF-α, GM-CSF, IL-5, IL-13, IL-10, TGF-ß, MIP-1α, MIP-1ß, RANTES, and nitric oxide (NO). More recently, these cells were also shown to produce IL-22 (37).

2- Role in pregnancy:

NK cells function in pregnancy through their interaction with fetal extravillous trophoblasts to remodel maternal vasculature, and increase fetal blood supply. NK cell-secreted IFN-γ plays a role in inducing placental vasculature (38). A failure of early trophoblast invasion and remodeling of the spiral arteries leads to poor blood supply to the placenta. This may result in pre-eclampsia, which is a disease that occurs in the third trimester of pregnancy (after 20 weeks). This disease is characterized by pregnancy-induced hypertension and proteinurea. Pre-eclampsia leads to maternal and fetal mortality worldwide and it is the most common cause of preterm birth. Furthermore, NK cells' hyperactivity is suspected of being responsible for recurrent miscarriages (three or more abortions) (39). Also, some studies have shown that females with recurrent miscarriages have significantly increased numbers of NK cells expressing activating KIR receptors compared to normal fertile women (40). The women carrying a high content of activating

KIR genes have about a threefold increased risk of developing recurrent miscarriages (40).

3. Controlling leukemia:

NK cells have a significant role in controlling the development of leukemia as they can kill leukemia cells growing in culture as well as *in vivo* in animal models (4). In addition, blocking of the interaction between inhibitory receptors and their MHC ligands in mice enhances their anti-tumor activity and control of tumor growth (41). Moreover, leukemia cells can express ligands for many activating KIR receptors. This means that NK cells can kill tumor cells by default if they are not inhibited by the engagement of inhibitory KIR receptors. The role of NK cells in controlling leukemia is also suggested by the fact that leukemia cells do not down regulate HLA-C and HLA-Bw4, which act as ligands for several inhibitory receptors of NK cells. On the other hand, leukemic cells down-regulate HLA-A and HLA-Bw6 (42). It is of interest that NK cells do not have any receptors that recognize these MHC antigens. In addition to the direct killing of tumor cells, the activated NK cells secrete IFN-γ, which is a potent cytostatic agent (43).

4. Role as memory cells:

Despite being effector cells of innate immunity, NK cells have been shown to have immunological memory. This feature of NK cells was discovered in RAG-/-mice, which lack both T and B cells. The researchers discovered that recall responses to hapten-induced hypersensitivity in these mice was mediated by NK cells (44). The exact mechanisms behind NK cell dependent memory or recall responses are not well defined. Another study using mouse models of cytomegalovirus infection showed that like T cells, NK cells bearing the virus-specific LY 49H receptor undergo four phases of the adaptive immune response (45). These phases had not been previously documented in NK cells. This ability of NK cells has major implications in the generation of immunological memory against pathogens, malignancy and vaccination strategies.

1.2.1- NK receptors (NKR)

NK cells do not have a well-defined single molecular structure to recognize target cells. Instead, they express a variety of molecular structures that bind to MHC and non-MHC molecules on the target cells. NKRs can be divided into inhibitory and stimulatory types depending on the nature of the signal they send to NK cells after binding to their specific ligands. Each NK cell expresses inhibitory and stimulatory NKRs. The human NKR can be divided into MHC and non-MHC-binding receptors.

1.2.1.1- MHC-binding receptors

These receptors recognize and bind MHC class I molecules. They include KIR, NKG2, and Ig-like transcript (ILT) families, as shown in Table 5.

1.2.1.1.1-CD94/NKG2 killer lectin-like receptor (KLR)-C (NKG2/CD94 family)

These are also known as the (NK Group 2) NKG2/CD94 family of receptors. The genes for these receptors are located on human chromosome 12p12.3–p13.2 in a region called the NK gene complex (NGC). These receptors are type II, C-type, lectin-like integral membrane glycoproteins. This family contains four receptors: A/B (KLR-C1), C (KLR-C2), E/H (KLR-C3), and F (KLR-C4). B and H represent splice variants of A and E genes, respectively (7).

Of these receptors, CD94/NKG2A carries two ITIMs in its long cytoplasmic tail. It is expressed on a subset of human NK cells having the $CD56^{high}$ $CD16^{low}$ phenotype as well as on the $CD56^{low}$ subset of NK cells, albeit at lower levels. This receptor has an inhibitory function. NKG2C has a short cytoplasmic tail, links noncovalently with a homodimer of DAP-12, and activates NK cells upon binding with its ligands. NKG2E has a charged amino acid (lysine) in its transmembrane region, but it does not associate with DAP-12. NKG2A and NKG2C are expressed on overlapping subsets of CD56+ NK cells. NKG2C and NKG2E act as activating receptors. NKG2F is a unique gene within

Table 5. MHC-binding receptors and their characterictics.

Gene family	Ligand	Expression	Function
KIR (CD158) family	HLA-C	NK, T	+/ -
(NKG2/CD94 family)	HLA-E	NK	+
ILT-2 (CD85j)	HLA-G	NK , Mac, DC	+/-
CD160 (BY55)	HLA-C, G	NK	+

Abbreviations: HLA: Human Leukocyte Antigen, NK: Natural Killer cells, T: T cells, Mac: macrophages, DC: dendritic cells. The signs + and – indicate activating and inhibitory functions, respectively.

Adapted from Iannello A et al., 2008 (reference 7).

the NKG2 family whose translated product contains a positively charged residue in its transmembrane region, an intracellular ITIM-like sequence and an extracellular domain (62 residues) that is truncated relative to other NKG2 molecules. The NKG2 receptors bind the non classical MHC class Ib molecule HLA-E (46-48).

1.2.1.1.2-ILT (CD85) family

ILT or Ig-like transcripts are also known by two other names: Leukocyte Ig-Like Receptor (LILR) and Macrophage Ig-like Receptors (MIR) (7, 49). This family consists of 13 members with either inhibitory or activating properties. They are expressed on monocytes, macrophages, dendritic cells, and some subsets of B and T cells. A single member of this family ILT2 is expressed on a subset of NK cells (49). The ILT family members bind to classical and non classical HLA class I molecules. They are located on human chromosome 19 close to the KIR gene cluster.

1.2.1.1.3-CD160 (BY55)

CD160 is a GPI anchored, Ig-like molecule expressed on the $CD56^{dim}$ subset of NK cells, γδ T cells, and a subset of CD8+ T lymphocytes. It binds HLA-G, HLA-C and other HLA molecules (50). CD160 positive NK cells and CTL usually accumulate in inflammatory conditions. The NK cell activation via CD160 leads to secretion of proinflammatory cytokines, which include IFN-γ, TNF- α and IL-6 (51).

1.2.1.2- Non MHC-binding receptors

These receptors recognize molecular structures other than MHC molecules. The most important members of this group are discussed below, and the list of all receptors and their characteristics is shown in Table 6.

1.2.1.2.1-NKG2D receptors (KLR-K1; CD314)

NKG2D are type II lectin-like receptors. They were first identified as a member of the NKG2 family. However, they are not typical members of this family. They are expressed on all human NK cells, and their expression is upregulated by IL-15 and IL-12. On the

reverse, certain other cytokines e.g., TGF-β and IL-10 decrease their expression. They are also expressed on resting and activated human CD8+ T lymphocytes (7, 52).

NKG2D receptors do not recognize and bind HLA-E, as do all members of the CD94/NKG2 family. Instead, they bind MICA, MICB and HCMV-induced ULBPs (53; reviewed in 54; shown in Figure 4). The MIC genes are highly polymorphic, and are located on human chromosome 6q25 outside the MHC locus. The ligands for NKG2D are usually not expressed under physiological conditions. However, they are induced on body cells in response to stress, DNA damage, infection and malignancy. The expression of these ligands on body cells flags them for destruction by NK cells via NKG2D (54, 55).
In addition to having different ligands, NKG2D also differs from other members of the NKG2 family; it does not need CD94 for expression on the cell surface (55).

1.2.1.2.2- Natural cytotoxicity receptors (NCRs)

There are three NCRs: NKp46 (CD335), NKp30 (CD337), and NKp44. NKp46 and NKp30 are expressed on resting and activated NK cells, whereas NKp44 is expressed only on cytokine-activated NK cells. The engagement of these receptors with their ligands triggers NK cell-mediated killing and secretion of IFN-γ. The ligands for the NCR mostly remain unknown (7). Only NK46 and NKp30 were reported to recognize the haemagglutinin antigen (HA) of the influenza virus (56).

1.2.1.2.3- SLAM-related Receptors (SRRs)

The signalling lymphocytic activation molecule (SLAM) family of receptors is expressed by a wide range of immune cells. Through their cytoplasmic domain, the SLAM family receptors associate with SLAM-associated protein (SAP) and SAP-related molecules. SLAM (CD150) is expressed on the surface of T cells (57,58).

The SRR family includes 2B4 (CD244), NTB-A (Ly108), and CD2-like Receptor on Activated Cytoxic Cells (CRACC; CD139). These receptors are related to SLAM, as they all use similar signalling molecules, SAP or related molecules. The genes for SSRs are located on human chromosome 1q22. They are expressed on NK cells, monocytes, basophils, γδ T cells and CD8+ T cells of the effector memory phenotype (7, 57).

1.2.2- NK cell Co-receptors

Co-receptors usually add to the strength of activating signal. These molecules themselves are unable to trigger NK cell functions. According to this definition, some co-receptors may be able to trigger NK cell functions under certain conditions and therefore may qualify as receptors. For example, the Lymphocyte Function-associated Antigen 1 (LFA-1) can trigger NK cell-mediated lysis under appropriate conditions, and could be considered as an activating receptor (59).

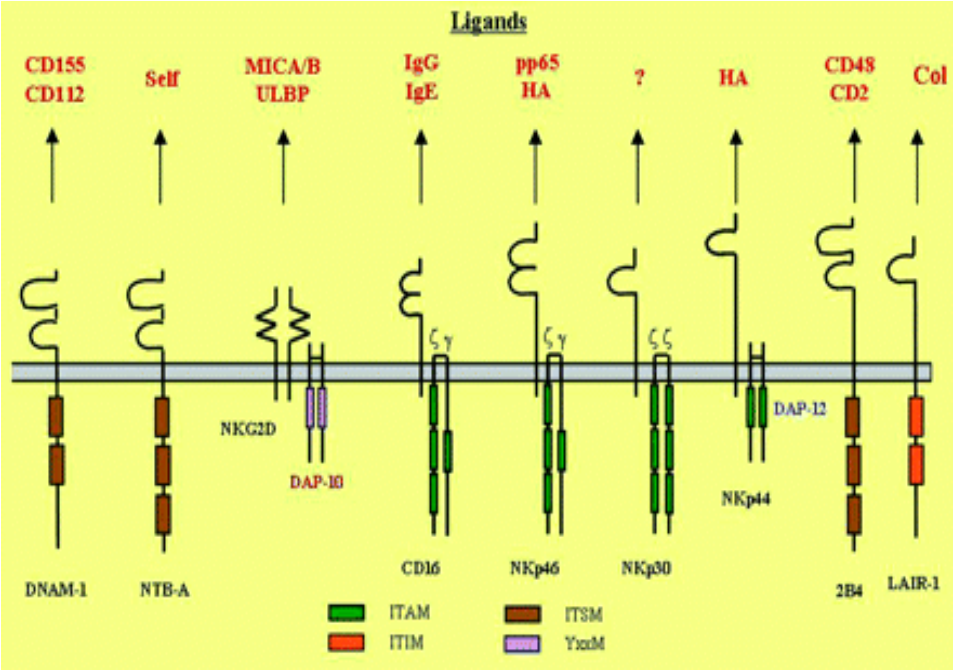
NK cells express several coreceptors, which bind to their cognate ligands on target cells and send co-stimulatory signals. These signals add to the overall strength of the activating signal. Adhesion molecules, such as integrins, selectins, and several Ig-like molecules usually act as co-receptors for NK cells. Table 7 lists NK cell co-receptors, their expression and function.

Table 6. Non MHC-binding NK cell receptors.

Receptor	Expression	Ligands	Function
NKG2D	NK,CD8+T	MICA, MICB, ULBPs	+
NKp46	NK	HA	+
NKp44	Activated NK	HA	+
NKp30	NK	?	+
NKR-P1 (CD161; KLR-B1)	NK, NKT, CTL	LLT-1	+/–
2B4(CD244)	NK , T, Monocytes, Basophils	CD48	+/–
KLR-G1	Mast cells, NK , CTL	Cadherens	–
FcRL	B cells, NK	IgG complexes	–
NKp80	NK	AICL	+
DNAM-1	NK, B cells	Nectin-2, PVR	+
Four Ig-like B7 homologues (4IgB7H or B7H)	NK,T	PD-1	+/–
SIGLEC-7	NK	Sialic acid	–
CEACAM-1 (CD66a)	NK	CEA and CEA-related Proteins	–
LAIR	Leukocytes	Collagen	–?

CEA: Carcinoembryonic antigen; CEACAM-1: CEA-related cell adhesion molecule; FcRL6: FcR-like protein 6; SAP: Signaling Lymphocyte Activating Molecule (SLAM)-Associated Protein; SIP: Stress-induced proteins [MICA, MICB, UL16-binding protein (ULBP)]; DNAM-1: DNAX accessory molecule 1; SIGLEC-7: Sialic acid-binding Ig-like lectin 7. The designations (+), (–), and (+/–) indicate that the function is activation, inhibition, and both, respectively. Redrawn from Iannello A et al., 2008 (reference 7).

Figure 4. Non-MHC-binding NK Cell Receptors.



ITSM: Immunoreceptor tyrosine-based switch motif; Col: Collagens; NTB-A: NK-T-B: NK, T-B cell antigen; LAIR-1: Leukocyte-associated Ig-like Receptors. The YxxM motif, when phosphorylated, recruits PI-3K. Not drawn to scale. The question mark (?) indicates that the ligand is unknown. Reproduced from Iannello A et al., 2008 (reference 7) after permission.

Table7. Human NK cell co-receptors, their expression and function.

Name	Ligand	Expression	Function
CD2 (LFA-2)	D58 (LFA-3) CD48 (weakly)	All NK	Costimulation, adhesion
LFA-1 (CD11a/CD18)	CD54 (ICAM-1–5)	All NK	Costimulation, adhesion Cytoskeleton rearrangement
CD8	MHC class I	Subset	Costimulation, adhesion
CD69	Unknown	Activated NK	Costimulation
CD56 (N-CAM)	Self	Subset	Homotypic adhesion
CD59	C8, C9	All NK	Adhesion, costimulation
CD57	Unknown	Subset	Marker of senescence
CD28	B-7	Fetal NK	Costimulation
CD27	CD70	Subset	Costimulation
CD44	Hyaluronic acid	Activated NK	Costimulation, adhesion
VLA-4	Fibronectin, VCAM-1 MAdCAM-1	Subset	Adhesion, diapedesis
VLA-5	Fibronectin	Subset	Adhesion, diapedesis

N-CAM: Neural cell adhesion molecule; VCAM: Vascular cell adhesion molecule; MAdCAM: Mucosa addressin cell adhesion molecule.

Adapted from Iannello A et al., 2008 (reference 7).

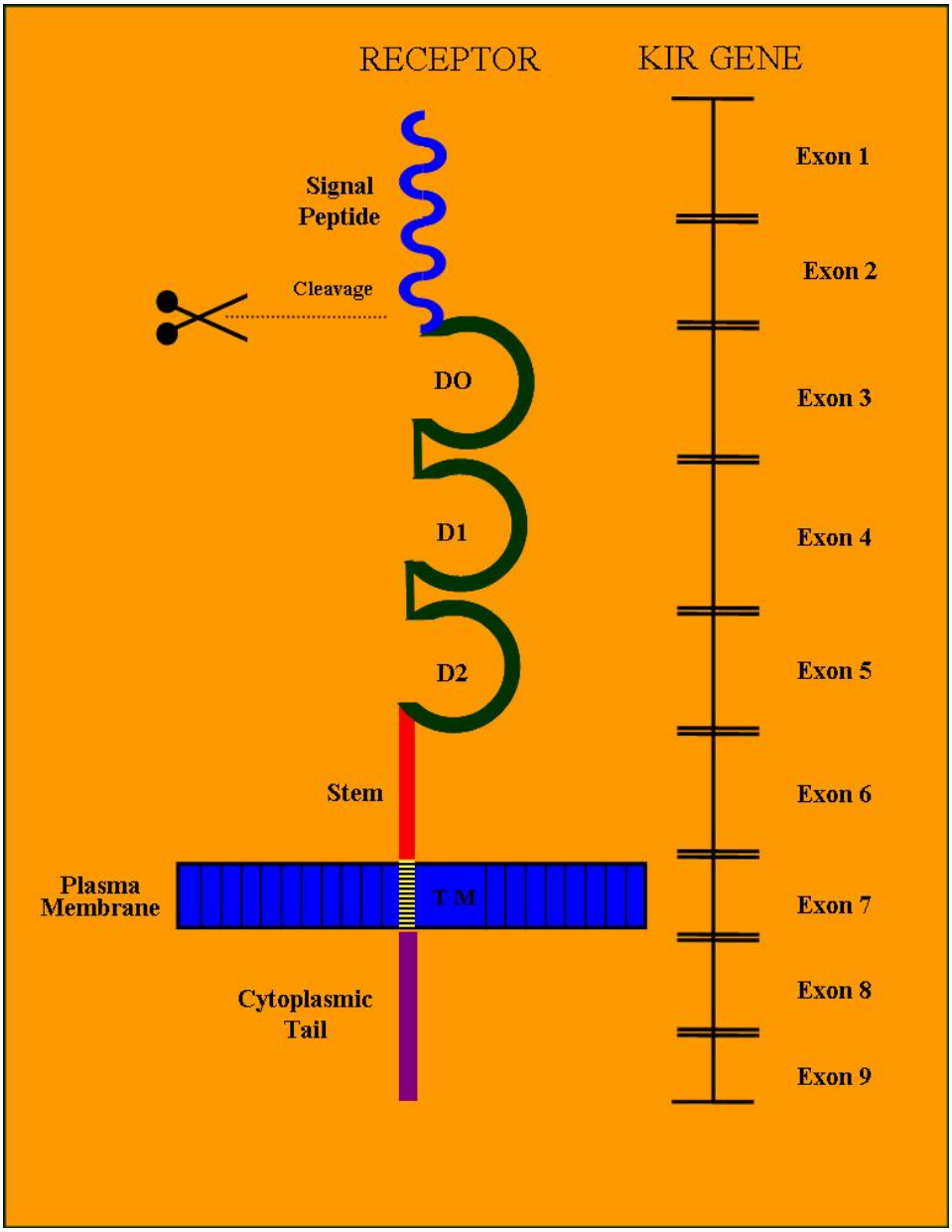
1.3- KIR family of NKRs

The KIRs (Killer-cell Immunoglobulin-like Receptors) are so named because they share structural homology to immunoglobulin molecules. They are type I integral membrane glycoproteins. They are usually expressed as monomers on the surface of natural killer (NK) cells. These receptors play an important role in controlling NK cell activities. To date, fifteen KIR genes, which include two pseudo genes, have been well described. These genes are located on human chromosome 19q13.4 in a tandem head to tail fashion in a short 150 kb region called the Leukocyte Receptor Complex (LRC). The structure of a typical KIR gene and the receptor is shown in Figure 5.

Each KIR has an extracellular region, a stem, a transmembrane region and a cytoplasmic tail. The extracellular region binds the receptor ligand and consists of two or three immunoglobulin (Ig)-like domains. The cytoplasmic tail may be short or long. Each long-tail KIR has two immunoreceptor tyrosine-based inhibitory motifs (ITIM) and is inhibitory in function. When the receptor binds to its ligands, the tyrosine residues in the tail become phosphorylated and recruit SH-2 domain-containing phosphatases: SHP-1 and 2. These phosphatases dephosphorylate several substrates involved in the NK cell activation cascade. Dephosphorylation transiently inhibits NK cells from triggering their effector functions (60).

The KIR receptors with a short cytoplasmic tail possess a charged amino acid (lysine) in their transmembrane regions and associate non-covalently with an adaptor protein called KARAP/DAP-12, which has immunotyrosine-based activating motifs (ITAMs) in its cytoplasmic tail. Upon binding to the MHC-ligands, the tyrosine residues in the ITAMs become phosphorylated, recruit several kinases and ultimately trigger NK cell-mediated killing and cytokine secretion. Each KIR gene has been named according to

Figure 5. The structure of a typical KIR gene and the receptor.



A typical KIR gene comprises nine exons shown here on the right side of the figure. Double horizontal lines in the gene indicate introns. The schematic structure of the encoded receptor is shown on the left. The part of the receptor encoded by each individual exon is also indicated. The scissor in the figure indicates cleavage site for the signal peptide. The letters N and C designate N- and C-terminals of the protein, respectively. D0, -1, -2: Extracellular Ig-like domains; TM: Transmembrane region. Not drawn to scale. Reproduced from Iannello A et al., 2008 (reference 7) after Publisher's permission.

the number of immunoglobulin-like domains in the extracellular regions and the length of the cytoplasmic tail present in its encoded receptor. In this taxonomy, 2D refers to two domain-, and 3D refers to three domain-receptor encoding gene. The letters (L) refer to long tail and (S) to short tail encoding genes. For example, KIR2DL1 means a gene that encodes a receptor having two extracellular domains and a long tail. The final number differentiates between individual KIR genes in each group.

The inhibitory KIR genes discovered so far include KIR2DL1, KIR2DL2/3(alleles), KIR2DL4, KIR2DL5a, KIR2DL5b, KIR3DL1, and KIR3DL2 (61, 62). The six activating genes include KIR2DS1-5 and KIR3DS1. All activating KIR genes represent independent loci on the chromosome. However, KIR3DS1 represents allelic variants of KIR3DL1 and encodes short tailed activating receptors (61, 63). KIR2DL4 is an unusual KIR; it is not expressed clonally on NK cells as other inhibitory KIRs are (64). It is expressed on all NK cells in all humans, and it expresses an inhibitory ITIM in its cytoplasmic tail but also expresses a charged amino acid (arginine) in its transmembrane region and interacts covalently with its signaling partner, the γ chain of FcεRI(64). In other words, it exhibits dual characteristics of an activating and an inhibitory KIR. The receptor interacts with HLA-G (a non classical MHC class Ib antigen).

1.3.1- The KIR ligands

The two-domain KIRs bind HLA-C antigens. These antigens are divided into group I and II, which are characterized by the presence of an asparagine and a lysine at position 80 in their amino acid sequences, respectively. KIR2DL1 and its allelic forms encode receptors that recognize group II HLA-C antigens (HLA-Cw1, 3, 7, 8, 13 and 14). On the other hand, KIR2DL2 and KIE2DL3 recognize group I HLA-C antigen (HLA-Cw2, 4, 5, 6, 17 and 18). It is noteworthy that all the known HLA-C molecules in humans (Cw1-18) are recognized by either KIR2DL1 or 2DL2/3 receptors (65-69). The affinity of different KIRs to bind their respective HLA-C ligands differ from one to another, e.g., KIR2DL1 binds with high affinity to their group II HLA-C ligands, whereas KIR2DL3 binds with its group I HLA-C ligands relatively weakly. The allelic variant KIR2DL2 binds with its HLA-C ligands with intermediate affinity. These binding characteristics have an important impact on NK cell functions. For example, in a person who is homozygous for

group II HLA-C and co-expresses KIR2DL1, NK cells will be under tight inhibition compared to a person who is homozygous for group I HLA-C and co-expresses KIR2DL3 (62-66). The three-domain KIR3DL1 and its activating allotype KIR3DS1 bind the HLA-Bw4 serospecificity molecules, which have isoleucine at position 80 (70, 71). Their binding to the HLA-Bw4 serospecific molecules that have threonine at position 80 is relatively weak. It is worth mentioning that HLA- B allotypes have two mutually exclusive serotypes, namely Bw4 or Bw6. HLA-Bw4 antigens could have threonine or isoleucine at postion 80 of their amino acid sequence. It is noteworthy that different KIR3DL1 allotypes also vary in their affinity for their HLA-Bw4 ligands. The activating short-tailed KIRs have been shown to bind HLA-ligands with very low affinities compared to the inhibitory KIR receptors (67). Table 8 lists KIRs, their distribution, signaling partners and ligands.

1.3.2- KIR haplotypes

KIR genes vary from one person to another. The degree of diversity in the KIR genes matches that found in HLA genes. There are two mechanisms that contribute to this diversity:

(1) KIR haplotypes differ in gene content.

(2) KIR genes are highly polymorphic.

Because of this diversity, unrelated individuals rarely have identical KIR genotypes. Based upon their gene contents, KIR haplotypes can be divided into two distinctive groups: A and B.

Table 8. Human KIRs (CD158) and their ligands.

Receptor	Distribution	Signaling partner	Ligand
(I) Activating KIR			
1. KIR2DS1 (p50.1)	NK	DAP-12	HLA-C II, ?
2. KIR2DS2 (p50.2)	NK	DAP-12	HLA-C I, ?
3. KIR2DS4 (p50.3)	NK	DAP-12	HLA-Cw4, ?
4. KIR2DS3, 5	NK	DAP-12	?
5. KIR3DS1 (p70)	NK	DAP-12	HLA-Bw4-I, ?
(II) Inhibitory KIR			
1. KIR2DL1 (p58.1)	NK, CTL	SHP-1, 2	HLA-C II
2. KIR2DL2/3 (p58.2)	NK, CTL	SHP-1, 2	HLA-C I
3. KIR3DL1 (p70; NKB1)	NK, CTL	SHP-1, 2	HLA-B Bw4
4. KIR3DL2 (p140)	NK, CTL	SHP-1, 2	HLA-A3, A11
5. KIR2DL4	NK	FcεR1γ-chain	HLA-G
6. KIR2DL5	NK, CTL	SHP-2	HLA-G?

Activating KIRs may be expressed on CD4+ T cells in some disease conditions but are rarely expressed on CTL. HLA-Bw4-I designates Bw4 allotypes having isoleucine at position 80. The question mark (?) indicates unknown and/or controversial ligands. All KIRs are expressed clonally on overlapping subsets of NK cells except KIR2DL4, which is expressed on all NK cells. Inhibitory KIRs are also expressed on the CTL of the effector/memory phenotype. Adapted from Iannello A. et al., 2008 (reference 7).

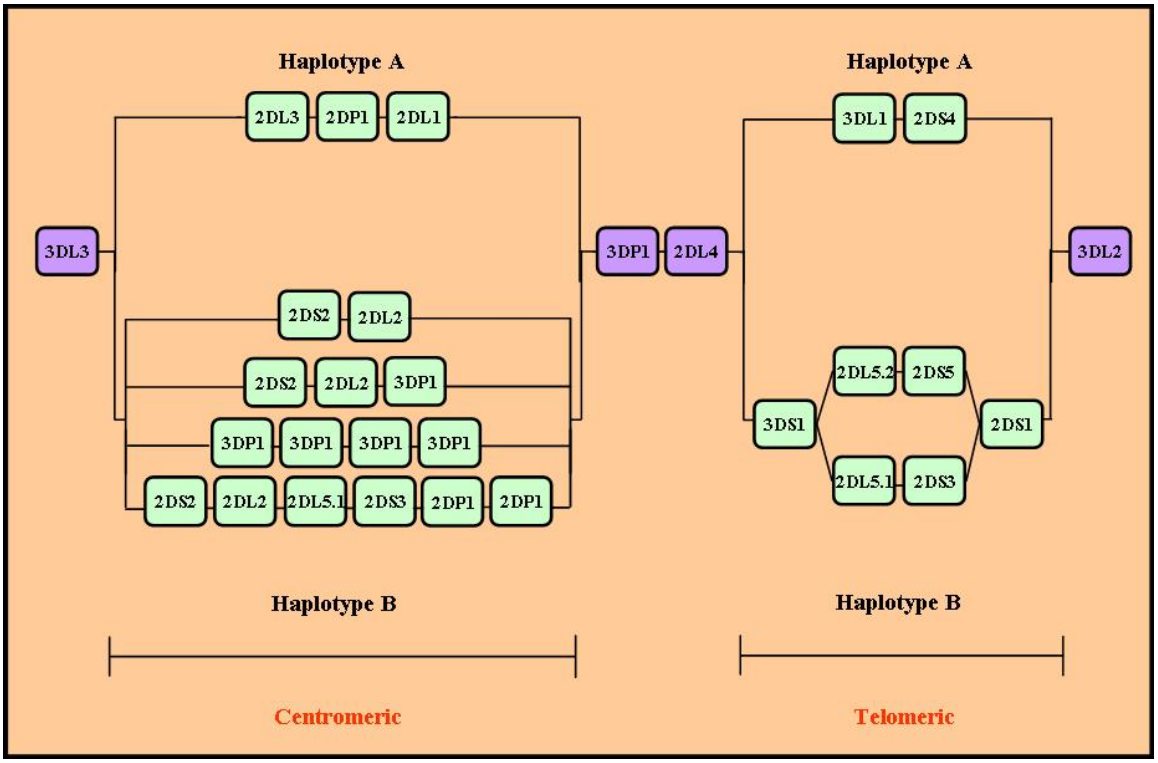
Group A haplotypes are less variable. They lack stimulatory KIR genes except for KIR2DS4. Interestingly, KIR2DS4 frequently carries a 22 bp deletion in its exon 5 and encodes a non-functional receptor (72). In general, the group A haplotypes are composed of a fixed content of seven KIR genes and two pseudogenes, and are diversified through allelic polymorphism (reviewed in 73).

Group B haplotypes have a variable number of KIR genes that are not present in group A haplotypes (e.g., KIR2DS1, 2DS2, 2DS3, 2DS5, 3DS1, and 2DL5). They often, but not always, lack KIR 2DS4. B haplotypes exhibit more diversity both in terms of gene content and allelic polymorphism. There are four genes that are found in both groups of haplotypes and are called framework genes. They include KIR3DL2 on the telomeric end, 3DL3 on the centromeric end, and KIR2DL4 and KIR3DP1 in the central region (Figure 6; 62, 65, 72, 73).

1.3.3- KIR-HLA epistatic interactions and disease outcome

The genes of the KIR family are highly polymorphic. KIRs bind epitopes, which are displayed on a subset of related MHC class I antigens. The KIR genes encode either activating or inhibitory receptors. The inhibitory receptors bind with their specific ligands on the target cell and inhibit the NK cell from killing the target cell as well as from secreting chemokines and cytokines. Each inhibitory KIR has a different affinity for its MHC ligand, which means that the level of inhibition exerted by a given KIR/ligand pair is different from the other pairs. It is of interest to note that the genes for the KIR ligands (e.g., MHC class I antigens) are also highly polymorphic. Furthermore, the genes for KIR and MHC class I antigens in humans are found on chromosomes 19 and 6, respectively,

Figure 6. KIR Haplotypes.



The framework genes (*3DL3*, *3DP1*, *2DL4*, and *3DL2*; in violet color) are present in each haplotype. The figure shows KIR genes present in centromeric and telomeric halves of the frequently found A and B haplotypes above and below the framework genes, respectively. Each box in the figure represents a *KIR* gene. *3DP1* is a pseudogene. Reproduced from Iannello A et al., 2008 (reference 7) after permission.

meaning that during meiosis they segregate independently from one another. This would create different combinations of KIR and MHC ligand genes for each individual, which would then affect the activity of their NK cells and their ability to control tumor growth. For example, an individual with a combination of KIR and MHC ligands that tightly inhibit NK cells may be more at risk for developing leukemia (66, 73).

There is also another NK activating receptor, NKG2D, which has very low genetic variability, but whose ligands (MHC class I heavy chain-related protein A: MICA, and MICB) are very polymorphic (55). Similarly to KIR and its ligands, NKG2D has different binding affinities for various forms of MICA and MICB. Therefore, different NKG2D ligands vary in their ability to activate NK and T cells in different individuals. Because of this, an individual's NKG2D ligands may control the level of activation of his/her NK cells, which can then affect his/her risk of developing leukemia.

1.3.4- Effects of KIR genotypes on human disease

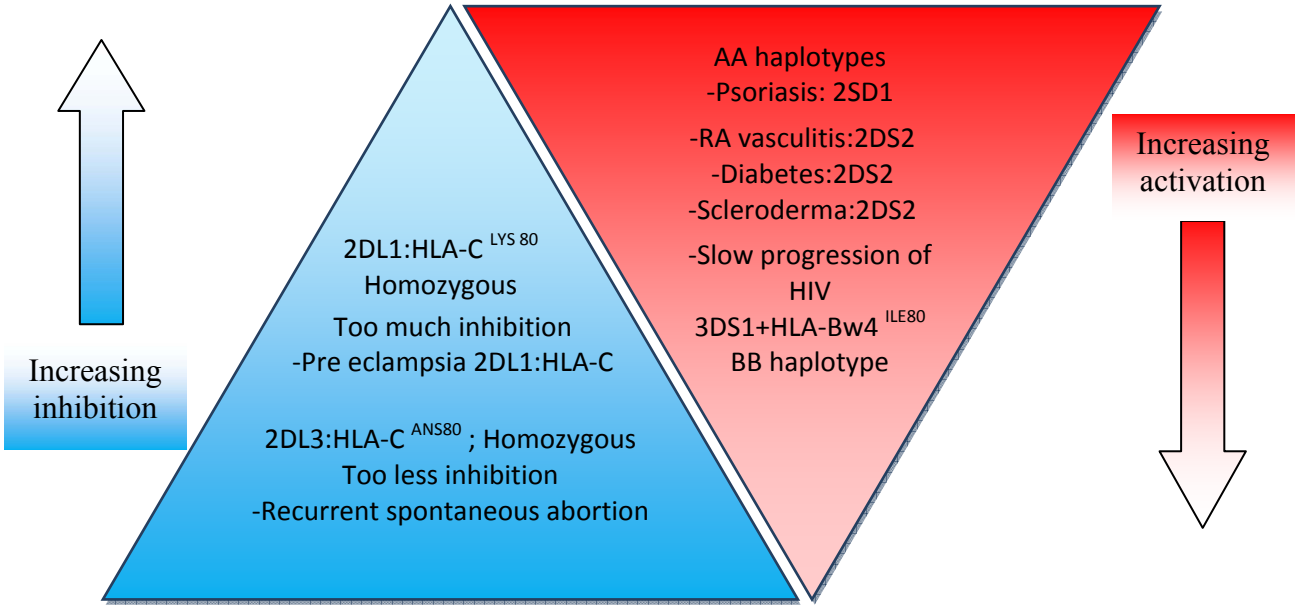
Genetic studies on the association of KIR with diseases have been mainly carried out in viral infections and autoimmune diseases. Figure 7 shows a model for the association of KIR with different diseases. According to this model, the individuals carrying KIR and HLA gene combinations (e.g., those homozygous for KIR2DL1 and HLA-C^{LYS80} genes, with no activating KIR gene) that inhibit NK cells relatively tightly would be less likely to develop autoimmune diseases. Such individuals may suffer from pre-eclampsia and are likely to be more susceptible to viral infections and malignancy. On the other hand, the individuals with the KIR-HLA gene combinations (e.g., KIR2DL3 and HLA-C^{ASP80} with one or more activating KIR genes) that inhibit NK cells to the minimum are likely to control viral infections and malignancy. However, this model predicts that such individuals would suffer more from autoimmune diseases and may experience repeated spontaneous miscarriages (73). In fact many studies concerning the associations of KIR and HLA genes with innate resistance/susceptibility to autoimmune diseases, malignancy, control of viral infections and reproductive success in humans have verified the predictions made by this model (10, 73- 78). Activating KIR genes, which encode

NK cell activating receptors, have also been reported to affect outcomes of many human diseases. In line with the model, several activating KIR genes have shown significant associations with autoimmune diseases. The first of these was KIR2DS2, which was associated with the development of vasculitis in rheumatoid arthritis (10). Another group found that patients with acute coronary syndrome share common features with those diagnosed with rheumatoid vasculitis (10, 79), indicating association of KIR genotypes and vascular disease.

1.3.5-KIR genes in leukemia

A few earlier studies described an association of KIR genes with leukemia (9,74, 80,81; reviewed in 82). Unfortunately all these studies were performed on adult leukemic patients. One group studied the relationship between certain inhibitory KIR-HLA interactions and chronic lymphoid and myeloid leukemia in the Belgian adult leukemic patients (9). Their study subjects mostly had other types of leukemia; only 8 cases were ALL. They found significant associations of certain inhibitory KIR haplotypes with some forms of leukemia; however the number of patients with ALL was too low to find any significant association. These workers conducted another study in Polish and German adults suffering from Myeloid or Chronic Lymphocytic Leukemia and found significant associations with certain combinations of inhibitory KIR and their HLA ligands (74). The study had no participants with ALL. Another study (80) reported significant association of the intact KIR2DS4 gene with innate resistance to CML in humans but not with resistance to ALL. The number of the leukemic patients with ALL was very low (only 21) in this study. Due to this low number of participants with ALL, the study had not enough power to detect associations between KIR2DS4 and ALL. In a relatively recent study in a Chinese population (81), researchers found that activating KIR genes were more common in CML patients than in healthy controls, although only the difference for the KIR2DS4 gene reached statistical significance. This study did not differentiate between the functional and mutant variants of the KIR2DS4 gene. The researchers also found a significantly decreased frequency of KIR2DS3 in ALL patients compared to the

Figure 7. Genetic model for association of KIR with human diseases.



This model is based on KIR genotypes and KIR:HLA combinations that are considered to provide different levels of inhibition and activation, and on disease studies in which an association with protection or susceptibility was associated with specific KIR genes, or specific KIR and HLA combinations.
Adapted from Williams AP et al., 2005 (reference 73).

control group (81). We were unable to find any study in the literature that studied the potential association of activating or inhibitory KIR genes in childhood leukemia.

CHAPTER 2

AIMS AND OBJECTIVES

The aim of this study was to investigate the associations between activating KIR genes and acute lymphocytic leukemia (B ALL), which is the most common form of leukemia affecting children under 15 years of age. As stated earlier, humans differ from each other with respect to the number of inherited activating KIR genes. They may have 0-6 of these genes. Since the receptors encoded by the genes enhance NK cell activation and overall immune competence of the individual, we hypothesized that their inheritance is likely to protect individuals from developing this cancer.

The specific objectives of the study were to compare the frequencies of activating KIR genes between B-ALL patients and healthy controls in French-Canadian children, and determine whether these genes showed significant association with resistance or susceptibility to this cancer in this population.

CHAPTER 3

RESULTS

The results obtained from the investigations conducted on the genomic DNA obtained from B-ALL patients and control subjects were compiled into a research article. The article is reproduced in this chapter.

Association of activating Killer-cell Immunoglobulin-like Receptor genes with decreased risk for developing B cell acute lymphoblastic leukemia in children

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ABSTRACT

Pre-B cell acute lymphoblastic leukemia (B-ALL) is the most frequent form of leukemia affecting children under 15 years of age. Evidence is accumulating that genetic factors play an important role in conferring susceptibility/resistance to leukemia. In this regard, the Killer-cell Immunoglobulin-like Receptor (KIR) genes are of particular interest. These genes are highly polymorphic and encode receptors that control functional activities of Natural Killer (NK) cells, which are known to kill leukemia cells and regulate immune responses both *in vitro* and *in vivo*. However, little is known concerning the role of these genes and/or their variants in conferring innate susceptibility/resistance to the childhood leukemia. In this study, using a case-control design, we have addressed this issue. Our results show that harbouring activating KIR genes confers protection from this cancer in French-Canadian children. The protection increases as the number of these genes increases in an individual. These results provide novel insights concerning pathogenesis as well as for potential immunotherapy in children affected with Pre-B ALL.

INTRODUCTION

Each year about 15,000 new cases of leukemia are diagnosed in North America. Acute lymphoblastic leukemia (ALL) represents about 95% of these cases occurring in children under fifteen years of age. About 85% of the ALL cases in children involve Pre-B cells (1). The pre-B ALL is also the most deadly form of leukemia occurring in children. Cumulative evidence suggests that the genetic make up of an individual plays an important role in determining innate resistance/susceptibility of an individual to the development of this leukemia. In this respect, several genetic determinants that influence cell proliferation and cell cycle progression have been shown to be associated with susceptibility/resistance to develop Pre-B ALL in children (2-5; reviewed in 6). However, little is known about the role of the Killer-cell Immunoglobulin-like Receptor (KIR) genes in the development of this leukemia.

The KIR gene family comprises sixteen genes located in a head-to-tail fashion on human chromosome 19q13.4 in a 150 kb region called "Leukocyte Receptor Complex or LRC" (7, 8). The genes in the family are highly polymorphic and are rapidly evolving. Of the sixteen KIR genes, seven inhibitory genes (KIR3DL1-3, KIR2DL1, KIR2DL2/3 and KIR2DL5A and B) encode receptors with long-tailed cytoplasmic tails and inhibit the functions of the immune cells. Each inhibitory receptor binds to public epitopes present in a group of related MHC class I antigens (9, 10). The KIR family also contains six activating genes (KIR3DS1, KIR2DS1-5) that encode receptors with short cytoplasmic tails. The binding of the activating receptors to their cognate ligands on a target cell results in activation of the immune cells. In the case of Natural Killer (NK cells), this activation leads to killing of the target cell as well as secretion of soluble mediators like IFN-γ and TNF-α. It is noteworthy that KIR haplotypes may vary from one another with respect to the number of activating KIR genes and have been divided into two groups: A and B. The group A haplotypes carry only one activating KIR gene, KIR2DS4, which is often mutated and encodes a non functional receptor. The group B haplotypes usually lack KIR2DS4 but contain 1-5 other activating KIR genes. Consequently, individuals differ from each other with respect to the number of inherited activating KIR genes (7, 8). Several studies have shown that KIR genotypes as well as the number of activating KIR

genes inherited by an individual influence his/her resistance/susceptibility to infectious agents, development of autoimmune diseases, reproductive efficiency, graft versus host disease (GVHD) and cancer (11-16; reviewed in 17). Certain activating KIR genes have been shown to protect humans from non-Pre B ALL (18). However, little is known concerning the impact of activating KIR genes on the innate resistance/susceptibility to develop the childhood leukemia pre-B ALL. We addressed this issue in this study and show here that all activating KIR genes protect children from developing pre-B ALL. Furthermore, we also observed that the individuals who harbour higher numbers of activating KIR activating were afforded more protection from this childhood leukemia.

MATERIALS and METHODS

Patient Population:

In order to investigate associations between activating KIR genes and pre-B ALL, we carried out a case-control study at the CHU Sainte-Justine Research Center, Montreal. Cases of pre-B ALL patients (N=102) were recruited from the Hemato-Oncology clinic of the hospital during 1989-2004 and included children diagnosed prior to age 18. They had the following characteristics: (a) girls to boys' ratio of 40:60; (b) age at diagnosis: 9% between 0-1 year, 70% between 2-10 years, and 21% between 10-18 years. Diagnosis of pre-B ALL was based on established criteria that included: histological examination of the blood and bone marrow smears, immunophenotyping of the lymphoblasts.

Given the potential heterogeneity in the distribution of KIR gene frequencies even within seemingly ethnically homogeneous populations, controls (N=250) were selected from different sources to enhance population representation. These included children visiting the orthopaedic department of the study hospital for minor fractures, their siblings, a sample of population-based children. Only controls without pre-B ALL, other cancers or autoimmune disorders were included. These controls have been previously utilized for replicating or confirming associations between a number of susceptibility genes and complex diseases such as inflammatory bowel diseases (IBD) (19-21). Both cases and controls were restricted to those who described themselves as French-Canadian, and were residing in Quebec at the time of recruitment. The relevant demographic, clinical and histopathological data were extracted from the medical records of these patients or from

their parents. Blood and/or saliva samples were collected from the participants as a source of DNA.

Genomic DNA from blood and saliva was extracted by salting out technique and/or commercial kits (Oragene). The quantity and quality of the extracted DNA samples were determined by UV spectrophotometry. The samples were coded and kept at the Banks maintained at the CHU Sainte-Justine Research Center.

The study was approved by the Institutional Ethics committee, and written informed consent was obtained from all the participants.

KIR Genotyping:

The genomic DNA samples from the patients and control subjects were analysed for the presence or absence of individual activating KIR genes using PCR and published sequence specific primers as described (22-29; see Table 1 in Appendix I for the primer sequences and expected sizes of the amplicons for the genes). Standard PCR protocols were used. The general procedure included initial denaturation at 95˚C for 5 minutes followed by 30-35 cycles, each comprising 30 seconds of denaturation at 95˚C, 30 seconds of annealing at 60-68˚C and 2 minutes of extension at 72˚C. The final cycle included extension for 10 minutes. The number of PCR cycles and annealing temperatures were adjusted for each gene in preliminary experiments. The presence or absence of the genes was determined by running the reaction products on 2-2.5% agarose gels. The gels were stained with ethidium bromide, scanned and imaged. The appearance of the amplicon band of the expected molecular weight (see Figure 1 in the Appendix II for a few typical agarose gel images) was considered as the presence of the gene in the genomic DNA sample. The DNA samples known to be positive or negative for the gene were used as positive and negative controls, respectively. The PCR reactions were also run without DNA template as a safeguard against false positive cases as well as for a housekeeping gene, GAPDH, as a control for false negative cases.

Despite all the precautions, the PCR reactions for a few samples (5 control and 2 B-ALL samples) did not succeed and were excluded from the analyses.

Statistical analysis:

Based on the genotyping, patients were classified as those in whom the gene was present or absent. As we could not distinguish between heterozygotes and homozygotes for the presence of a gene, it was not possible to examine deviation from Hardy-Weinberg Equilibrium for the studied genes. Initial analysis included examining the distribution of the genes in cases and controls using Chi-square tests. Subsequently, logistic regression analysis was carried out. A single model for each gene as the independent variable was first fit. As many of the KIR genes are in high linkage disequilibrium with each other, a multivariate model including all 5 genes was then fit to assess the independent effect of each gene after controlling for the effects of other genes. In addition to the effect of individual genes, we also assessed the combined effects of harbouring 1 or more activating genes in comparison with not harbouring any of the genes. For this an analysis considering the carriage of activating genes as a continuous variable as well stratified into categories ($\leq$1, between 2-3 and $\geq$4) was also carried out. Odds ratios (OR) and corresponding 95% confidence intervals (95% CI) were estimated. All analyses were carried out using STATA software (version 10; STATA Corp, TX, USA).

RESULTS:

A total of 100 cases and 245 controls were successfully genotyped. Table 1 shows the comparison of the distribution of individual activating KIR genes between patients and controls as well as results from univariate analysis. The frequencies of all the 6 activating KIR genes were lower in the patients compared with the controls. Consequently, all of these genes were associated with significantly dcreased risk for developing pre-B ALL. Strongest associations were noted with the KIR2DS2 gene wherein the presence of the gene decreased the risk for developing pre-B ALL by ~80% (OR=0.19, 95% CI=0.11-0.34, p-value= 5.4×10^{-9}). When a multivariate logistic model was fit, after accounting for the effects of other genes, the protective association between the KIR2DS2 gene persisted and was of similar magnitude (OR=0.23, 95% CI=0.13-0.42, P=2.7×10^{-6}), indicating that the protective effects conferred by the gene were independent of other KIR genes that could be in high LD (linkage disequilibrium) with it. Associations with

the KIR2DS1 and KIR2DS3 genes however were no longer evident in the multivariate analysis, suggesting that the effects of these genes were not independent but were as a result of their being in high LD with other KIR activating genes. The KIR2DS1 gene also seemed to confer protection, but this association did not achieve statistical significance.

As mentioned in the introduction, KIR2DS4 is frequently mutated and shows a 22-bp deletion, which results in the expression of a non-functional receptor. In the analysis shown in Table 1, we counted the individuals showing only this 22-bp mutant form as being negative for the gene. The individuals were considered positive for the gene when non-mutated amplicon band was seen on the gel with or without the presence of the mutated band.

We also took into consideration whether the individuals had only mutant form, only wild type functional form, had both functional and mutated genes or had neither functional nor mutated genes. We compared the cases and controls with respect to the frequencies of these four KIR2DS4 genotypes. As shown in Table 2, we observed that children who had an active and a mutant gene were protected from risk for pre-B ALL (OR=0.35, 95% CI= 0.18-0.69, p= 0.003). There were suggestions that those who carried 2 active genes were also protected from pre-B ALL however the magnitude of the protection was lower than that observed for children who carried 1 mutant and 1 active gene.

There existed a statistically significant trend towards increased protection with increasing numbers of inherited activating KIR genes. The analysis showed that with each additional activating gene, the risk decreased by 0.435 (OR: 0.4350601, SE: 0.050248, z: -7.21, P>z : 0.000, and 95% CI: 0.3469271-0.5455824). The effects of inheriting multiple activating genes on protection from the childhood leukemia are shown in Table 3. The results suggest that individuals who harboured increasing number of the genes were less susceptible for pre-B ALL. The strongest protection was observed in individuals who harboured any 4 or more KIR activating genes (OR=0.07, 95% CI= 0.03-0.18, P=3.60 x 10^{-9}).

DISCUSSION

In this case-control study we investigated whether activating KIR genes were associated with susceptibility/resistance to pre-B ALL in a French-Canadian population. We

observed that all six activating KIR genes tended to confer protection from acquiring pre-B ALL ($P<0.05$; Table 1). However, in a multivariate analysis four of these genes (KIR2DS2, KIR2DS4, KIR2DS5 and KIR3DS1) conferred protection independent of the effects of the other genes. There were also suggestions that the protective effects of the activating genes were additive.

To the best of our knowledge, this is the first study that has described the protective effects of activating KIR genes on an individual's susceptibility to develop pre-B ALL in a French-Canadian population. A few earlier studies, focussed on adult populations, have described the associations of KIR genes with other leukemias in different human populations. For example, one group (30, 31; reviewed in 32) has shown potential associations of certain inhibitory KIR-HLA gene interactions, but not of activating KIR genes, with chronic lymphoid (CLL) and myeloid leukemias (CML) in the Belgian population. No associations with ALL were noted; however, given the small number of ALL patients evaluated (n=8), the study had limited power to demonstrate meaningful associations. Furthermore, the researchers did not discriminate between functional and mutant variants of the KIR2DS4 gene. Another study conducted in Polish and German individuals discriminated between functional KIR2DS4 and its mutant variants (16). The researcher found a protective effect of the gene in CML but not in ALL patients. Similar to the previous study, they also had limited power (n= 21) to detect significant associations if any with ALL. All the patients in the study were of Polish origin, while controls comprised individuals from Polish and German descents. Given the widespread heterogeneity in KIR gene frequencies, confounding due to ethnicity cannot be ruled out in this study. A more recent study in Chinese populations (18) has shown that activating KIR genes were more frequent in CML patients than in healthy controls, although the difference for only KIR2DS4 genes reached statistical significance. The study also did not discriminate between mutant and functional variants of the gene. The researchers also found a significantly ($P=0.029$) decreased frequency of KIR2DS3 in ALL patients compared to the control group.

It is noteworthy that all the previous studies (16, 18, 30, 31) have attempted to examine the association between KIR genes and adult-onset leukemia. These studies did not include childhood leukemias. Further, comparison is hampered given that early-onset

leukemia may involve different susceptibilities and differing etiologies vis-à-vis adult-onset leukemia. Furthermore, even within childhood leukemias, etiologies for ALL may differ between the different forms (Pre-B ALL and ALL with unspecified cell types). In order to reduce this heterogeneity and given that Pre-B ALL is the predominant form of all childhood leukemias (85% of the leukemias occurring in children under 15 years of age; 1, 6) we focussed on this phenotype. In this context, we excluded cases of childhood leukemia of T cell phenotype (T-ALL) or of unspecified cell phenotypes to avoid concerns about their potentially different etiologies.

KIR gene frequencies are known to vary between and within populations. Thus the choice of the control population is of paramount importance. Our healthy controls were drawn from a variety of sources so that they represented the population frequencies of the selected genes. Secondly, both cases and controls were restricted to a common ancestry reducing potential ethnic heterogeneity. It is noteworthy that the KIR gene frequencies within the control populations are reflective of the distribution within other populations of similar ancestry (Belgian and French) (http://www.allelefrequencies.net), further demonstrating that bias if any, in the selection of controls was limited. Thus, a relatively large number of patients, more relevant control donors (with respect to ethnicity) and a precisely defined leukemic condition may have been responsible for the results obtained in our study. Our findings of significant protection conferred from pre-B ALL will thus require confirmation in an ethnically similar population with a similar disease phenotype.

It is noteworthy that KIR genes mainly control functional activities of NK cells, which are important effector cells of the innate immune system. They can efficiently kill most tumor cells in the body without prior sensitization and amplification (9, 33-35). In particular, these cells kill target cells of hematopoietic origin more efficiently than target cells of non-hematopoietic origin. They have been shown to kill autologous and heterologous leukemia cells in *in vitro* assays (35, 36). The protective role of activating KIR genes from childhood leukemia suggests that the individuals with these genes are likely to a) recognize and kill the leukemic cells via these receptors on their NK and/NKT cells, b) are likely to have lower threshold of activation of their NK and other immune cells (e.g., $CD8^+$ T cells) and provide better immune surveillance against malignancy, and c) the NK cells with activating receptors can better induce anti-cancer adaptive immunity.

These mechanisms may not be mutually exclusive. In fact as mentioned above, NK cells have been demonstrated to kill autologous leukemic cells *in vitro* (32). The role of these cells in controlling leukemia is further supported by the reports that infusion of mismatched alloreactive NK cells (which bear activating KIR and/or inhibitory KIR whose cognate HLA ligands are missing in the recipients) into leukemia patients provides NK cells expressing activating KIR genes may prove more beneficial in children suffering from Pre-B ALL.

Unlike their homologous inhibitory receptors, activating NK cell receptors bind to specific HLA- class I ligands with very low affinities. In fact it is believed that these activating receptors may bind to the ligands that are expressed de novo in cancer or virus-infected cells and/or they may recognize foreign peptides bound with HLA-class I peptides. In the case of KIR2DS4, the receptor was described to bind an unidentified ligand expressed by primary melanoma cells (38).

Interestingly, the KIR3DS1 gene, which has been shown to be protective for Pre-B ALL in this study, has been associated with protection from infection with the type 1 Human Immunodeficiency Virus (HIV-1) as well as with delayed progression of the infection towards AIDS (15, 39). In fact, KIR3DS1 is the collective name given to several allelic variants of KIR3DL1. These variants encode a short-tailed activating version of the receptor. KIR3DL1 is one of the most polymorphic genes in the KIR family. It exists in 59 allelic variants of which 13 encode short-tailed activating version of the receptor and hence are collectively designated as KIR3DS1, whereas the remaining 46 allelic variants encode long-tailed inhibitory version of the receptor and are designated as KIR3DL1 (7, 8, 9). Researchers have discovered that KIR3DL1 receptors bind HLA-Bw4 allotypes that have an isoleucine (Ile) at position 80 in their α-1 domain. The activating versions of the receptor were shown to bind the HLA-Bw4 allotypes with threonine (Thr) at position 80. However, the subsequent studies have not confirmed these observations (7, 8, 10). Therefore, like other short cytoplasmic tail activating KIR, the exact ligands for KIR3DS1 remain unknown. In fact, even in the cases, where some activating KIR receptors have been shown to bind MHC class I ligands, the binding had been of low affinities (40; reviewed in 9). Our results suggest that the presence of activating KIR genes may facilitate activation of NK cells, $CD8^+$ T lymphocytes (CTL) and NK-T cells

in cancers and viral infections. Hence they may provide protection from viral infections and malignancy. Interestingly our results about Pre-B ALL patients fit with this paradigm.

In conclusion, our study demonstrates important protective effects of KIR gene variation and B-ALL. Larger studies are required to replicate these findings in different ethnic populations.

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Table 1. The comparison of frequencies of activating KIR genes in Pre-B ALL patients and healthy controls

Gene	Patients N (%) N=100	Controls N (%) N=245	OR (95% CI)	P-values	OR_{adj} (95% CI)*	P_{adj}-values*
2DS1	28 (28.3)	102 (41.6)	0.55 (0.33-0.92)	0.02	0.65 (0.37-1.17)	0.15
2DS2	20 (20.0)	138 (56.3)	0.19 (0.11-0.34)	5.4×10^{-9}	0.23 (0.13-0.42)	2.7×10^{-6}
2DS3	14 (14.1)	83 (33.9)	0.32 (0.17-0.60)	3.7×10^{-4}	0.64 (0.31-1.30)	0.22
2DS4[a]	38 (39.2)	139 (56.7)	0.49 (0.30-0.79)	0.004	0.48 (0.28-0.82)	0.008
2DS5	19 (19.2)	104 (42.4)	0.32 (0.18-0.56)	7.5×10^{-5}	0.37 (0.20-0.70)	0.002
3DS1	16 (16.0)	101 (41.2)	0.27 (0.15-0.49)	1.57×10^{-5}	0.44 (0.23-0.84)	0.014

* Logistic regression estimates after adjusting for the effects of other genes.
The superscript "a" indicates that KIR2DS4 was considered absent when the PCR amplified only the 32-bp deleted amplicon. It was considered present when the non-mutant amplicon was observed on the gel with or without the mutant band.
Please note that the prefix KIR is not shown with the gene names.

Table 2. Comparison of frequencies of mutant and functional KIR2DS4 genes in Pre-B ALL patients and healthy controls

Gene Type	Patients	Controls	OR (95% CI)	P-value
Absent[a] or Mutant[b]	59 (60.8)	106 (43.3)	Reference	
Active/Mutant	13 (13.4)	66 (26.9)	0.35 (0.18-0.69)	0.003
Active/No Mutant	25 (25.8)	73 (29.8)	0.62 (0.35-1.07)	0.086

a: Absent means that no band (mutant or full length) was detected in the genome.
b: Mutant means the presence of a 32-bp deleted non functional gene.

Table 3. Comparison of frequencies of different numbers of activating KIR genes between Pre-B ALL patients and healthy controls

Number of genes	Patients	Controls	OR (95% CI)	P-value
<=1	60 (60.0)	48 (19.6)	Reference	
2-3	33 (33.0)	122 (49.8)	0.22 (0.13-0.37)	2.8×10^{-8}
>=4	7 (7.0)	75 (30.6)	0.07 (0.03-0.18)	3.6×10^{-9}

CHAPTER 4

DISCUSSION

In a case control study, we examined whether activating KIR genes were associated with susceptibility/resistance to pre-B ALL. We investigated 100 cases of B-ALL, which were in French-Canadians residing in the Province of Quebec. Furthermore, all of our cases were of B-ALL type. We excluded T-cell ALL and other ALL cases that were not of B-cell origin. Since ALL cases originating in children in different cell lineages may differ from each other with respect to their etiologies and could be a confounding factor, we decided to focus on the ALL cases originating in the B-cell lineage. It is noteworthy that B-ALL represents 85% of the leukemia occurring in children under 15 years of age (1-3). Our healthy control group consisted of 245 children drawn from the general population and belonging to the same ethnic origin as the cases. We observed that all activating KIR genes have a tendency to be associated with protection from childhood leukemia, as the frequencies of all these genes were significantly lower in patients than in the controls ($P<0.05$). However, in analyses for independent effects of each gene, four of these genes (KIR2DS2, KIR2DS4, KIR2DS5 and KIR3DS1) conferred statistically significant protection from this type of childhood leukemia.

To the best of our knowledge, this is the first study that has described significant associations of activating KIR genes with an individual's innate resistance to developing B-ALL in a French-Canadian population. We could not find any report in the literature that has investigated the association of KIR genes (activating or inhibitory ones) with

childhood leukemia. There are a few studies that have reported the associations of KIR genes with AML, CML, CLL or ALL in adults. One of the studies (9) investigated potential associations of 11 KIR genes, 2 NKG2/CD94 genes and different KIR-HLA interactions with different types of leukemia in Belgian adults. The study included a relatively small number of patients: 8 ALL, 29 AML, 20 CML and 39 CLL patients. Nevertheless, the study found that certain inhibitory AB KIR haplotypes were more frequent in leukemia patients as compared to the control subjects. The authors concluded that leukemia develops in individuals whose NK cells are more tightly inhibited by their inherited KIR and HLA genes. The same group of researchers performed another study on this subject (74). The cases in this study were all adults and comprised 31 CLL and 48 CML. They found significant associations of certain KIR-HLA combinations with these leukemia types. The authors concluded that KIR-HLA-mediated NK cell control is an important factor in the development of CLL and CML in adult humans. Another group of researchers (80) investigated the potential association between leukemia and KIR2DS4 polymorphism in Polish and German adult individuals. The gene is often present on the group A KIR haplotypes and is often mutated and non-functional (72). The workers differentiated between functional KIR2DS4 and its mutant 22 bp-deleted variant in their study. The study found significant associations of the functional gene with innate resistance to developing CML but not ALL. A drawback of this study is that it had a relatively small number of ALL cases (only 21). Due to this relatively small number of ALL patients, the study had too low power to detect any potential association between this activating KIR gene and ALL. Furthermore, all of the patients in the study were of Polish origin, while the controls comprised individuals from Polish and German descent. It is possible that mixed ethnic origins of the control population may have also affected the results.

Recently, a study on the potential associations between KIR genes and different types of leukemia has been reported in a Chinese population (81). The researchers found increased frequencies of activating KIR genes in CML patients versus those in healthy controls, although the difference reached statistical significance only for the KIR2DS4 gene. This study, however, did not differentiate between the functional and mutant variants of the KIR2DS4 gene. It is noteworthy that about 80% of Caucasians harbor a 22

bp deletion in exon 5 of the gene, which renders the gene non functional (7, 72). The frequency of the mutant KIR2DS4 in Chinese is not known. Had the researchers taken into consideration this mutation, their results could have been more reliable. The researchers also found a significantly (P=0.029) decreased frequency of KIR2DS3 in ALL patients compared to the control group. However, the study included only adult ALL patients, and not children.

Taken together, the studies in adults suggest that KIR genes especially the activating ones as well as certain KIR-HLA gene interactions play an important role in determining innate resistance/susceptibility of human adults to developing leukemia (reviewed in 82). For various reasons, the results from our study cannot be compared with those from earlier studies. The main reason is the difference in the patient population: all of the previous studies were based upon adult patients whereas our study is based only on children less than 15 years of age. Furthermore, all of the previous studies did not focus on one type of leukemia. In contrast, our study has focused on one well-defined childhood leukemia i.e., B-ALL. We have even eliminated childhood leukemias of T cell origin (T-ALL). Furthermore, we have a larger number of leukemia cases as compared with the previous studies. Thus, a homogenous group of patients with respect to ethnicity, better defined childhood leukemia and a relatively large number of cases and controls may have been responsible for detecting significant associations between activating KIR genes and B-ALL in our study.

The demonstration of significant associations between activating KIR genes and a reduced risk for developing B-ALL suggests that NK cells have a role in controlling this cancer. It is worth noting that NK cells were discovered as a result of their ability to kill certain tumour cell lines (83). NK cells recognize and eliminate cells that fail to express self-MHC molecules. In particular, these cells kill target cells of hematopoietic origin more efficiently than target cells of non-hematopoietic origin. They have been shown to kill autologous and heterologous leukemia cells in *in vitro* assays (83, 84). The cytolytic activity of human NK cells is regulated by overall strengths of inhibitory and activating signals emanating from a diverse array of inhibitory and activating receptors and coreceptors. These receptors bind to their cognate ligands present on the surface of cancer cells. KIR receptors play a predominant role in deciding whether a NK cell kills or spares

a target cell (65, 66). The protective role of activating KIR genes from childhood leukemia suggests that the individuals with these genes are likely to:

a) Recognize and kill leukemic cells via these receptors on their NK and/NKT cells.

b) Have a lower threshold of activation of their NK and other immune cells (e.g., CD8+ T cells) and provide better immune surveillance against malignancy.

c) The NK cells with activating receptors can better induce anti-cancer adaptive immunity.

These mechanisms may not be mutually exclusive. In fact, as mentioned above, NK cells have been demonstrated to kill autologous leukemic cells *in vitro* (84, 85; reviewed in 82). The role of these cells in controlling leukemia is further supported by reports that the infusion of mismatched alloreactive NK cells (which bear activating KIR and or inhibitory KIR whose cognate HLA ligands are missing in the recipients) into leukemia patients provides significant graft versus leukemia effects (82). In a study investigating the influence of donor and recipient KIR genotypes on the outcome of hematopoietic cell transplantation between HLA-matched siblings in CML patients (84), the researchers found that the best survival rate was associated with the donor lacking and the recipient having group B KIR haplotypes (group B haplotypes are comprised of more than one activating KIR gene); the poorest survival rate was associated with the donor having and the recipient lacking group B KIR haplotypes. The latter combination was also associated with increased relapse and acute GVHD. The results from stem cell transplantation in leukemic patients strongly suggest a role of NK cells as well as of KIR receptors and their ligands in deciding the outcome of the graft and leukemic control.

In our results, we have found that KIR3DS1 has a protective effect in ALL. A previous study (76) has shown that the presence of an activating KIR3DS1 along with the co-expression of its ligand, i.e., the HLA-Bw4 allotypes, confers resistance to the development of AIDS in HIV-infected patients. It is worth pointing out that KIR3DS1 represents allelic variants of KIR3DL1, which exist in 59 allelic variants, 13 of which encode a short tailed activating receptor while the other 46 allelic versions encode long tailed inhibitory versions of the receptor. KIR3DL1 receptors bind HLA-Bw4 allotypes that have an isoleucine (Ile) at position 80 in their α-1 domain. On the other hand, the exact ligand for KIR3DS1 remains uncertain. Some studies suggest that KIR3DS1 binds

the HLA-Bw4 allotypes with threonine (Thr) at position 80. However, several reports were unable to verify these results (reviewed in 73).

As is the case for KIR3DS1, the ligands for most other activating KIRs remain unknown. It is believed that the ligands for these receptors may be induced on some tumor cells. In this respect, KIR2DS4 was reported to bind an uncharacterized ligand on certain melanoma cells (87). Considering the protective role of activating KIR genes in childhood leukemia, it would be important to search for ligands for these receptors.

It is noteworthy that activating KIR genes have been shown to protect humans from several viral infections, e.g., HCV and HIV (76, 78). Our results suggest their protective role in childhood leukemia. They are in agreement with the paradigm that activating KIR genes and less inhibitory KIR-HLA interactions protect humans from infectious agents and malignancy by enhancing NK cell activities and overall immune competence of the host. However, this protection comes with a price, as many of the activating KIR genes have also been shown to enhance an individual's susceptibility to autoimmune diseases like arthritis and type 1 diabetes (10, 77).

Our results may bear important implications for B-ALL. They may provide impetus to discovering ligands for activating KIRs. It is tempting to speculate that B-ALL cells may express ligands for these receptors. The discovery of these receptors may lead to novel KIR-based drugs for treating leukemia in humans.

Another implication of our study is that the results may be used to identify children at higher risk for developing this cancer. These results predict that children lacking activating KIR genes are at high risk for developing leukemia. An early detection and treatment of the cancer has better prognosis for the patients (21, 88).

The results from our study may have implications in bone marrow grafts in B-ALL patients. These results suggest that infusions of bone marrow stem cells from donors having activating KIR genes may be more effective in eliminating the cancer. Furthermore, infusions of NK cells expressing activating KIR genes may prove to be more beneficial in children suffering from this cancer. It is noteworthy that alloreactive NK cells, whether infused into recipients or develop in recipients due to mismatches between donors and recipients, reduce graft-versus-host disease, kill leukemic cells and diminish relapses in bone marrow transplants (66, 89).

The protective role of activating KIR genes from childhood leukemia is likely to be modulated by the presence of inhibitory KIR genes and their MHC class I ligands. Studies are currently underway in our laboratory to investigate the potential impact of the inhibitory KIR on the protective effects of the activating KIR genes.

Finally, we have demonstrated the association of activating KIR genes with reduced risk for B-ALL in French-Canadian children. Further studies are needed to replicate these results in ethnically different populations.

CHAPTER 5

CONCLUSIONS

Based upon our case control study, in which we used genomic DNA from 100 B-ALL children and 245 healthy controls, we conclude that activating KIR genes (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1) are associated with a decreased risk for developing this childhood leukemia. The frequencies of all of these genes were significantly reduced in the B-ALL cases as compared to those observed in healthy children. Furthermore, the number of these genes shows a significant linear association with resistance to developing this cancer. This finding suggests additive effects of the activating KIR genes in providing protection from developing this cancer in children. Furthermore, we also stratified the individuals in both cases and controls in three categories, i) as having 0 or 1, ii) having 2 or 3, and iii) having more than 4 activating KIR genes. When we compared the frequencies of the individuals between cases and controls in the three categories, the difference between the frequencies of the genes in the patients and controls increased as the number of the genes examined increased, i.e., from category i to iii. We conclude from this data that the risk for developing B-ALL increases as the number of inherited activating KIR decreases in children.

In a univariate logistic regression analysis, all activating KIR genes were associated with a decreased risk for developing B-ALL. However, in a multivariate analysis, only

four of the genes (KIR2DS2, KIR2DS5, KIR2DS4, and KIR3DS1) showed significant association with decreased risk for developing this cancer. In this analysis, KIR2DS1 and KIR2DS3 lost significance. However, the frequencies of these two genes were still lower in cases compared with the healthy controls. It is highly probable that if a larger study would be conducted with a larger number of cases, the results for these two genes could also be significant.

The association of activating KIR genes with reduced risk for childhood leukemia strongly suggests the involvement of NK cells in controlling this cancer. NK cells bearing activating KIR are likely to kill B-ALL cells. These predictions can be tested in *in vitro* experiments. Our results also suggest that B-ALL cells are likely to express ligands for activating KIRs. Finding these ligands should be an area of high priority research. For this purpose, the use of B-ALL cells may be more promising. Their discovery may lead to novel KIR-based therapies for B-ALL in the future.

Our results also bear implications for bone marrow transplantations that are often carried out in leukemic children. They suggest that bone marrow stem cells from the donors that are positive for one or more activating KIR genes may prove to be more successful in controlling leukemia. Furthermore, our results also suggest that infusions of NK cells expressing activating KIR may be used as a therapeutic strategy in controlling and treating childhood leukemia. Such NK cells may be enriched from autologous or heterologous sources.

Further studies should be carried out to replicate these results in other ethnic populations. If validated, the results may be helpful in the early identification of children who are at-risk for developing this most frequent childhood leukemia.

CHAPTER 6

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87. **Katz G, Gazit R, Arnon TI, et al**. *MHC class I-independent recognition of NK activating receptor KIR2DS4.* 2004, J Immunol, 173(3): 1819-1825.

88. **Smith MA, Gloecker Ries.** *Childhood cancer: Incidence, survival, and mortality.* Philadelphia : Lippincott Williams and Wilkins, 2002.

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APPENDIX 1

Table 1. Sequence-specific primers used for PCR amplification of activating KIR genes.

Gene	Primer sequences	Amplicon size	Reference
KIR2DS1	CTTCTCCATCAGTCGCATGAA AGAGGGTCACTGGGAGCTGAC	102	1
KIR2DS2	CGG GCC CCA CGG TTT GGT CAC TCG AGT TTG ACC ACT CA	240	1
KIR2DS3	CTA TGA CAT GTA CCA TCT ATC CAC AAG CAG TGG GTC ACT TGA C	190	1
KIR2DS4	CTG GCC CTC CCA GGT CA TCT GTA GGT TCC TGC AAG GAC AG	199 or 221	2
KIR2DS5	TGA TGG GGT CTC CAA GGG TCC AGA GGG TCA CTG GGC	125	1
KIR3DS1	AGC CTG CAG GGA ACA GAA G GCC TGA CTG TGG TGC TCG	300	1

All primer sequences are given in 5' to 3' orientations.

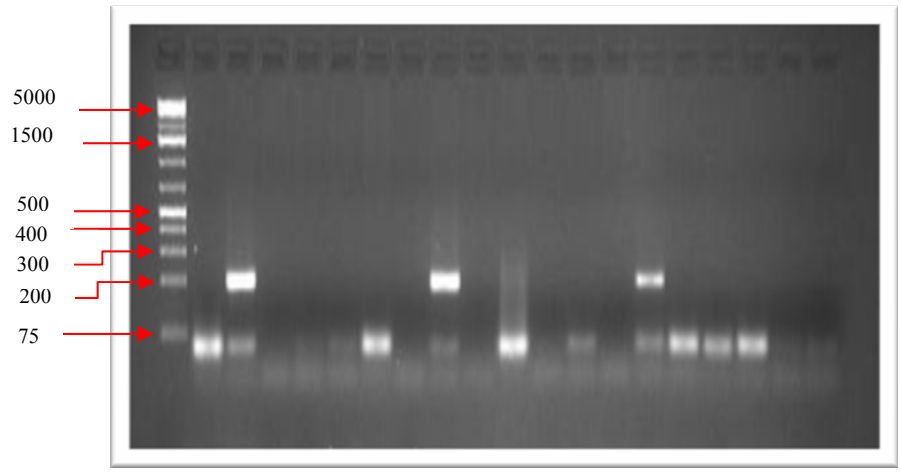
1- **Zhi-ming L, Yu-lian J, Zhao-lei F, Chun-xiao W, Zhen-fang D, Bing-chang Z, Yue-ran Z**. Polymorphisms of Killer Cell Immunoglobulin-like Receptor Gene: Possible Association with Susceptibility to or Clearance of Hepatitis B Virus Infection in Chinese Han Population. 2007, Croat Med J, 48(6): 800-806.

2- **Maxwell LD, Williams F, Gilmore P, Meenagh A, Middleton D**. Investigation of killer cell immunoglobulin-like receptor gene diversity: II. KIR2DS4. 2004, Hum Immunol, 65(6): 613-621.

APPENDIX 2

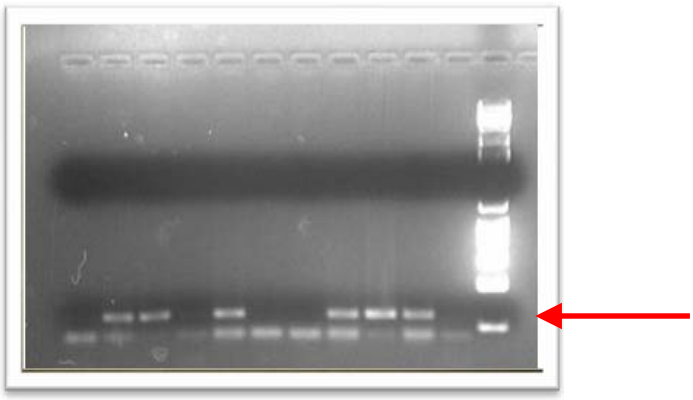
Figure 1. Images of representative agarose gels for different KIR genes.

A.KIR2DS3



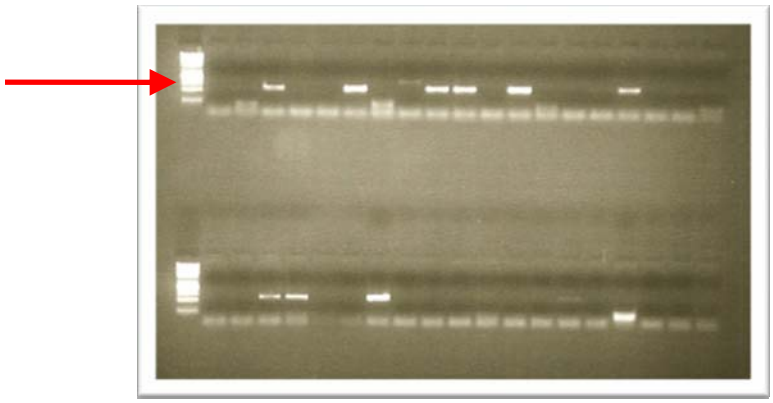
The Figure shows molecular weight markers in lane1(GeneRuler from Fermentas Cat. No. SM1331). The negative and positive controls are shown in lane 2 and 3, respectively. The other lanes contain different samples. The presence of the expected 190 bp band indicates presence of the gene.

B. KIR2DS1



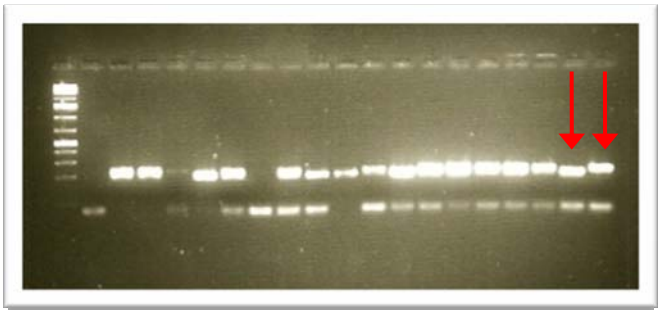
The arrow indicates position of the expected band for KIR2DS1(102 bp). The first three lanes (from right to left) represent Molecular Weight Marker ladder (Fermentas Cat. No. SM1331), a negative and a positive control, respectively. The other lanes indicate different samples. The expected band for the gene (upper one) is clearly visible in positive samples.

C. KIR2DS2



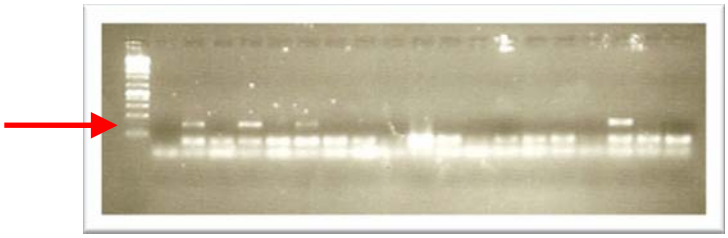
The arrow indicates position of the expected band for KIR2DS2 (240 bp). The first four lanes represent Molecular Weight Marker ladder (Fermentas Cat. No. SM1331), two negative and a positive control, respectively. The other lanes indicate different samples. The upper band of the expected size (240 bp) is clearly visible in positive samples.

D. KIR2DS4



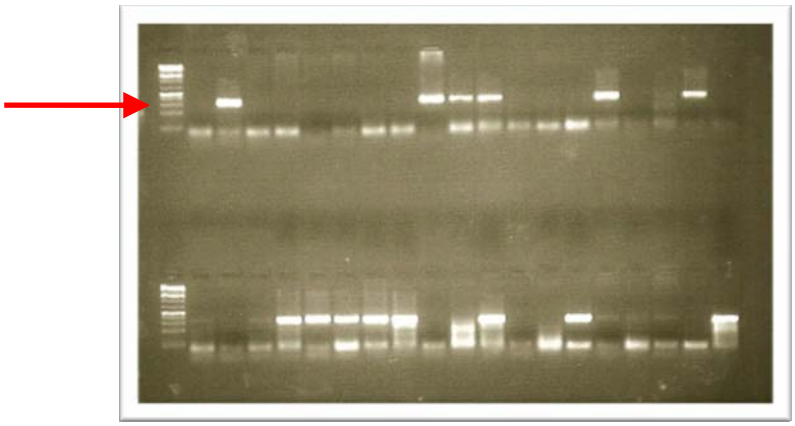
The amplicons for the KIR2DS4, 221 bp for the intact gene and 199 bp for the mutant gene, are shown by right and left arrows, respectively. The first three lanes represent Molecular Weight Marker ladder (Fermentas Cat. No. SM1331), a negative and a positive control, respectively.

E. KIR2DS5



The arrow indicates position of the expected band for KIR2DS5 (125 bp). The first three lanes represent Molecular Weight Marker ladder (Fermentas Cat. No. SM1331), a negative and a positive control, respectively. The other lanes indicate different samples. The upper band of the expected size is clearly visible in positive samples.

F. KIR3DS1



The arrow indicates position of the expected band for KIR3DS1 (300 bp). The first three lanes represent Molecular Weight Marker ladder (Fermentas Cat. No. SM1331), a negative and a positive control, respectively. The other lanes indicate different samples. The upper band of the expected size (300 bp) is clearly visible in positive samples.