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Genetic distribution of Natural Killer Cell Receptors (Killer Immunoglobulin-like Receptors) in Familial Mediterranean Fever, Recurrent Tonsillitis and Lymphoma in Lebanese patients.

By

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Amira Sabbagh

To my parents, sister and the

closest to my heart

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ABSTRACT

Natural killer (NK) cells are crucial effectors of the immune system response being fundamental in defenses against tumor growth and certain cytopathic viruses. The first identified receptors are a group of human leukocyte antigen (HLA) termed killer immunoglobulin Ig-like receptors (KIRs). Genotypic profiles of the Natural killer cell Immunoglobulin-like Receptors (KIR) have been reported to vary among different ethnic groups and variable clinical entities. This study represents the first report on its distribution among patients with Familial Mediterranean Fever (FMF), Recurrent Tonsillitis and Lymphoma in Lebanese patients. The study population consisted of unrelated Lebanese patients diagnosed at the American University of Beirut Medical Center with FMF (n=56), Recurrent Tonsillitis (n=34) and Lymphoma (n=37). Their DNA was typed using a Sequence Specific Primer (SSP) technique for the presence of 16 KIR gene and pseudogene loci and was compared to the general Lebanese population control samples. Results show that the distribution of KIR genes was not significantly different at the 5% level between the cases and controls (Lebanese population), except for a pseudogene KIR 3DP1*003, that was significantly more prevalent among FMF patients than the general Lebanese population as well as for a the KIR 2DS5 gene that was also significantly more prevalent among lymphoma patients as compared to the general Lebanese population. Six new KIR profiles were identified in this study among FMF patients. These results raise an interesting question of whether or not KIR genotype is involved in the predisposition to or pathogenesis of FMF, Recurrent Tonsillitis and Lymphoma. Additional research must be performed especially at the translational/protein level to detect the biological implications of these 2 genes and whether or not they are expressed and whether their expression has a role in the pathogenesis of the disease.

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LIST OF ABBREVIATIONS

CTL: Cytolytic T lymphocyte

EBV: Epstein-Barr virus

FL: Follicular Lymphoma

FMF: Familial Mediterranean Fever

HD: Hodgkin's disease

HL: Hodgkin lymphoma

HLA: Human Leukocyte Antigen

IL: Interleukin

ITAM: Immunoreceptor Tyrosine-based Activation Motif

ITIM: Immunoreceptor Tyrosine-based Inhibition Motif

KARAP: Killer-cell Activating Receptor Associated Protein

KIR: Killer Immunoglobulin Ig-like Receptor

LCR: Leukocyte Receptor Complex

MEFV: MEditerranean FeVer

MHC: Major Histocompatibility Complex

NCR: Natural Cytotoxicity Receptors

NHL: Non-Hodgkin lymphoma

NK: Natural killer

NPC: Nasopharyngeal Carcinoma

TCR: T-cell receptors

INTRODUCTION

1.1. Natural Killer Cells (NK cells)

1.1.1. Introduction:

Natural killer (NK) cells represent a subset of lymphoid population, accounting for approximately 10–20% of peripheral blood lymphocytes. NK cells are considered to be part of the innate immunity as they do not need prior sensitization to exert their activity. They possess antigen-binding receptors in their membrane. The receptors on these cells can recognize antigens that are associated with either class I or class II Major Histocompatibility Complex (MHC) molecules on the surface of cells. The MHC is a set of molecules displayed on cell surfaces that are responsible for lymphocyte recognition and "antigen presentation". The MHC molecules control the immune response through recognition of "self" and "non-self" and, consequently, serve as targets in transplantation rejection. NK cells were originally identified on a functional basis due to their ability to lyse certain tumors without any previous stimulation *in vitro or in vivo*, unlike other lymphocytes; therefore, representing crucial effectors of the innate immune response (Kim, Iizuka, Aguila, Weissman, and Yokoyama, 2000). They are mainly limited to the peripheral blood, spleen, bone marrow and liver. Generally, they are not typically found in lymph nodes or lymphatics; however, they can migrate to these tissues in the early phases of various inflammatory processes. NK cells are fundamental in defenses against tumor growth in vivo and prevent the rapid propagation of metastatic tumors at least in a murine model (Kim et

al., 2000) as well as against certain cytopathic viruses, primarily herpesviruses (Biron, Nguyen and Pien, 1999). It was shown that NK cells, when activated, function by releasing cytokines and chemokines which will stimulate inflammatory responses, alter hematopoiesis, control monocyte and granulocyte cell growth and function, as well as select the type of subsequent adaptive responses (Trinchieri, 1989). Despite this knowledge on NK cells, they remained an ambiguity for many years, especially with reference to their nature and mode of action (Moretta L et al., 2002). However, due to advances in molecular characterization of different cell types of the innate immune system; NK cells in particular are nowadays becoming a field of major interest in immunology (Moretta L et al., 1994; Trinchieri, 1989).

1.1.2. NK cell inhibition via MHC class I-specific inhibitory receptors:

Molecular studies in mice (Ljunggren and Karre, 1990; Yokoyama and Seaman, 1993) and in humans (Ciccone et al., 1992; Colonna, Borsellino, Falco, Ferrara and Strominger, 1993; Moretta A et al., 1990; Moretta L et al., 1992;) permitted the identification of a number of novel receptors specific for the major histocompatibility complex (MHC) class I named Natural Killer cell Receptors (NKRs). These NKRs differ from the T-cell receptors (TCR) in that they send inhibitory rather than activating signals. As suggested by Ljunggren and Kärre (1990), the "missing self" hypothesis explains the mechanism by which the receptors are responsible for the discrimination by NK cells between normal cells and cells enduring tumor transformation or viral infection mice. It seems that the lack of expression of self MHC class I molecules, which is a frequent event in tumor transformation and viral infection, would result in lysis of these target cells since NK cells recognize MHC molecules via surface receptors sending signals that inhibit

the NK cell function (Moretta A et al., 1996). The first identified is a group of human leukocyte antigen (HLA) class I-specific inhibitory receptors termed killer immunoglobulin Ig-like receptors (KIRs) that are responsible for recognition of different allelic determinants of the HLA-A, B and C groups (Lanier, 1998, Long, 1999; Moretta A et al., 1996).

1.1.3. NK cells triggering via receptors mediating natural cytotoxicity:

NK cells require an "off" signal that will prevent them to attack normal cells, which led to the implication of an "on" signal mediated by triggering receptors. The first identified activating receptors in humans were also denoted as to be from the KIR gene family (Biassoni, Cantoni, and Falco, 1996; Bottino, Sivori, Vitale, 1996; Trowsdale et al., 2001). Studies led to the identification of three distinct human receptors that have been referred to as natural cytotoxicity receptors (NCRs). Figure 1 shows the different receptors (activating and inhibitory) and ligands involved in human NK cell–mediated cytotoxicity.

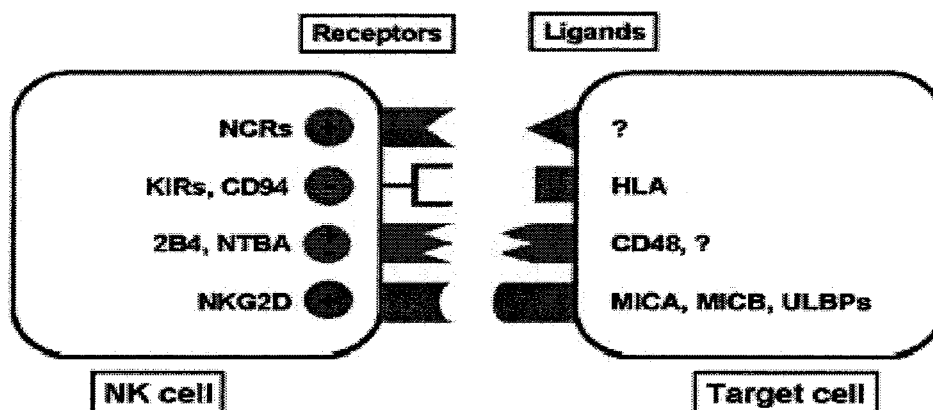

Figure 1. Receptors and ligands involved in human NK cell-mediated cytotoxicity.

NK cell activation is the final result of the engagement of a number of receptors that have opposite functions. A simplified model of the surface receptors and their ligands involved in NK cell activation (green) or inactivation (red) is shown. In the absence of inhibitory signals, activating NK cell receptor

ligation with molecules on the target cell results in NK cell triggering and target cell lysis. This event occurs in MHC class I HLA-defective cells, such as tumors or virus-infected cells. In the case of normal cells that express MHC class I, the interaction between inhibitory receptors and MHC class I delivers signals that overcome NK cell triggering, thus preventing target cell lysis. The + and - symbols denote activating or inhibitory signals, respectively.
NB: In this figure, only Inhibitory KIRs are shown.

1.2. Killer Immunoglobulin Ig-like Receptors (KIRs)

1.2.1. KIR Repertoire:

Killer immunoglobulin-like receptors (KIR) were first recognized by their potential to allocate destruction of specific cells by NK cells. To date, 16 distinct KIR gene and pseudogene loci (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DS1, KIR2DP1, and KIR3DP1) have been identified and are clustered in the 1 MB leukocyte receptor complex (LRC) region on human chromosome 19q 13.4 (Figure 2) (Marsh et al., 2003; Wilson, Torkar and Trowsdale, 1997). Studies have revealed an extensive diversity at the KIR gene locus, generating remarkable diversity in the observed KIR phenotypes among various populations (Uhrberg et al., 1997).

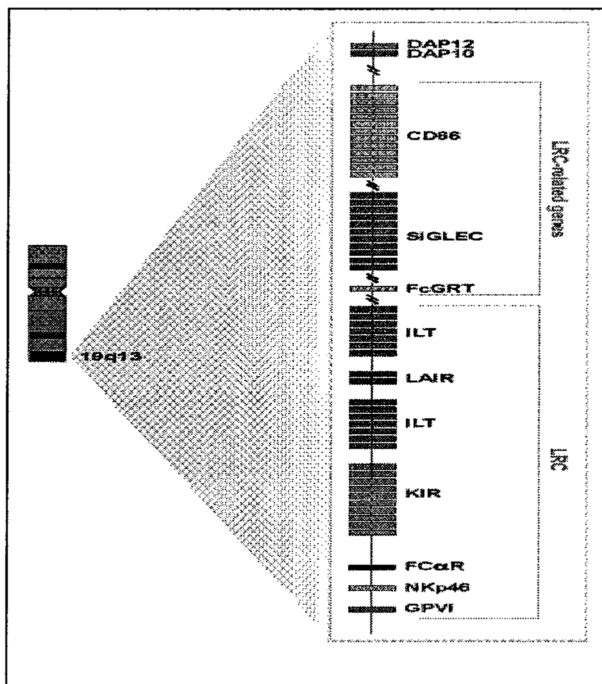

Figure 2. Map of the Leukocyte Receptor Complex.

The leukocyte Receptor Complex is located on the human chromosome 19q 13.4 and at least 40 members of the Immunoglobulin superfamily are incorporated within the LRC, including the KIR genes.

1.2.2. Mode of action:

Activation signals are transmitted by small transmembrane-anchored adaptor proteins that possess immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains. NK cells express these ITAM-bearing adaptor proteins. KIRs have been identified to pair with these adaptor proteins. Ligation of an ITAM-bearing receptor complexes results in the recruitment and activation of a tyrosine kinase, expressed on all NK cells (Lanier, Corliss, Jun, Leong and Phillips, 1998). Paradoxically, the mechanism for the inhibitory NK cell receptors was determined long before understanding the activation signaling pathway. All of the inhibitory KIRs receptors possess immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic domains. These ITIMs will get phosphorylated and will recruit and activate two phosphatases. These phosphatases will switch off the activating signals initiated via the various triggering NK cell receptors (Lanier et al., 1998; Long, 1999; Moretta A et al., 1996).

1.2.3. Nomenclature:

A subcommittee of the WHO Nomenclature Committee for Factors of the HLA System assigned the naming of the alleles of the genes encoding the KIRs (Marsh et al., 2003). The first digit following the KIR acronym corresponds to the number of Ig-like domains in the molecule and the ''D'' denotes ''domain.'' The D is followed by either an ''L'' meaning ''Long'' cytoplasmic tail, and ''S'' indicating a ''Short'' cytoplasmic tail, or a ''P'' for ''pseudogenes'' The final digit indicates the number of the gene encoding a protein with this structure. Where two or more genes have analogous structures and have similar sequences, they are usually assigned the same

number but distinguished by a final letter, as the KIR2DL5A and KIR2DL5B genes (Gomez-Lozano, Gardiner, Parham and Vilches, 2002).

1.2.4. KIR Ligands:

It has been shown that ligands of several inhibitory KIR genes are actually subsets of HLA class I molecules (Vilches and Parham, 2002). A more precise summary of the known ligands of KIR are listed in Table 1 (Carrington and Norman, 2003).

Table 1. KIR Ligand Specificity

2DL1 and 2DS1	2DL2/3 and 2DS2	3DL1/S1	3DL2	2DL4	2DS4
HLA-C grp 2	**HLA-C grp 1**	**HLA-B Bw4**	**HLA-A**	**HLA-G**	
C*02	C*01	B*08	A*03		C*04
C*04	C*03	B*13	A*11		
C*05	C*07	B*27			
C*06	C*08	B*44			
		B*51			
		B*52			
		B*53			
		B*57			
		B*58			

The table shows the specific interaction between the several KIRs and the HLA ligands.

1.2.5. Exon-Intron Structure of the KIR genes:

A constant characteristic of the KIR gene family is the arrangement of the exon-intron sequence. The genomic structure of the KIR genes has been identified after cloning a region of the chromosome 19q13.4. The distances between the introns vary widely whereas the margins of the exons are preserved. The KIR signal sequence is expressed by the first two exons, whereas exons 3 to 5 encode the Ig-like domains D0, D1, and D2. The exons 6 and 7 encode the linker and the transmembrane regions, and the last exons in the sequence encode the cytoplasmic domain (Wilson et al., 1997).

1.2.6. KIR Gene Order and Haplotypic Diversity:

The order of the KIR genes along the chromosome has been determined and two distinct haplotypes are recognized: A and B (Hsu et al., 2002; Uhrberg et al., 1997; Witt et al., 1999) (Figure 3). In general, the genes are arranged in a head-to-tail manner and each gene is about 10-16 kb in length separated by a 2kb sequence. Group A haplotypes include KIR3DL3, KIR2DL3, KIR2DP1, KIR2DL1, KIR3DP1, KIR2DL4, KIR3DL1, KIR2DS4, and KIR3DL2 genes. They have only one activating receptor, have low variation in gene content but have extensive allelic variability. In contrast, all other haplotypes are described as haplotype B and have between two and five activating receptors have substantial gene content variability but are less polymorphic than the group A genes. The haplotype is considered AA when only group A genes are present. Similarly, it is assumed BB only when group B genes are present. However, when both are encountered, then the haplotype is considered AB.

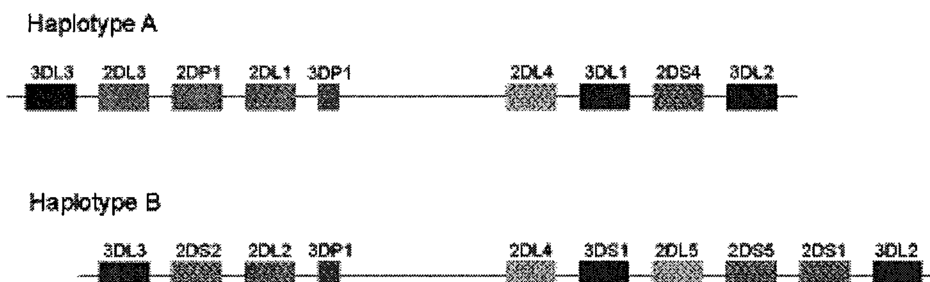

Figure 3. Gene order of two fully sequenced KIR haplotypes.

The order of the KIR genes along the chromosome has been determined for two distinct haplotypes. The genes are organized in a head-to-tail fashion, and each gene is roughly 10–16 kb in length with a sequence of about 2 kb separating each pair of genes, except for a 14 kb stretch of unique sequence upstream of 2DL4.

1.2.7. Phylogenetic Evolution:

Several KIR genes have been identified in all primates and were revealed to be rapidly evolving (Hershberger, Shyam, Miura and Letvin, 2001; Khakoo et al., 2000; Sambrook et al., 2005; Vilches et al., 2002). Comparison of KIR in several primate species, including, common chimpanzee, pygmy chimpanzee, and rhesus monkey, confirmed a remarkable change in the KIR-gene family over a few million years (Hershberger et al., 2001; Khakoo et al., 2000; Rajalingam et al., 2001). In chimpanzee, the closest living species to humans, ten KIR genes have been identified, and only three of them seem to be direct orthologues of human KIR (Khakoo et al., 2000).

1.2.8. Diversity:

Population studies have revealed that KIR gene frequencies and genotype content differ significantly among diverse ethnic groups. In addition, the frequencies of A and B haplotypes have also been found to differ between populations including Caucasoid (Hsu et al., 2002; Norman, Stephens, Verity, Chandanayingyong and Vaughan, 2001; Toneva et al., 2001; Uhrberg, Parham and Wernet, 2002), African (Denis et al., 2005; Norman et al., 2002), North Indian (Rajalingam et al., 2002), Japanese (Yawata et al., 2002), Korean (Whang, Park H, Yoon and Park M.H., 2005), Greek (Niokou, Spyropoulou-Vlachou, Darlamitsou, Stravropoulos-Giokas, 2003), Chinese (Jiang, Zhu, Lv and Yan, 2005), Australian Aborigines and Vietnamese (Toneva et al., 2001), and Palestinian (Norman et al., 2001). In the Lebanese normal population, only one study, conducted by Mahfouz, Rayes, Mahfoud, Bazarbachi and Zaatari (2006), reported the KIR genotype and haplotype frequencies (Mahfouz, Rayes, Mahfoud, Bazarbachi

and Zaatari, 2006). Another Lebanese report compares the latter results to the frequencies obtained in the Palestinian population (Norman et al., 2001) and studies the allelic diversity between these two Arab populations which has shed additional light on the importance of these genes in delineating a possible geographic genetic demarcation among different ethnicities or even different communities among the same or close ethnic groups (Rayes et al., 2007).

1.2.9. Disease association:

Studies have also shown the effect of the presence or absence of specific KIR genes in human disease including hepatitis C virus infection (Khakoo et al., 2004), HIV-1 infection (Gaudieri et al., 2005), cervical neoplasia (Carrington, Wang and Martin, 2005), malignant melanoma (Naumova et al., 2005), psoriatic arthritis (Nelson et al., 2004; Williams F et al., 2005), psoriatic vulgaris (Luszczek et al., 2004), type 1 diabetes mellitus (Santin, de Nanclares, Calvo, Gaafar and Castano, 2006), Rheumatoid Arthritis (Kogure et al., 2007), nasopharyngeal carcinoma (Butsh Kovacic et al., 2005) and preeclampsia (Hiby et al., 2004). Most of these studies have reported strong correlation between the expression of KIR genes and the disease.

1.3. Familial Mediterranean Fever (FMF)

Also known as paroxysmal polyserositis, Familial Mediterranean Fever (FMF), is an autosomal recessive inflammatory disorder predominantly affecting people living in or originating from areas around the Mediterranean Sea, mainly Jews, Armenians, Turks, Arabs and Italians (Chen et al., 1998; Daniels, Shohat T., Brenner-Ullman and Shohat M., 1995; Touitou, Ben-Chetrit and Notrnicola, 1998;). It is characterized by recurrent attacks of inflammation of serosal membranes

and of fever, resulting in acute abdominal, chest, or joint pain. The major complication of FMF is the development of renal amyloidosis, ultimately leading to kidney failure. FMF notoriously has been hard to diagnose until mutations in the MEFV (for MEditerranean FeVer) gene have been identified. The gene is located on chromosome 16 and encodes a leukocyte- and monocyte-specific inflammatory regulator. It is suggested that mutations in the MEFV gene are responsible for the autoinflammatory phenotype of FMF (Centola et al., 2000). It has been considered that immunological abnormalities in the T and B cells, in addition to certain cytokines, take part in the pathogenesis of the disease (Bagci, Toy and Tuzun, 2004; Musabak et al., 2004). It has been suggested that there is a continuous elevation of cytokine levels in FMF patients when compared to a control group (Bagci et al., 2004). It has also been found that secreted levels of some cytokines are higher in attack-free FMF patients than those in controls. This is probably due to the misregulation of cytokine transcriptional pathways (in attack-free FMF patients) (Notarnicola, Didelot, Seguret, Demaille and Touitou, 2002). If MEFV gene mutations are known and the pathophysiology is clear, why are we suspecting a role for KIR? Due to the incomplete penetrance of the MEFV mutations, it is possible that KIR genotype may be a factor influencing penetrance and thus, looking at the various genotypes of this surface receptor is worth a research investigation (Tunca et al., 2002).

1.4. Recurrent Tonsillitis

The tonsils and the adenoids, also known as nasopharyngeal tonsils, are mostly composed of lymphoid tissue, which is found throughout the gastrointestinal tract and on the base of the tongue. This lymphoid tissue is composed of all types of lymphocytes, which are necessary to

support humoral and cell-mediated immune responses (Bernstein, 1992). Since it is in touch with the environment, it appears to have a crucial role in the immune defense against bacteria and other antigens.

Acute tonsillitis/pharyngitis is an inflammation of the pharyngeal tonsils, and is considered as one of the most common illnesses resulting in a symptom referred to as sore throat. Most of the episodes are infectious. The etiologic agents may be viral or bacterial, with the bacterial agents accounting for up to one third of acute tonsillitis episodes. These bacterial agents include Staphylococcus aureus, Haemophilus influenzae, group A Streptococcus and Klebsiella pneumoniae (Barzilai, Miron and Sela, 2001). Recurrent Tonsillitis is usually due to chronic inflammation of the tonsils. It is considered to be recurrent when it occurs three times in six months or four in 12 months (Alho et al., 2007). Heritability of recurrent tonsillitis has been questioned and it was shown that there is evidence for a substantial genetic predisposition for recurrent tonsillitis (Kvestad et al., 2005). In clinical practice, such evidence was noticed as well, and it seems that recurrent tonsillitis runs in some families and gets transmitted from one generation to the other within these families. This raises the suspicion of a genetic predisposition in these patients (Kvestad et al., 2005).

1.4. Lymphoma

The WHO classification of hematological malignancies recognizes three major categories of lymphoid neoplasms: Non-Hodgkin lymphoma (NHL) which could be categorized as either B cell neoplasm or T and NK cell neoplasm, and Hodgkin lymphoma (HL).

Mature B-cell neoplasms comprise over 85% of non-Hodgkin lymphomas. The two most common types are B-cell lymphoma and follicular lymphoma (FL), and comprise 50% of all non-Hodgkin lymphomas.

1.5.1. Follicular Lymphoma

Follicular Lymphoma (FL) is a neoplasm of follicle center B cells. FL is the second most common form of non-Hodgkin's lymphoma, accounting for 35% of adult NHL and 22% worldwide. It affects mostly adults, with a median age of 59 years and a male:female ratio of 1:1.7 (The Non-Hodgkin's Lymphoma Classification Project, 1997). The major sites of involvement include lymph nodes, but spleen, bone marrow, peripheral blood and Waldeyer's ring can also be involved. The disease could be either indolent, gradually progressive over a period of several years, presenting with lymphadenopathy; or it could progress rapidly, frequently transforming into an aggressive type of lymphoma with early death (Johnson et al., 1995). The median survival rate is of 8 to 10 years.

The major molecular defect of neoplastic cells in FL is the t(14;18)(q32;q21) translocation which is seen in the majority of cases. This translocation will trigger the rearrangement of the bcl-2 oncogene resulting in the constitutive expression of the anti-apoptotic protein bcl-2, thus inhibiting apoptosis and eventually leading to progression to a follicular lymphoma (Knutsen, 1997; Korsmeyer, 1992). The translocation t(14;18) is not associated with either a better or a worse prognosis. However, it is suggested that the analysis of this translocation in this particular disease is important in terms of studying the ethno-geographic differences in the incidence of such tumors. In fact, it was deduced from several studies that the frequency of this translocation

varies considerably across different geographic regions, and may vary from up to 89% in the American follicular lymphoma to around 30% in the Japanese lymphoma (Amakawa, Fukuhara and Ohno, 1989; Gulley, Dent and Ross, 1992). A study conducted by Mahfouz, Shammaa, Tawil, and Zaatari (2006) found a low frequency of t(14;18) in Lebanese follicular lymphoma patients (45.2%, 19 out of 42 cases), one of the lowest frequency among several surrounding and regional countries in the Middle East (Mahfouz, Shammaa, Tawil and Zaatari, 2006).

Recently, immunological responses generated by neoplastic cells in FL have been corroborated to have a crucial role in the clinical characteristics and prognosis of the disease. In fact, NK cells have been implicated in natural resistance to tumors, particularly lymphomas. Several studies investigated the distribution of the murine monoclonal antibody, HNK-1, which reacts with all the human NK cells in the peripheral blood. Follicular lymphomas were found to be highly infiltrated with HNK-1+ cells compared to other types of B-cell malignant lymphoma, all types of T-cell malignancies, and Hodgkin's disease, which were shown to be very poor in HNK-1+ cell reaction (Mori, Yamaguchi, Morita and Mohri, 1985). It is suggested that a joint genetic and immunologic data might facilitate a better understanding of the development of FL, and thus improve the treatment options to acquire a higher survival rate.

1.5.2. Hodgkin Lymphoma

Hodgkin Lymphoma (HL), also known as Hodgkin's disease (HD), account for approximately 30% of all lymphomas. It differs from other lymphomas in terms of both specific pathological and epidemiological features. It is known that both genetic and environmental factors are crucial factors in the pathogenesis of HL (Cartwright and Watkins, 2004). However, an infectious agent

has been suspected as a precursor to HL. In particular, considerable evidence has implicated the Epstein-Barr virus (EBV), a herpesvirus that was found to be associated with several lymphoid and epithelial malignancies. A biologic explanation of this association between EBV and HL is that EBV renders the human B-lymphocytes immortal and turns them into tumor cells. (Thorley-Lawson and Gross, 2004). The suspicion that EBV has an etiological role in the pathogenesis of HL is further supported by the presence of the EBV antigens in Reed–Sternberg cells in approximately 40 to 50 percent of HL patients. In deed, epidemiologic studies have shown that a history of infectious mononucleosis-a clinical manifestation of EBV infection- triples the risk of developing HL. Antibody titers to the EBV antigen was found to be higher in HL patients than control subjects at the time of diagnosis as well as years before and after diagnosis (Mueller, 1987; Mueller et al., 1989). These observations indicate a causal association between infectious mononucleosis–related EBV infection and EBV-positive Hodgkin's lymphoma in young adults. It is then deduced that a malfunction of the immune system against EBV infection may contribute to the development of HL. The NK cells are known to control the early phase of primary viral infections, including EBV (Williams H et al., 2005). This association is well illustrated by the development of Epstein-Barr virus–driven lymphoproliferative disorder in a child with a specific NK cell deficiency (Eidenschenk et al., 2006).

Purpose of the Project:

The purpose of this study was to establish the role of the NK cells in these three different diseases, Familial Mediterranean Fever, Recurrent Tonsillitis and Lymphoma in Lebanese patients. So far no data exist worldwide linking KIR genotype to these specific diseases. This

was done by DNA typing using Sequence Specific Primer (SSP) technique for the presence of 16 KIR gene and pseudogene loci among the patients as compared them to the general Lebanese population, and by presenting a statistical analysis of the findings. Obviously, each of these clinical entities belongs to a category that might indicate a different immunological and inflammatory response and the role of KIR genotype is to be investigated.

Chapter 2

Materials and Methods

2.1. Study Population

The study population consisted of unrelated Lebanese patients diagnosed at the American University of Beirut Medical Center from 2004-2006 with FMF (n=56), Recurrent Tonsillitis (n=34) and Lymphoma (n=37). Demographic data of these patients are presented in separate tables (Appendix 1). In addition, we used as control population the 120 healthy unrelated Lebanese individuals previously reported by Mahfouz, Rayes, Mahfoud, Bazarbachi, and Zaatari (2006) in the same ethnic group (Mahfouz, Rayes et al., 2006). The latter were randomly selected from the HLA healthy bone marrow donors' bank and represent the various districts of Lebanon.

2.2. DNA extraction and KIR Genotyping

PEL-FREEZ kits (Pel-freez/Dynal, Norway) were used for DNA extraction from 2-3 mls of collected peripheral blood. The DNA material was properly labeled and stored at -80^{0}C.

Primer mixes were purchased from PEL-FREEZ/DYNAL company (Oslo, Norway) as part of the *KIR Genotyping SSP kit* which is a PCR-based method designed to detect the absence or presence of the following 16 gene loci of KIR (variants also tested): *2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1,* and *3DP1*. Two variants for *KIR2DL5* were typed *KIR2DL5A*001* and *KIR2DL5B*002/003/004* and two variants for *KIR2DS4* were tested and reported as *KIR2DS4*001/002* and *KIR2DS4*003-006*. In addition,

two variants for the pseudogene *KIR3DP1* were tested: *KIR3DP1*001/002/*004 and *KIR3DP1*003*. The primer mixes are shown in Appendix 2.

KIR genotyping was performed as recommended by the manufacturer, and detailed instructions were strictly followed. Briefly, 25µl of DNA were added to 150µl of PCR buffer and 2.4µl of Taq DNA polymerase and dispensed as aliquots of 8µls into a supplied 96-well plate for a total reaction volume of 23µl in each well (reaction + paraffin oil already present in each well). The thermocycling steps include an initial heating step at 95°C for 1 minute, followed by 30 cycles of 94°C for 20 seconds, 63°C for 20 seconds, and 72°C for 90 seconds. A final holding step was performed at 4°C. Electrophoresis of the 2% Gel was done in ethidium bromide and visualization performed under UV light transillumination.

2.3. Gel Analysis

Appendix 3 shows the explanation of the analysis of the gel.

Haplotypes' identification was based on the internationally assigned haplotypes A and B. When a patient shows positivity for only group A or group B genes, then this patient is categorized as AA or BB respectively. However, when the patient shows positivity for both group A and B genes, then this patient is categorized as AB. Different possibilities of the 3 genotypes can be encountered and some have been already identified by previously published data. When a new haplotypes is found, the ()n -meaning new- was assigned to it.

2.4. Statistical Analysis

SPSS 15.0 was used to conduct statistical analysis. Genetic expression was expressed as number and frequency. Chi-square was used to test for association between group (case vs control) and genetic expression. In case one of the categories contained less than 5 patients, Fisher's exact test was conducted. P-value less than 0.05 was considered statistically significant.

Results

Appendix 3 shows the distribution of the assumed KIR haplotypes among the FMF, the recurrent tonsillitis and the lymphoma patients respectively. The corresponding number of genes in each profile with the total number of individuals in every profile is also displayed.

Tables 2, 4 and 6 represent the gene frequencies for the FMF, the recurrent tonsillitis and the lymphoma patients, respectively, as compared to the general Lebanese population. In addition, the chi-squared p-values were determined for every single gene.

Tables 3, 5 and 7 represent the frequency distribution of the AA, AB, and BB genotypes as well as the frequency of the A and B haplotypes among the FMF, the recurrent tonsillitis and the lymphoma patients, respectively, compared to the general population.

3.1 Distribution of KIR Locus frequencies among FMF patients

The 56 FMF patients have the following demographic characteristics: 28 (50%) were males and 28 (50%) were females. The average mean age was 41.7 years (range= 19-64).

Figure 4 shows the gel results of one FMF patient. This patient has positive bands for A1, B1, C1, D1, E1, F1, G1, H1, A2, B2, C2, E2, G2, H2, A3, B3, C3 and D3 and negative bands for D2, F2, and E3. After gel analysis on the gel worksheets, the KIR genes present in this patient can be obtained.

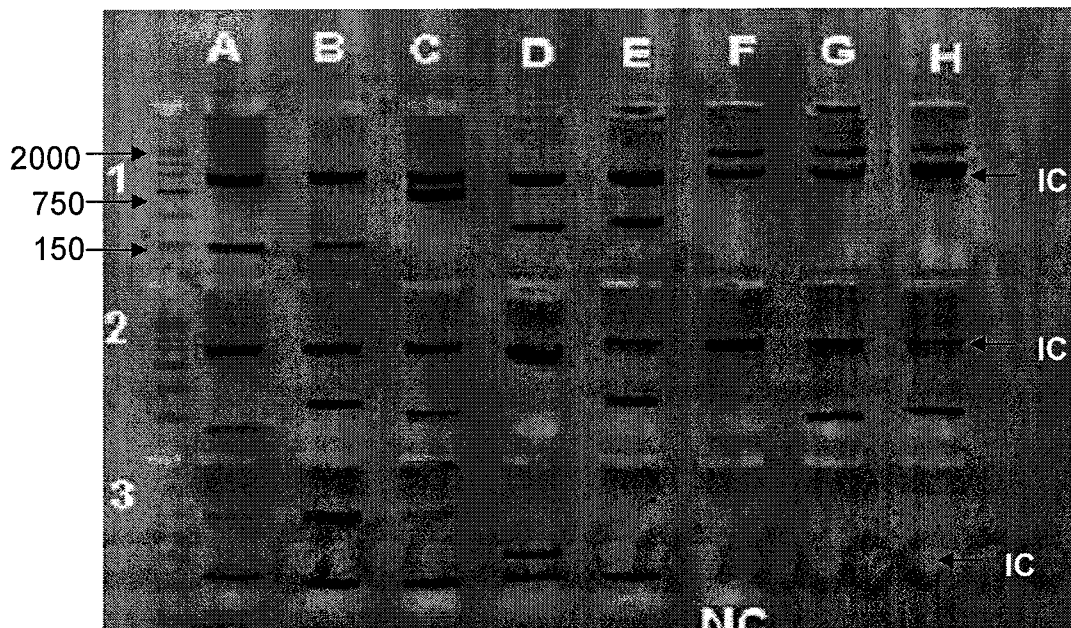

Figure 4. Gel Electrophoresis of KIR genotyping for one FMF patient.
On each well location, an internal control (IC) should appear. If a band is present at the specific size for each well, then the band is considered positive. F3 is the negative control (NC) which is negative.
IC: Internal Control, Scale 150-2000 bp

As seen in the general population, all FMF patients with both haplotype A and B maintained three genes, *KIR3DL3, KIR3DL2*, and *KIR2DL4*. We observed 19 different KIR genotypes among the FMF patients compared to 24 in the general Lebanese population with six new profiles not previously reported in our population (referred as "()n" in Appendix 4). The KIR genotypes among the FMF patients contained between 7 and 15 genes with an average number of 11.5 KIR loci per individual (excluding the pseudogenes, *KIR2DP1* and *KIR3DP1*).

In table 2, the results show that the distribution of KIR genes was not significantly different at the 5% level between the cases (FMF patients) and controls (Lebanese population), except for a pseudogene KIR 3DP1*003. It was significantly more prevalent among FMF patients than the general Lebanese population (p-value = 0.0314).

	Gene	Lebanese population		FMF Patients		P-value
		N	%	N	%	
Inhibitory KIR	2DL1	119	99.2	54	96.4	0.2122
	2DL2	71	59.2	31	55.3	0.6339
	2DL3	106	88.3	46	82.1	0.2741
	2DL4	120	100	56	100	1.00
	2DL5A	43	35.8	20	35.7	0.9878
	2DL5B	46	38.3	25	44.6	0.4279
	3DL1	115	95.8	55	98.2	0.3910
	3DL2	120	100	56	100	1.00
	3DL3	120	100	56	100	1.00
Non Inhibitory KIR	2DS1	49	40.8	25	44.6	0.7998
	2DS2	71	59.2	33	58.9	0.9761
	2DS3	45	37.5	25	44.6	0.3686
	2DS4*001/002	47	39.2	5	8.9	0.1678
	2DS4*003-006	96	80.0	49	87.5	0.5296
	2DS5	37	30.8	21	37.5	0.3834
	3DS1	43	35.8	19	33.9	0.8051
Pseudo-genes	2DP1	116	96.7	54	96.4	0.9356
	3DP1*001/002/004	33	27.5	11	19.6	0.2551
	3DP1*003	120	100.0	54	96.4	**0.0314**

Table 2. KIR Gene frequencies.

Table 3 shows that the group A haplotype was as frequent (50.9%) as the group B haplotype (49.1%) with a ratio of A:B of 1:1. The genotype distribution among both groups is significantly different (p=0.007).

The frequency of the genes versus sex was determined and there was no significant difference between males and females in FMF patients (data not shown).

KIR Genotype	Lebanese population No.	%	FMF patients No.	%	p-value
AA	31	25.8	17	30.4	**0.007**
AB	75	62.5	23	41.1	
BB	14	11.7	16	28.5	
Total	120	100	56	100	
KIR Haplotype					
A	137	57	57	50.9	0.28
B	103	43	55	49.1	
Total	240	100	112	100	

Table 4. Frequency of KIR haplotype and genotype groups.

3.2 Distribution of KIR Locus frequencies among Recurrent Tonsillitis patients

The 34 recurrent tonsillitis patients have the following demographic characteristics: 19 (55.9%) were males and 15 (44.1%) were females. The average mean age was 5.2 years (range= 3-11). Figure 5 shows the gel results of one patient. This patient has positive bands for A1, C1, D1, E1, F1, A2, C2, E2, G2, A3, B3, and D3 and negative bands for the remaining bands. The F3 is the negative control and is negative.

Figure 5: Gel Electrophoresis of KIR genotyping for one recurrent tonsillitis patient.

Similarly to the general population, all tonsillitis patients have the *KIR3DL3, KIR3DL2*, and *KIR2DL4* genes for both haplotypes A and B. We noted 25 different KIR genotypes among the group of patients compared to 24 in the general Lebanese population (Appendix 4). The KIR genotypes among the tonsillitis patients contained between 8 and 14 genes with an average number of 11 KIR loci per individual (excluding the pseudogenes, *KIR2DP1* and *KIR3DP1* and their variants).

For best statistical analysis and comparisons between the patients and controls, we have used a selective sample (68 cases) representing the controls already studied by Mahfouz et al in 2006 in the general Lebanese population (120 cases). The selection was done by the random computer selection method which randomly selected the 68 patients out of the 120 without any bias. We have done this because the total number of tonsillitis patients is only 34 and thus, appropriately, the control must be twice as larger.

The data obtained and shown in Table 4 demonstrate a non significant difference at the 5% level between the cases (tonsillitis patients) and controls (general population) when comparing the distribution of their KIR genes.

	Gene	**Lebanese population**		**Tonsillitis Patients**		**P-value**
		N	**%**	**N**	**%**	
Inhibitory KIR	2DL1	67	98.5	34	100	1.00
	2DL2	42	61.8	20	58.8	0.77
	2DL3	60	88.2	26	76.5	0.12
	2DL4	68	100	34	100	1.00
	2DL5A	24	35.3	15	44.1	0.39
	2DL5B	26	38.2	12	35.3	0.77
	3DL1	64	94.1	32	94.1	1.00
	3DL2	68	100	34	100	1.00
	3DL3	68	100	34	100	1.00
Non Inhibitory KIR	2DS1	28	41.2	16	47.1	0.57
	2DS2	42	61.8	19	55.9	0.57
	2DS3	27	39.7	18	52.9	0.20
	2DS4*001/002	24	35.3	11	32.4	0.77
	2DS4*003-006	54	79.4	24	70.6	0.32
	2DS5	19	27.9	11	32.4	0.64
	3DS1	23	33.8	14	41.2	0.47
Pseudo-genes	2DP1	65	95.6	34	100	0.55
	3DP1*001/002/004	19	27.9	6	17.6	0.26
	3DP1*003	68	100	33	97.1	0.33

Table 4. KIR Gene frequencies.

Figure 6 shows the gel results of a lymphoma patient. This patients has positive bands for A1, B1, C1, D1, E1, F1, A2, B2, E2, F2, G2, H2, A3, B3, C3, D3, E3. The negative control, F3, is negative.

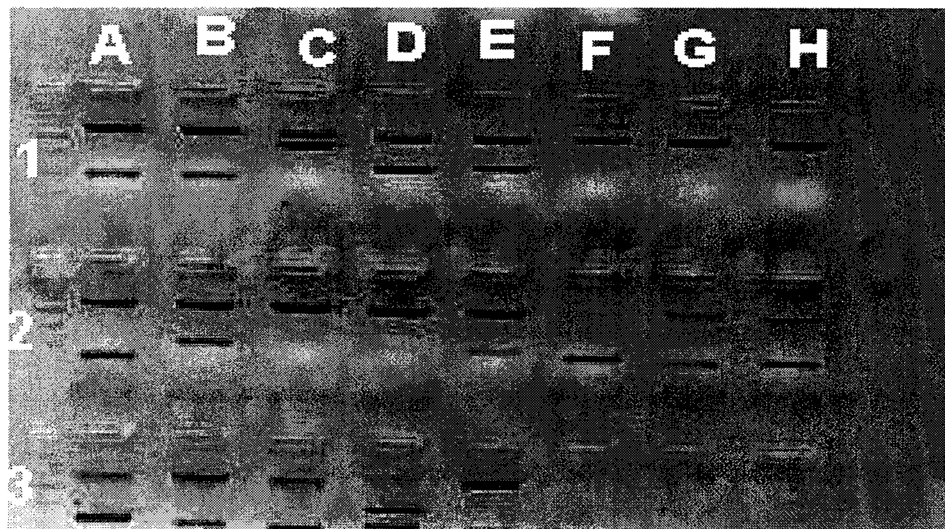

Figure 6: Gel electrophoresis of KIR genotyping for one lymphoma patient

As compared to the FMF and tonsillitis patients, and also similar to what has been described in the general population, all lymphoma patients maintained the genes *KIR3DL3, KIR3DL2*, and *KIR2DL4* for both haplotypes A and B. In this category of patients, we noted 27 different KIR genotypes among the group of patients compared to 24 in the general Lebanese population (Appendix 4). The KIR genotypes among the lymphoma patients contained between 7 and 15 genes with an average number of 11 KIR loci per individual (excluding the pseudogenes, *KIR2DP1* and *KIR3DP1* and their variants).

Except for *KIR 2DS5*, the data obtained and shown in table 6 demonstrate a non-significant difference at the 5% level between the cases (lymphoma patients) and controls (general population) when comparing the distribution of their KIR genes. The KIR 2DS5 was significantly more prevalent among lymphoma patients as compared to the general Lebanese population (p-value = 0.03). However, no difference was found between males and females in FMF patients.

	Gene	Lebanese population		Lymphoma Patients		P-value
		N	%	N	%	
Inhibitory KIR	2DL1	119	99.2	37	100	1.00
	2DL2	71	59.2	25	67.6	0.53
	2DL3	106	88.3	32	86.5	0.72
	2DL4	120	100	37	100	1.00
	2DL5A	43	35.8	11	29.7	0.66
	2DL5B	46	38.3	15	40.5	0.88
	3DL1	115	95.8	35	94.6	0.66
	3DL2	120	100	37	100	1.00
	3DL3	120	100	37	100	1.00
Non Inhibitory KIR	2DS1	49	40.8	18	48.6	0.50
	2DS2	71	59.2	24	64.9	0.74
	2DS3	45	37.5	14	37.8	0.88
	2DS4*001/002	47	39.2	8	21.6	0.45
	2DS4*003-006	96	80.0	26	70.3	0.09
	2DS5	37	30.8	17	45.9	**0.03**
	3DS1	43	35.8	15	40.5	0.88
Pseudo-genes	2DP1	116	96.7	37	100	0.57
	3DP1*001/002/004	33	27.5	8	21.6	0.53
	3DP1*003	120	100	37	100	1.00

Table 6. KIR Gene frequencies.

Interestingly, and from the results in Table 7, it is noted that the group A haplotype was twice as frequent (66.2%) as the group B haplotype (33.8%) with a ratio of A:B of 2:1 (compared to the ratio of 1.3:1 reported for the general population as described by *Mahfouz, Rayes et al.*, 2006). The genotypic distribution was not found to be significantly different between the patients and the controls.

	Lebanese population		**Lymphoma patients**		**p-value**
KIR Genotype	**No.**	**%**	**No.**	**%**	
AA	31	25.8	12	32.4	0.09
AB	75	62.5	25	67.6	
BB	14	11.7	0	0.0	
Total	120	100	37	100	
KIR Haplotype					
A	137	57	49	66.2	0.15
B	103	43	25	33.8	
Total	240	100	74	100	

Table 7. Frequency of KIR haplotype and genotype groups.

DISCUSSION & CONCLUSIONS

KIR receptors have been studied in the setting of a variety of clinical conditions including HIV and hepatitis C, melanomas, diabetes, and psoriatic arthritis.

In this research work, we tested unrelated Lebanese patients with different clinical diseases (Familial Mediterranean Fever, Recurrent tonsillitis and Lymphoma – including both Hodgkins and non-Hodgkin's types) for 16 KIR genes and pseudogenes using PCR-SSP typing and compared them to the general population. The choice of these clinical entities pertains mainly to the immunopathophysiology of the disease. For example, FMF is a genetic condition and is not really called "immunological" disease, while Lymphoma on the other hand, mainly involves our immune systems and has previously been reported in the international medical literature to involve a battery of cytokines, interleukins, and growth factors – mainly a burst of the immune system. As a typical in-between example, recurrent tonsillitis involves the tonsils as a first line defense system against a variety of viral and bacterial agents, thus, a pathway for immunological response initiation and activation – like in lymphoma patients - but at the same time, it is not a "genetic" condition unlike the case in FMF, meaning that there is no genetic defect behind the pathogenesis of the disease (as so far is being known). This is contrary to FMF where the MEFV gene mutations are incriminated in this clinical condition.

In the FMF patients, 56 patients were included in this work. We found 19 distinct gene profiles, six of which have not been previously described among the "control cases" (selected from healthy bone marrow donors). The average number of KIR loci per patient is comparable to the general Lebanese population (an average of 11 KIR loci); therefore, there is no difference in the gene content among the FMF patients and controls (Mahfouz, Rayes et al., 2006). This indicates that the KIR locus gene content itself may not be of any significance in disease pathophysiology. In reference to Table 3, the only statistically significant difference between the FMF patients and healthy controls as per the distribution of activating and inhibitory genes, is found in a pseudogene (KIR 3DP1*003). This is quite interesting at this level especially in terms of the p-value (0.03) since pseudogene are not supposed to be expressed. Therefore, much more research must be performed especially at the translational/protein level to detect the biological implications of this finding and whether or not this is truly a pseudogene and thus the result is only a statistical "artifact". Another statistically significant finding is related to the difference in the genotypic profiles among FMF patients and control cases with a p-value of 0.007, whereby there is a major increase in the BB genotype in the FMF patients unlike its lowest prevalence in the general population (28.5% vs. 11.7%). This is important since the B haplotype does not contain a high number of activating genes, thus, is an increase in inhibitory KIR genes – through a homozygous BB genotype – involved in the pathogenesis of FMF? An interesting study by Moffett and Hiby (2007) demonstrated a link between the *AA* genotype and the way the maternal immune system may be operating in pregnancy to regulate placentation (Moffett and Hiby, 2007). It was found that combination of maternal KIR AA genotype with a fetal HLA-C is

associated with an increased risk of preeclampsia. Therefore, we are suggesting that the same rule might be applying in case of Familial Mediterranean Fever patients.

In the recurrent tonsillitis patients, we noted 25 different KIR genotypes among the group of patients compared to 24 in the general Lebanese population. The KIR genotypes among the tonsillitis patients contained between 8 and 14 genes with an average number of 11 KIR loci per individual. The average number of KIR loci was similar to the general population, with an average of 11 KIR loci; therefore, the gene content of the KIR repertoire in the tonsillitis patients does not have any role in the predisposition to the disease, similarly to what we have described for the FMF patients. In terms of genotypic profile, in addition to the fact that the group *A* haplotype was twice as frequent (69.1%) as the group *B* haplotype (30.9%) with a ratio of A:B of 2:1, we are observing in this group of patients a decrease in a the BB genotype. *KIR3DS1* has been correlated to cervical neoplasia (Carrington et al., 2005) whereby it is suggested that it might simply mark another nearby gene that is directly involved in pathogenesis of the disease, rather than being directly involved in the disease process, thus having this KIR marker implies having the other pathogenesis gene. It is important to note that a KIR gene (or haplotype) conferring protection against one disease may actually predispose to another. This has been largely studied through genetic epidemiological data and research which showed that certain KIR genotypes expected to result in an activating phenotype are associated with protection against some infectious diseases (Khakoo et al., 2004; Martin, Gao et al., 2002) but are also associated with susceptibility to other autoimmune pathogenesis (Martin, Nelson et al., 2002; van der Slik et al., 2003; Yen et al., 2001). Therefore, the important data obtained from such a study showed

that cervical neoplasia may more closely resemble autoimmune disease, in spite of its infectious etiology. This is what we are suggesting in the recurrent tonsillitis group of patients whereby the increase in *AB* genotype (mainly referring to an increase in *A* haplotype – the rich in activating KIR genes) may lead to the direct involvement of natural killer cells -depending on their receptors genotypes- into a repetitive immune response against a viral or bacterial threat leading to an altered inflammatory process in a "recurrent" tonsillitis form.

In addition, we have included two types of lymphomas in this work: Hodgkin and non-Hodgkin. Natural killer cells have been implicated in natural resistance against tumors, especially lymphomas (Mori, Mohri, Morita, Yamaguchi and Shimamine, 1983). In addition to genetic and environmental factors, infectious agents like Epstein-Barr virus (EBV) have been incriminated in Hodgkin's lymphoma. The association between viral infection and the activity of NK cells is well illustrated by the development of Epstein-Barr virus–driven lymphoproliferative disorder in a child with a specific NK cell deficiency (Eidenschenk et al., 2006). In this category of patients, the noted 27 different KIR genotypes (compared to 24 in the general Lebanese population) highly indicates the "individuality" and "personalized genetic make-up" of each patient greatly represented in the difference in the KIR genotypic profile. As compared to the general population, with an average of 11 KIR loci, there is no difference in the gene content among the lymphoma patients similar to what was described for the FMF and recurrent tonsillitis. This indicates as well that the KIR locus gene content itself may not be of any significance in disease pathophysiology and it is rather the under-expression or over-expression of a certain specific gene (or pseudogene) – whether activating or inhibitory – that really may be implicated. A very

interesting finding, though the sample size is very low, is the total absence of the *BB* genotype in these patients. However, since it is not apparent to be statistically significant, it is important to promote for the recruitment of a larger number of patients and really check whether the *A* haplotype is a "risk factor" or not. An important study involved the role of adaptive immune response in the etiology of Nasopharyngeal Carcinoma (NPC) whereby KIR genes were investigated (Butsh Kovacic et al., 2005). Here, the authors of this research proposed two distinct hypotheses that could explain the possible role of KIRs in the etiology of NPC. First, they suggested that the increase in activating KIRs (and/or reduction in inhibitory KIRs) may be protective against NPC as a consequence of increased cytolysis of EBV-infected cells by activated NK cells. Otherwise, and as a second proposal, they postulated that activating KIRs (and/or reduction in inhibitory KIRs) might increase risk of NPC through nonspecific inflammatory responses such as oxidative DNA damage, triggered by activated NK cells. The study supported the second proposal. This is exactly what we referred to in the recurrent tonsillitis and lymphoma patients research study conducted in this work. In addition, in this study, it was found that the risk of developing NPC is augmented with the increase in number of activating KIRs, especially in EBV positive subjects. This finding supports the theory that activation of innate immune effector cells increases the risk of virally associated cancers such as NPC. Furthermore, the activating KIR2DS1 gene was found to be associated with psoriasis vulgaris when combined with a certain locus on the HLA-C. This association of KIR2DS1 with the disease suggests a stimulatory role of KIR2DS1 on recognition of a specific HLA-C locus in susceptibility to psoriasis vulgaris (Luszczek et al., 2004). This association might be considered as a true association since KIR2DS1 gene has also been found to be a potential contributor to

psoriatic arthritis, indicating a possible role for this gene in relation to susceptibility to this disease (Nelson et al., 2004; Williams F et al., 2005). Another activating gene, KIR2DS2, was noted to be related to rheumatoid arthritis, type 1 diabetes mellitus, and scleroderma. KIR2DS5, which we have found to be associated with Lymphoma, has previously been correlated to type 1 diabetes mellitus. All these findings imply that expression of these activating genes might have a role in the pathogenesis of the disease.

To conclude, this is the first study (national and international) on typing KIR genes and pseudogenes in Familial Mediterranean Fever, recurrent tonsillitis, and Lymphoma in which KIR general haplotypes and genotypes were determined. Future studies should be conducted to monitor the increase in *AB* genotype in recurrent tonsillitis, lymphoma, and other clinical entities. The increase in *BB* genotype needs to be further studied in terms of translational research whereby the proteins/receptors products of the KIR genes must be investigated in FMF patients. In addition, since the *A* haplotype by itself is predominant among such patients; the increased content of the activating genes in this haplotype must be investigated and researched in the future. With all the mysteries it holds, this unique cell circulating in our peripheral blood system and called Natural Killer may have a lot of hiding details to explain its “killing” function and whether it is doing it “naturally” or via specific interaction and induction of over- or under-expression of its internal genes. Till then, this work opens the door for broader research in the field of Killer Cell Immunoglobulin-Like Receptors, KIRs.

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Appendix 1

FMF Patients

ID	Sex	Age	Date received	Haplotype
1	M	25	23-Nov-05	AA
2	F	34	23-Nov-05	AA
3	F	33	23-Nov-05	AB
4	M	58	24-Nov-05	AB
5	F	45	24-Nov-05	AB
6	F	52	2-Dec-05	AA
7	F	37	2-Dec-05	AB
8	F	56	2-Dec-05	AB
9	F	43	7-Dec-05	AA
10	M	54	8-Dec-05	AB
11	M	49	8-Dec-05	AB
12	M	35	8-Dec-05	AA
13	F	44	8-Dec-05	AB
14	M	64	8-Dec-05	AB
15	F	57	8-Dec-05	AA
16	M	34	8-Dec-05	AA
17	F	46	9-Dec-05	AA
18	M	24	12-Dec-05	AB
19	M	37	12-Dec-05	AB
20	M	46	13-Dec-05	AB
21	F	53	15-Dec-05	AA
22	F	51	12-Dec-05	AA
23	M	25	6-Jan-06	AB
24	F	19	4-Jan-06	AA
25	M	24	4-Jan-06	AB
26	F	33	6-Jan-06	AB
27	F	44	8-Jan-06	AB
28	M	45	17-Jan-06	AB
29	M	55	17-Jan-06	AB
30	M	24	18-Jan-06	AB
31	M	35	9-Oct-06	AA
32	F	34	21-Dec-05	AA
33	F	42	22-Feb-06	AB
34	F	38	24-Feb-06	BB
35	F	47	27-Feb-06	AA
36	F	36	22-Dec-05	BB
37	M	25	3-Mar-06	BB
38	M	41	7-Mar-06	AB

39	M	32	10-Mar-06	AA
40	M	33	14-Mar-06	AA
41	F	45	15-Mar-06	AB
42	M	62	20-Mar-06	BB
43	M	50	21-Mar-06	AB
44	F	27	24-Mar-06	AB
45	M	38	24-Mar-06	AB
46	F	49	25-May-06	AA
47	M	50	25-May-06	AB
48	F	42	21-Feb-06	BB
49	M	31	6-Jun-06	BB
50	F	24	23-Nov-05	AA
51	F	39	23-Nov-05	AB
52	M	40	23-Nov-05	AB
53	F	59	24-Nov-05	AB
54	F	61	24-Nov-05	AB
55	M	54	2-Dec-05	AB
56	M	56	2-Dec-05	AB
57	M	39	2-Dec-05	AB

Recurrent Tonsillitis Patients

ID	Sex	Age	Date received	Haplotype
1	F	3	1-Mar-06	AA
2	F	4	3-Mar-06	AA
3	M	3	3-Mar-06	AB
4	M	8	17-Mar-06	AB
5	F	5	18-Mar-06	AB
6	M	5	18-Mar-06	AA
7	M	7	27-Mar-06	AB
8	F	6	1-Apr-06	AB
9	M	3	21-Apr-06	AA
10	M	4	24-Apr-06	AB
11	F	9	25-Apr-06	AB
12	M	5	24-May-06	AA
13	F	4	26-Jun-06	AB
14	F	4	28-Jun-06	AB
15	F	7	28-Jun-06	AA
16	M	4	1-Jul-06	AA
17	M	6	4-Jul-06	AA
18	F	4	11-Jul-06	AB
19	F	7	30-Aug-06	AB
20	F	6	26-Sep-06	AB
21	M	3	6-Oct-06	AA
22	M	11	6-Oct-06	AA
23	F	5	6-Oct-06	AB
24	M	10	20-Oct-06	AA
25	M	4	20-Oct-06	AB
26	F	3	3-Nov-06	AB
27	M	4	15-Dec-06	AB
28	M	5	18-Dec-06	AB
29	M	5	31-Jan-07	AB
30	F	4	15-Feb-07	AB
31	M	5	21-Feb-06	AA
32	M	4	23-Feb-07	AA
33	F	2	23-Feb-07	AB
34	M	8	21-Mar-07	BB

Lymphoma Patients

ID	Sex	Age	Date received	Disease	Haplotype
1	M	30	20-Feb-07	Hodgkin's lymphoma	AA
2	M	20	8-Mar-07	Hodgkin's lymphoma	AB
3	M	26	21-Sep-06	Hodgkin's lymphoma	AB
4	F	53	15-Sep-06	Hodgkin's lymphoma	AA
5	M	46	6-Dec-06	Hodgkin's lymphoma	AB
6	F	27	30-Aug-06	Hodgkin's lymphoma	AA
7	M	27	25-Sep-06	Hodgkin's lymphoma	AB
8	M	60	1-Jun-07	Hodgkin's lymphoma	AA
9	M	30	11-May-07	Hodgkin's lymphoma	AA
10	M	36	21-Feb-07	Hodgkin's lymphoma	AB
11	M	86	23-Feb-07	Hodgkin's lymphoma	AA
12	M	74	28-Mar-07	Hodgkin's lymphoma	AB
13	M	28	4-Apr-07	Hodgkin's lymphoma	AB
14	M	33	19-Apr-07	Hodgkin's lymphoma	AB
15	M	23	19-Apr-07	Hodgkin's lymphoma	AA
16	M	35	24-Apr-07	Hodgkin's lymphoma	AA
17	F	30	27-Apr-07	Hodgkin's lymphoma	AB
18	F	40	2-May-07	Hodgkin's lymphoma	AB
19	M	50	31-May-06	Follicular lymphoma	AB
20	M	42	05-Jun-06	Follicular lymphoma	AB
21	F	57	07-Jun-06	Follicular lymphoma	AA
22	F	56	14-Jun-06	Follicular lymphoma	AB
23	M	47	26-Jun-06	Follicular lymphoma	AB
24	F	54	28-Jun-06	Follicular lymphoma	AB
25	M	67	29-Jun-06	Follicular lymphoma	AB
26	F	53	11-Jul-06	Follicular lymphoma	AB
27	F	57	12-Jul-06	Follicular lymphoma	AB
28	F	54	07-Sep-06	Follicular lymphoma	AA
29	F	57	15-Sep-06	Follicular lymphoma	AB
30	M	39	22-Dec-06	Follicular lymphoma	AA
31	F	83	23-Jan-07	Follicular lymphoma	AB
32	F	67	22-Mar-07	Follicular lymphoma	AA
33	M	65	26-Mar-07	Follicular lymphoma	AB
34	M	39	28-Mar-07	Follicular lymphoma	AB
35	M	45	28-Mar-07	Follicular lymphoma	AB
36	M	48	12-Apr-07	Follicular lymphoma	AB
37	M	38	5-Apr-07	Follicular lymphoma	AB

Appendix 2

Primer Mix Specificity Table

The sense and antisense primers for each specific allele are already incorporated in the kit. The table shows the specific primer mixes in addition to the internal control sizes and the PCR product sizes for each allele.

Well #	Primer Mix ID	KIR Allele Specificity	Sense primer 3'end	Sense primer 3'end location	Antisense primer 3'end	Antisense primer 3'end location	App. Internal control size (bp)	App. PCR product size (bp)
1	KM001A	2DL1*001-006	GAA	459	GCG	563	800	145
2	KM002	2DL2*001-005	CCA	692	ACA	796	800	145
3	KM003	2DL3*001-006	CTG	1100	CAA	1119	800	510
4	KM004	2DL4*00101/00102/0010301/0010302/00104/00201/00202/003-007/0080101-0080103/0080201/0080202/009-011	TTA	685	TCT	872	800	230
5	KM005	2DL5A*001/005, 2DL5B*002-004	TCA	681	GAG	895	800	257
6	KM019	2DL5A*001/005	TCA	22	GAT	244	800	1753
7	KM020	2DL5B*002/004	GTA	5'UT	GAT	244	800	1893
8	KM023	2DL5B*002/003/004	TCG	22	GAT	244	800	1772
9	KM008B	2DS1*001	GAG	663	GAC	624	800	100
		2DS1*002-004	GAA	663				
10	KM009	2DS2*00101-00103/002-005	GTA	488	ATG	654	800	207
11	KM010A	2DS3*00101-00103	CCT	745	CCT	867	800	162
12	KM011	2DS4*00101/00102/00103/002	CTA	755	GGA	932	800	215
13	KM017A	2DS4*003/004/006	ATC	769	GGA	932	800	200
14	KM012B	2DS5*001-005	ACC	492	GGG	613	800	160
15	KM013	3DL1*00101/00102/002/003/00401/00402/005-009/01501/01502/016-019	CAA	476	CAA	566	800	129
16	KM014A	3DL2*001-013	ATG	448	CAA	559	800	150
17	KM015	3DL3*001/00201/00202/003/004	ATG	448	GTA	611	800	203
18	KM016A	3DS1*010-014	GAG	422	GGA	556	800	170
19	KM006	2DP1*001/002	CAT	156	TAC	282	800	171
20	KM007A	3DP1*001/002/004	TGC	55	TAC	229	200	975
		3DP1*00301/00302	GAG	5'UT				344
21	KM021	3DP1*001/002/004	TGC	55	TAC	229	200	975
22	KM000	Negative Control					NONE	200
								800

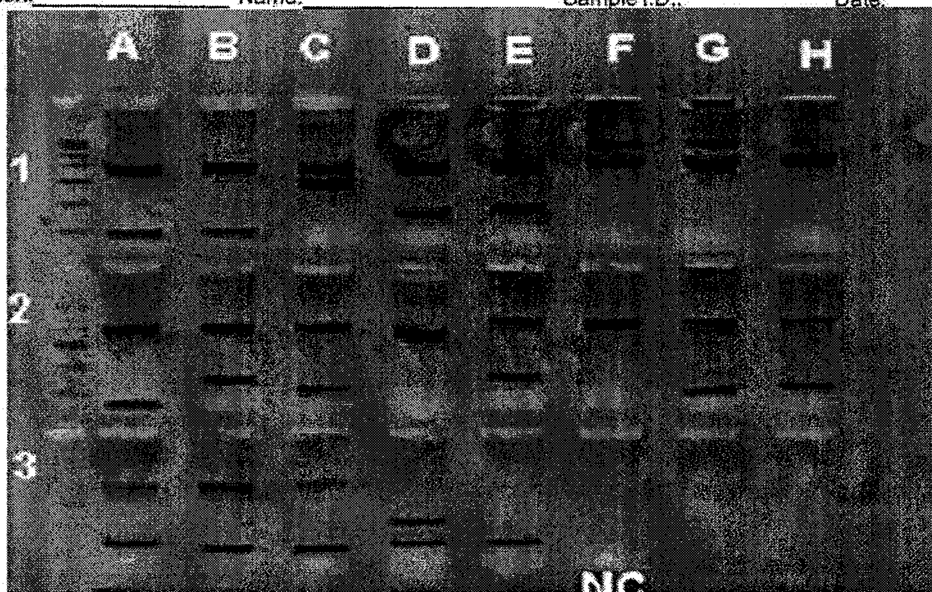
Appendix 3

Gel Documentation Form
KIR Genotyping SSP Kit

Dynal Biotech
Telephone: 800.558.4511
Fax:414.357.4518
www.dynalbiotech.com

Kit Name: KIR Genotyping SSP Lot#: 003 Batch#: ______ Exp. Date: ________ Tested by: ________

Institution: ______________ Name: ______________ Sample I.D.: ________ Date: ________



Well location	A1	B1	C1	D1	E1	F1	G1	H1		A2	B2	C2	D2	E2	F2	G2	H2	A3	B3	C3	D3	E3	F3	G3	H3
Lane number	1	2	3	4	5	6	7	8	M	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
Size (bp)	145	145	510	230	257	1753	1893	1772		100	207	162	215	200	160	129	150	203	170	171	975 344	975			
Test 1																									

Well location	A4	B4	C4	D4	E4	F4	G4	H4		A5	B5	C5	D5	E5	F5	G5	H5	A6	B6	C6	D6	E6	F6	G6	H6
Lane number	1	2	3	4	5	6	7	8	M	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
Size (bp)	145	145	510	230	257	1753	1893	1772		100	207	162	215	200	160	129	150	203	170	171	975 344	975			
Test 2																									

Well location	A7	B7	C7	D7	E7	F7	G7	H7		A8	B8	C8	D8	E8	F8	G8	H8	A9	B9	C9	D9	E9	F9	G9	H9
Lane number	1	2	3	4	5	6	7	8	M	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
Size (bp)	145	145	510	230	257	1753	1893	1772		100	207	162	215	200	160	129	150	203	170	171	975 344	975			
Test 3																									

Well location	A10	B10	C10	D10	E10	F10	G10	H10		A11	B11	C11	D11	E11	F11	G11	H11	A12	B12	C12	D12	E12	F12	G12	H12
Lane number	1	2	3	4	5	6	7	8	M	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
Size (bp)	145	145	510	230	257	1753	1893	1772		100	207	162	215	200	160	129	150	203	170	171	975 344	975			
Test 4																									

	Positive Lanes	Failed Lanes	Contamination (lane 22)
Test 1	____________	____________	☐ Yes ☐ No

Sheet number 1. The circles correspond to the positive bands on the gel and identify the lane number on sheet number 2.

KIR Genotyping SSP Kit - WORKSHEET
Code no.: 78930-3

Dynal Biotech
Telephone: 800.558.4511
Fax: 414.357.4518
www.dynalbiotech.com

Institution ______	Purpose of Test ______	Taq Lot# ______
Sample I.D. ______	DNA Extraction Method ______	Lot# 003 Batch# ______
Name ______	DNA Conc. (ng/µl) ______	Expiration Date ______
Ethnic Origin ______	Tested by ______	Test Date ______
Donor/Patient ______	Reviewed by ______	Review Date ______

Typing Result (Check genes present)

2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4*001/002	2DS4*003-006	2DS5	3DL1	3DL2	3DL3	3DS1	2DP1	3DP1*001/002/004	3DP1*003
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Typing Result	Allele Specificity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
+	2DL1*001-006	1																				
+	2DL2*001-005		2																			
+	2DL3*001-006			3																		
+	2DL4*00101/00102/0010301/0010302/00104/00201/00202/003-007/0080101-0080103/0080201/0080202/009-011				4																	
+	2DL5A*001/005					5	6															
+	2DL5B*002/004					5		7	8													
+	2DL5B*003					5			8													
+	2DS1*001-004									9												
+	2DS2*00101-00103/002-005										10											
+	2DS3*00101-00103											11										
—	2DS4*00101/00102/00103/002												12									
+	2DS4*003/004/006													13								
—	2DS5*001-005														14							
+	3DL1*00101/00102/002/003/00401/00402/005-009/01501/01502/016-019															15						
+	3DL2*001-013																16					
+	3DL3*001/00201/00202/003/004																	17				
+	3DS1*010-014																		18			
+	2DP1*001/002																			19		
—	3DP1*001/002/004 (975bp)																				20	21
+	3DP1*00301/00302 (344bp)																				20	
	Product Size (bp)	145	145	510	230	257	1753	1893	1772	100	207	162	215	200	160	129	150	203	170	171	344/975	975
	Lane Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
	Failed Controls																					
	False Positive																					
	False Negative																					

Sheet number 2. For each well location encircled, a line is drawn on the corresponding lane number. A gene is considered present only when all the respective lanes are positive. Lanes 5 and 6 are both positive; therefore, gene 2DL5A is considered present. On the contrary, lane 20 is positive however, lane 21 is negative; therefore, gene 3DP1 is considered absent in this patient.

Appendix 4

Distribution of the assumed KIR haplotypes among the 56 FMF patients.

N	Haplotype	2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	2DS1	2DS2	2DS3	2DS4*001/002	2DS4*003-006	2DS5	3DL1	3DL2	3DL3	3DS1	2DP1	3DP1*001/002	3DP1*003
17	AA																			
5	AB																			
5	AB																			
4	AB																			
3	AB																			
1	AB																			
1	AB																			
7	BB																			
2	BB																			
1	BB																			
1	BB																			
1	BB																			
1	BB																			
1	(AB)n																			
2	(AB)n																			
1	(AB)n																			
1	(BB)n																			
1	(BB)n																			
1	(BB)n																			

Distribution of the assumed KIR haplotypes among the 34 Recurrent Tonsillitis patients.

N	Haplotype	2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4*001/002	2DS4*003-006	2DS5	3DS1	2DP1	3DP1*001/002	3DP1*003
3	AA																			
2	AA																			
2	AA																			
2	AA																			
2	AA																			
2	AA																			
1	AA																			
2	AB																			
2	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			
1	BB																			

Distribution of the assumed KIR haplotypes among the 37 Lymphoma patients.

N	Haplotype	2DL1	2DL2	2DL3	2DL4	2DL5A	2DL5B	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4*001/002	2DS4*003-006	2DS5	3DS1	2DP1	3DP1*001/002/004	3DP1*003
1	AA																			
1	AA																			
6	AA																			
1	AA																			
1	AA																			
2	AA																			
2	AB																			
1	AB																			
1	AB																			
2	AB																			
1	AB																			
1	AB																			
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1	AB																			
2	AB																			
1	AB																			
1	AB																			
1	AB																			
1	AB																			

The blue box designates that the locus was detected, and the white box designates that it was absent. For each haplotype, the total number of individuals observed was shown and is designated by N. The ()n represent new haplotypes found in our patients, not yet identified in the literature.