Implications of genetic diversity on expression of LILRB1 in Natural Killer cells and susceptibility to HCMV infection in transplant patients

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Immunology

Medical Microbiology and Immunology
University of Alberta

Abstract:
Leukocyte Ig-like Receptor 1 (LILRB1) is an inhibitory receptor expressed on a number of immune cells. The expression profile on NK cells is variable between people, with a range of 25-80% of NK cells expressing LILRB1. We previously uncovered a correlation between genotype within the 5' end of the LILRB1 gene and frequency of expression on NK cells. This thesis research expands on our previous work in elucidating the regulatory mechanisms involved in LILRB1 gene expression as well as the role of the expression correlated SNPs.

We first set out to characterize the distal promoter of LILRB1 used by NK cells, a distal region not yet characterized at the time this research began. We identified JunD as an activator of this promoter and thus a regulator of LILRB1 gene expression. Next we focused on the role of the SNPs within the previously identified expression-correlated haplotypes. We discovered that the haplotypes extended to the distal promoter and correlated with expression in the distal promoter region, indicating that SNPs in either promoter region could be responsible for the expression level. Interestingly, the expression of LILRB1 on B cells appears to be unchanged regardless of donor genotype, which led us to investigate the potential reason. We found that there are differences in both the factors in the nuclear environment that are able to bind the core promoter and also in methylation of the DNA in the regulatory regions. Upon further examination, we found that the two haplotypes of the distal promoter show no difference in activity by reporter assay while the proximal promoter haplotypes differ. This led us to focus on identification of a role for the proximal promoter in NK cells. We found that the proximal promoter is active in NK

cells under activating conditions. Finally, we examined some of the potential implications of the genetic diversity of LILRB1 observed within the population. We focused on the role of LILRB1 diversity in the context of Human Cytomegalovirus (HCMV) infection, as LILRB1 is targeted by this virus by the production of a mimic of LILRB1's endogenous ligand, MHC-I, called UL18. We found that one LILRB1 protein variant, associated with the low expressing haplotype, binds UL18 with a higher affinity than the high expressing haplotype. Additionally, we found a trend wherein the low expressing promoter haplotype as well as the rare allele at position 927 in the gene, which produces a non-synonymous change from Isoleucine to Threonine, are correlated with increased susceptibility to CMV disease post-organ transplant.

This is the first research examining LILRB1 genetic regulation in NK cells in any detail. With this research, we have begun to uncover the role of LILRB1 genetic diversity in the context of a specific disease, HCMV infection. Understanding the regulation of the observed variable LILRB1 expression on NK cells will help to further uncover the role of this variability in the context of both health and disease.

Acknowledgements

First, I would like to thank my supervisor, Debby. She was a great mentor who gave me freedom to follow my ideas but was still always there to help and support me anytime I needed it. I started as a technician in the lab and Debby allowed me to design a PhD project that interested me and we both got to learn along the way!

Thank you to my committee members, Dr.'s Edan Foley and Jim Smiley, for all of their ongoing support throughout my graduate studies. Thanks as well to the many other people in the department who have helped along the way. My project had some diverse parts with a lot of new techniques to work out and everyone was always happy to provide advice and reagents.

I would also like to thank all of the friends I've made along the way in MMI. Many have graduated and left to pursue their next steps but we still keep in touch and their friendship has been extremely helpful throughout my degree. Special thanks to Adriana, Chad, Dave, Deanna, Elsa, Kristin and Lis.

Finally, thanks to my family, especially my mom, Karen and sister, Courtney. They are always there through everything and most importantly, are always able to make me laugh.

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Abbreviations:

cDNA – Complementary DNA
ChIP – Chromatin Immunoprecipitation
CMV – Cytomegalovirus
DNA – Deoxyribonuleic Acid
dsRNA – Double Stranded RNA
EMSA – Electrophoretic Mobility Shift Assay
HCMV – Human Cytomegalovirus
HIV – Human Immunodeficiency Virus
HLA – Human Leukocyte Antigen
IE – Immediate Early
Ig – Immunoglobulin
IL - Interleukin
Kb - Kilobase
kDa – Kilodalton
KIR – Killer Ig-Like Receptor
LILR – Leukocyte Ig-Like Receptor
LRC – Leukocyte Receptor Complex
MCMV – Mouse Cytomegalovirus
MFI – Mean Fluorescence Intensity
MHC – Major Histocompatibility Complex
mRNA – Messenger Ribonucleic Acid
NK – Natural Killer
PBMC – Peripheral Blood Mononuclear Cells
PCR – Polymerase Chain Reaction
PIR – Paired Ig-Like Receptor
qPCR – Quantitative Polymerase Chain Reaction
RACE – Rapid Amplification of cDNA Ends
RNA – Ribonucleic Acid
RTPCR – Reverse Transcription Polymerase Chain Reaction
siRNA – Silencing Ribonucleic Acid
SNP – Single Nucleotide Polymorphism
TNF – Tumor Necrosis Factor

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CHAPTER 1: Introduction

1.1 General overview:

The innate immune system is the first line of defense against invading pathogens. Innate immunity functions to fight off pathogens or at least keep them minimized until the adaptive immune system has time to mount a specific immune response. Immunity is a constant balance between activation and inhibition wherein unregulated levels of either signal can have severe repercussions for the organism as a whole. Too little activation and infection can go uncontrolled while too little inhibition can result in uncontrolled immune activation and auto-immunity (1, 2). Differential expression of immune genes is emerging as a key cause of differential responses to pathogens. A common theme within the innate immune system of vertebrates is families of paired receptors (3). These receptor families have both activating and inhibitory members with highly similar extracellular or binding domains but diverse intracellular signalling components to create a balance in signals. One of these families of immune receptors is the Leukocyte Ig-like Receptor (LILR) family encoded on chromosome 19 in humans within the leukocyte receptor complex (LRC) at position 19q13.4 (4, 5). This thesis is focused on the genetic regulation and diversity of a member of the LILR family, called LILRB1, and the selective influence of polymorphisms on expression in NK cells.

1.2 The LRC encoded LILR gene family

The LRC in which the LILR gene family is encoded is conserved throughout vertebrate species. Two of the main human receptor families of significance encoded by the LRC are the KIR and LILR groups. The gene of focus for this thesis will be a

member of the LILR family so while KIRs will be used as a comparison since they are much more extensively studied than the related LILRs, the KIR family will not be described in detail.

The LRC is variable between species, likely due to a number of extensive gene duplications and inversions. The human LILR gene family is made up of 11 genes and two pseudogenes. This locus encodes 5 activating LILRs, 5 inhibitory LILRs, one soluble LILR and two pseudogenes. The LILR gene family is greatly expanded in primates as compared to rodents where there is a single inhibitory family member of the homologous PIR genes (4, 6-10), which exemplifies the diversity in gene content of the LRC between species. The LILR family was expanded substantially by an inverted duplication, which is apparent when examining the gene family (Figure 1.1). With this duplication event, each LILR family member was able to further diversify and specialize in function. Many of these functions are still not known or understood. LILR expression is largely concentrated within the myeloid compartment of the immune system. For a few of the LILR receptors, the ligand is still unknown. However, a group of the LILRs including LILR-B1, B2, A1, A2 and A3 are labelled group 1 LILRs and bind to or are predicted to bind to MHC-I (reviewed in (11)). The related KIR genes are also a large family of activating and inhibitory immune receptors, however, their expression is restricted to NK cells and subsets of T cells. Additionally, KIR receptors bind MHC as well but each with high specificity for a particular subset of HLA molecules as they bind the polymorphic α1 and α2 domains while LILR receptors bind the highly conserved α3 and β2m regions

of the MHC ligand (Figure 1.2) (12-14). LILRB1 and B2 are known to bind to all classical, as well as some non-classical HLA molecules (reviewed in (15)).

In humans, only one of the LILR genes, LILRB1, is expressed on *ex vivo* NK cells and it has a variable expression pattern between individuals. A comparative approach to understand this expression can be used by examining other species where LILRB1 homologues are present. In rats, the inhibitory member of the PIR family, PirB, is expressed on NK cells (6). Interestingly however, in mice, PirB is not found on resting NK cells but is present on myeloid and B cells (7, 16). Human LILRB1 is also expressed on other lymphocyte lineages, including B cells and subsets of T cells, differentiating it from other LILR family members which are largely isolated to the myeloid compartment of the immune system. LILRB1 is the focus of this research, therefore a more detailed description of this gene/receptor follows.

1.3 Leukocyte Ig-like Receptor 1 (LILRB1/ILT-2/LIR-1/CD85j)

LILRB1 was discovered when it was identified as a receptor for an MHC-I mimic produced by Human Cytomegalovirus (HCMV) called UL18 (10). Though part of a large family of highly related genes, LILRB1 has qualities that differentiate it from the other LILR family members. As previously mentioned, LILRB1 has a distinct cell lineage distribution, with expression on B cells and a fraction of NK cells and T cells, in addition to the more common myeloid expression pattern of other LILR family members (reviewed in (15)). As mentioned, this family of receptors is extremely condensed in rodents, making functional studies of individual LILR receptors difficult in a mouse model. Deletion of the only inhibitory member in mice,

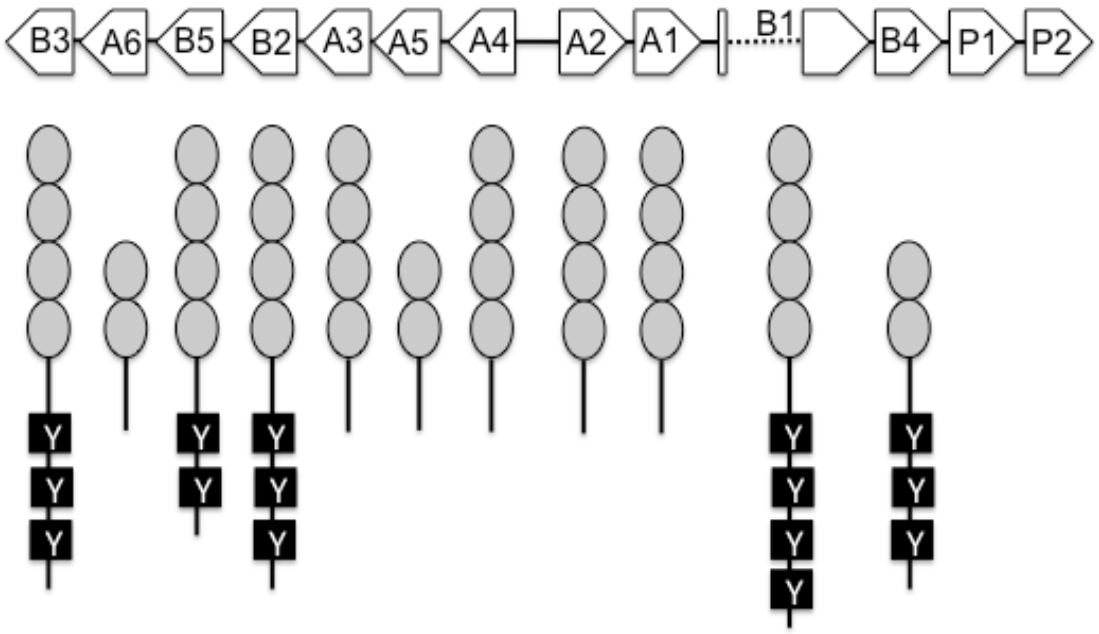


Figure 1.1 Schematic of the LILR gene locus and receptors.

The LILR family genes are shown at the top. The genes are denoted without the LILR prefix. The "A" indicates activating receptor function or secreted, the "B" indicates inhibitory function, the P indicates a pseudogene. The arrow points in the direction the gene is transcribed. A corresponding schematic of each receptor is shown below each gene. The grey ovals represent Ig domains. The black boxes with "Y" are present in inhibitory receptor tails and represent Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) or ITIM-like sequences.

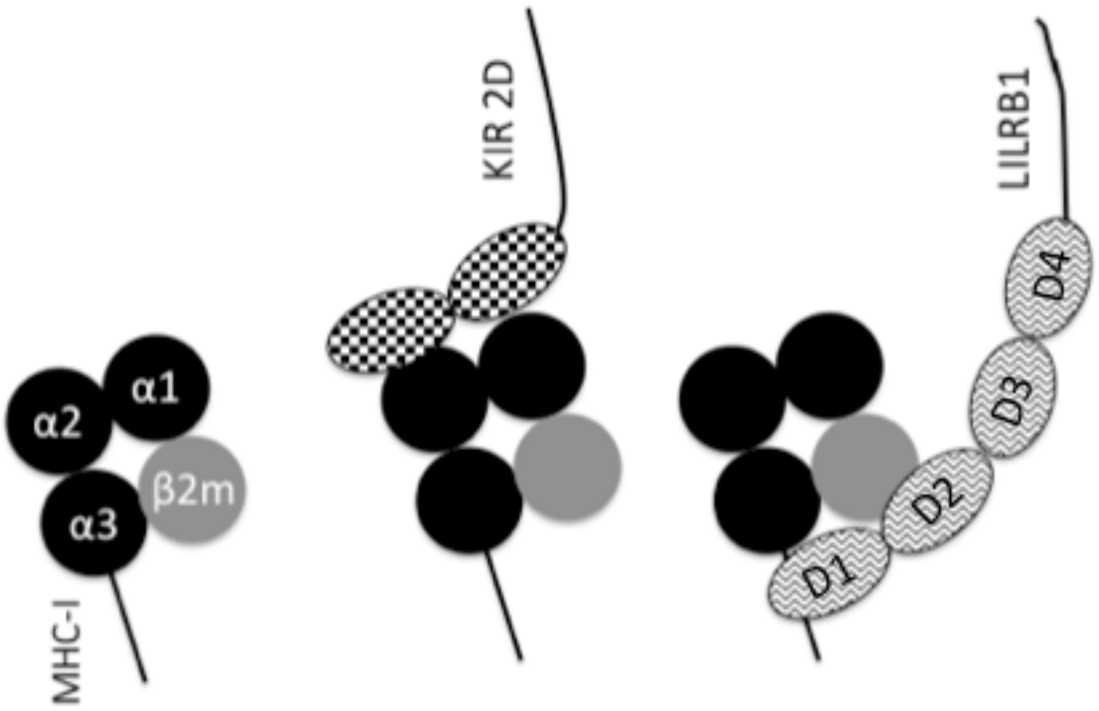


Figure 1.2 KIR and LILRB1 binding to MHC-I molecules.

The protein domains of MHC-I alone is shown on the left. The three domains of the alpha chain shown in black are labelled in association with the beta 2 microglobulin (β2m) subunit. The middle complex depicts a generic KIR receptor interacts with polymorphic α1 and α2 domains. The right complex depicts LILRB1 binding to MHC-I. The D1 and D2 domains of LILRB1 contact the more conserved α3 and β2m regions.

PirB, leads to Th2-skewed immune responses, enhanced susceptibility to bacterial infections and excessive graft-versus-host reactions (17-19). While this may provide insight into some of the roles of the inhibitory LILRs in general, the PirB knockout does not account for specialized functions of the five individual inhibitory LILRs in humans. Additionally, since PirB is not expressed on NK cells in mice, this model does not shed any light on the role of LILRB1 on NK cells.

1.3.1 Gene organization

The LILRB1 gene is made up of 16 exons and spans more than 20Kb (Figure 1.3). The other LILR genes are much smaller in size due to the lack of the large, first intron unique to LILRB1 (Figure 1.1). This intron makes up approximately 50% of the whole gene, which is very unusual in this highly compact region of the genome. The large first intron is preceded by a short non-coding exon. This upstream portion of the LILRB1 gene has only recently been discovered and characterized (20-22). The characterization of the upstream exon and regulatory region are discussed further in Chapters 3 and 4.

1.3.2 Function of LILRB1 in various cell lineages

LILRB1 can inhibit an array of activation signals in different LILRB1-expressing cell lineages. In B cells, binding of LILRB1 can inhibit class switching to IgG and IgE and down-regulate production of IL-8, IL-10, and TNF-alpha by inhibiting several activation pathways (23). In T cells, engagement of LILRB1 inhibits activation through CD3/TCR resulting in decreased CTL killing of targets, decreased

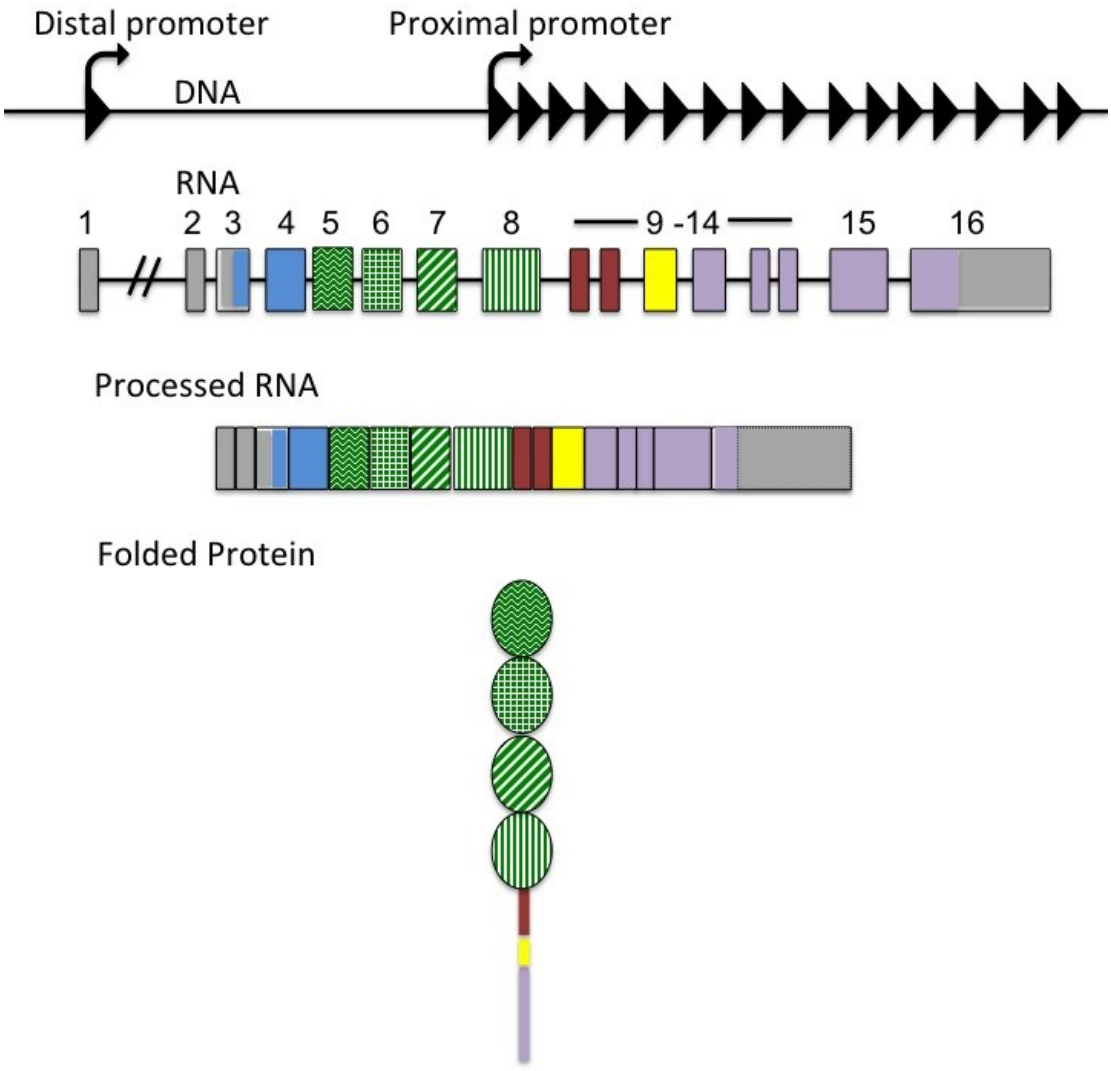


Figure 1.3. Human LILRB1.

The intron/exon organization of the LILRB1 gene is shown at the top with the exon represented by the arrowheads (drawing not to scale). The corresponding primary RNA and processed or spliced mRNA are shown below. The exons in the primary and mRNA are colour coded to indicate what each exon codes for in the LILRB1 protein. The colour coding is as follows: grey indicates untranslated region, blue indicates signal peptide, the green patterns indicate Ig domains 1 through 4, red indicates the stem, yellow indicates the transmembrane region, and purple indicates the cytoplasmic tail. The cartoon of the folded protein/receptor is indicated at the bottom and has the same colour coding to indicate the exon each domain is derived from.

proliferation of stimulated CD4+ T cells and decreased calcium mobilization (24). In monocytes, LILRB1 ligation down-regulates activation through Fc receptors (25). Ligation of LILRB1 during differentiation of monocyte derived dendritic cells results in decreased production of IL-10 and TGF-beta (26). Additionally, LILRB1 ligation can inhibit activation signals in monocyte derived dendritic cells through the human osteoclast-associated receptor (OSCAR), leading to inhibition of calcium mobilization, decreased production of IL-12 and IL-8 and weakened ability to induce T cell proliferation (27). In NK cells, engagement of LILRB1 by UL18 inhibits NK cytotoxic activities such as degranulation and target cell lysis (28).

1.3.3 LILRB1 cell lineage expression profile and regulation of expression

LILRB1 is expressed on monocytes, dendritic cells, B cells and subsets of T and NK cells (CITA). LILRB1 expression on NK cells is extremely variable between different people, ranging from 25-80% of NK cells expressing LILRB1 on their surface (22, 29). This expression pattern is similar to the variegated expression of the related KIR genes. Expression frequency of individual KIR genes is related to allelic differences (30, 31). Some alleles are expressed highly on the surface while others have little to no surface expression on NK cells. Therefore, in this thesis I investigated whether LILRB1 NK-specific variegated expression may be regulated by genetic variation by a mechanism similar to KIR.

1.3.3.1 Lineage specific regulation of expression

A study examining LILRB1 transcription examined its regulation in myeloid cells. Using two monocyte cell lines, a core proximal promoter was identified (32). This core promoter can be activated by transcription factors Sp1 and PU.1 (32). The distal promoter is used primarily by lymphocytes versus the proximal promoter, which is primarily used by myeloid cells (20, 21). The resulting LILRB1 protein is unchanged by the change in promoter usage as exon 1 encodes untranslated sequence. However, exon 1 contains several non-functional ATG sequences that result in translational stuttering, making translation from the distal transcript less efficient (21).

Interestingly, my analysis of the upstream promoter and exon 1 regions shows it is present in old world monkeys, cows and rats, however are not apparent in the examined new world monkey or mice (Figure 1.4). Evidence for the distal promoter being critical for expression on NK cells lies in the observation that PirB is expressed on NK cells in rats, who have this upstream promoter, but not mice (6, 16). Additionally, a transgenic mouse was made with a large insert including multiple human LILR and KIR genes (LILRB1, B4 and A1 and 10 KIR genes) and LILRB1 was expressed on all mouse NK cells (33). In this transgenic mouse, the whole LILRB1 gene was present, including the upstream exon and promoter. This suggests the distal genetic elements, such as the upstream promoter and exon, drive the expression of LILRB1 in NK cells and explains why PirB is not expressed in NK cells. This result also suggests that mechanisms controlling the variegated expression on NK cells are human specific and lie outside this sequence.

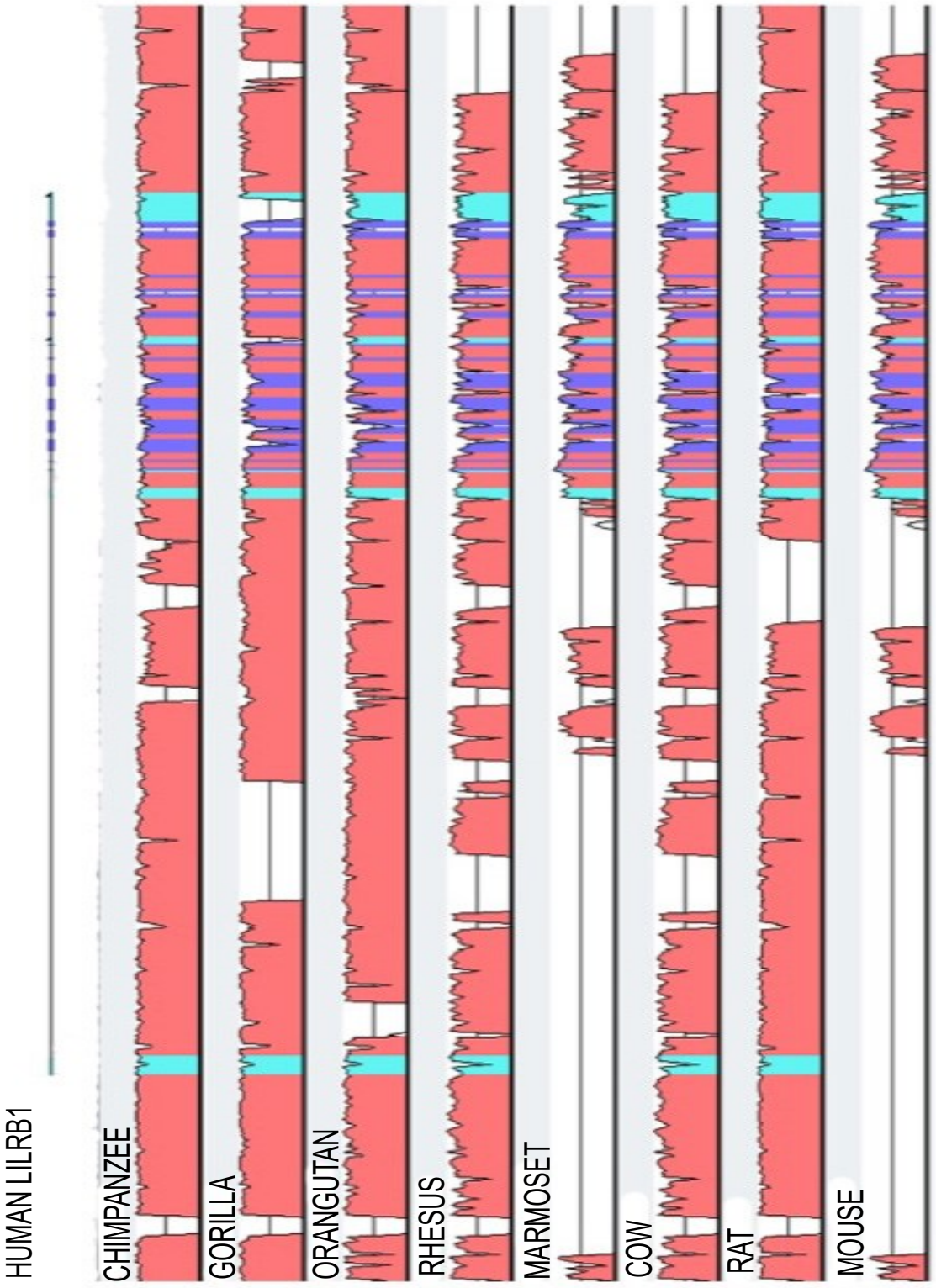


Figure 1.4. Multispecies alignment of LILRB1 genes (using vista browser).

The image was derived using all species available in the Vista database. The height of the curve at each point indicates the conservation with human LILRB1. Turquoise indicates non-coding exons or UTRs, blue indicates coding exons and pink area indicates introns and regions outside the gene. The arrow points to the distal first exon that is conserved in rat and cow, but not the more closely related marmoset or mouse.

1.3.3.2 Polymorphism of LILRB1

Ten non-synonymous SNPs were identified within the LILRB1 gene in the first published study of diversity within the coding sequence of LILRB1 (34). In a Japanese population, three LILRB1 haplotypes were uncovered in the 5' end of the gene, one of which, when expressed in combination with a particular MHC genotype, is correlated with susceptibility to rheumatoid arthritis (35).

In a previous study I performed as a technician in the Burshtyn group, I examined how diverse the LILRB1 gene is between people and assessed the role of polymorphism in expression on NK cells (29). We discovered that the LILRB1 gene was substantially more polymorphic than previously thought, with 85 single nucleotide polymorphisms (SNPs) in the gene from the proximal promoter to the end of the coding sequence, inclusive of introns, within a sample of 11 donors (29). The region examined in this study of polymorphism excluded what is now known as exon 1 as well as most of intron 1 (Figure 1.5). This was due to the fact that this region of the gene was not yet known about. We determined that SNPs within the 5' end of the gene including the proximal promoter and exon 2 through 3 are correlated with expression of LILRB1 on NK cells but not B cells (Figure 1.6) (29).

1.3.3.3 Environmental signals altering expression of LILRB1

While people often maintain a stable level of LILRB1 expression on NK cells over time, much like what has been observed for KIR genes (reviewed in (36)), we have also found some changes in the expression on NK and T cells over time in normal healthy donors (37). Perturbations in LILRB1 expression profiles on NK and T cells

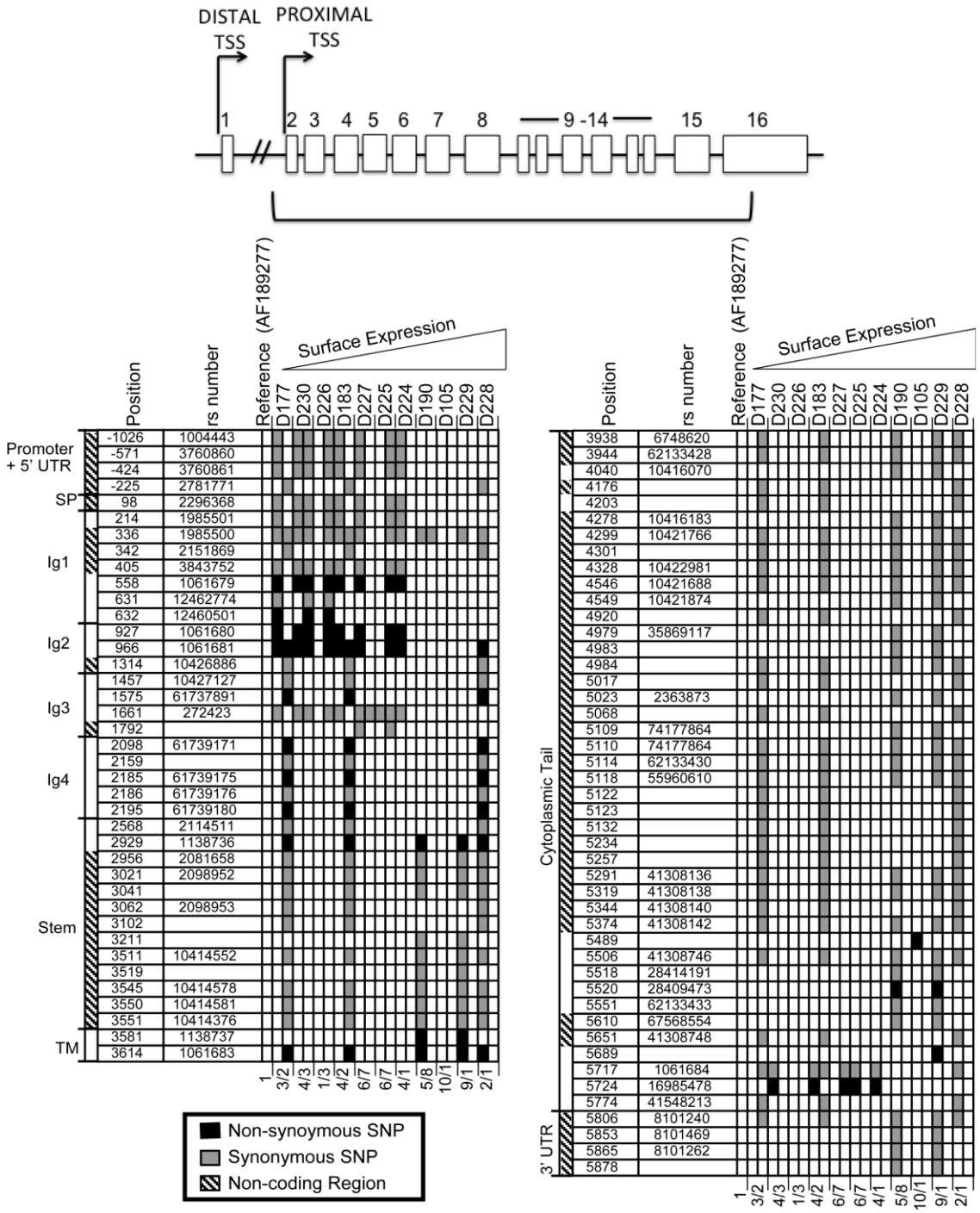


Figure 1.5. LILRB1 genetic diversity in a panel of 11 donors.
Adapted from Davidson et. al (29). The region of the LILRB1 gene indicated in the top diagram was sequenced in 11 donors and the results shown in the lower figure. Position in LILRB1 relative to the translational start site and rs numbers are indicated. Donors are in order from lowest to highest expression frequency on NK cells.

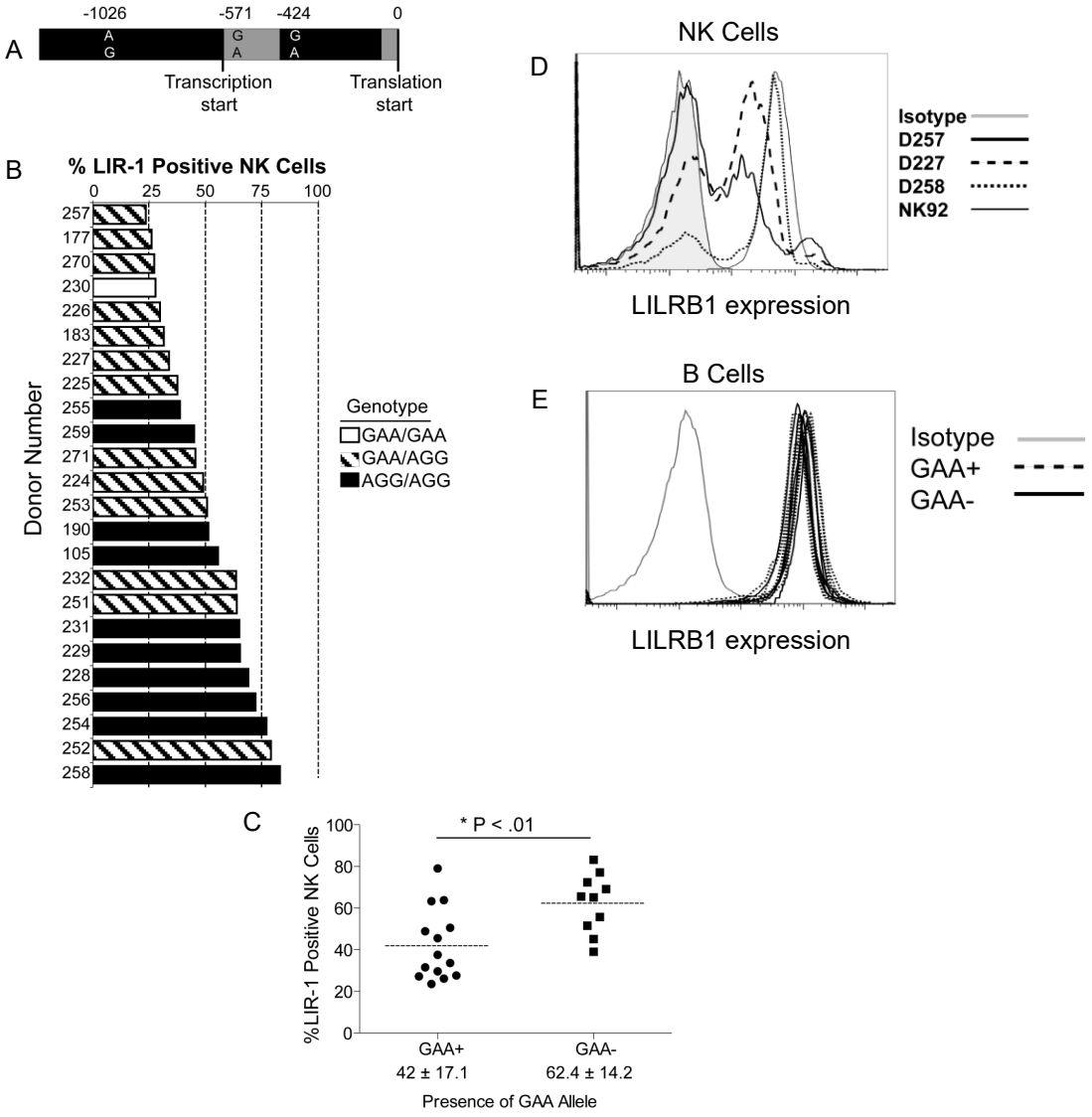


Figure 1.6 Correlation of LILRB1 genotype with NK cell surface expression.

Adapted from Davidson et. al. (29) A) Positions of the three polymorphisms making up the two proximal promoter haplotypes. B) Genotype of 24 donors graphed from lowest to highest percentage of LILRB1+ NK cells. C) Panel of 24 donors divided into presence or absence of the GAA allele. D) LILRB1 expression on NK cells from 3 donors (D257 and 227 are GAA+, D258 is GAA-). E) LILRB1 expression on B cells from 12 donors (7 GAA+, 5GAA-).

have also been reported in several situations including post transplant HCMV disease, HIV infection and first trimester pregnancy (38-41). It is possible that either LILRB1 positive subsets are expanding, LILRB1 is being upregulated, or that there is a combination of both occurring. In addition, we have observed that IL-2 and/or IL-15 cause some enhancement of surface expression of LILRB1 on NK and T cells *in vitro* (22). We also noted a potential role for IL-2 in regulating promoter activity in one NK-like cell line (37). Chapter 3 of this thesis includes data regarding culturing of *ex vivo* NK cells in activating conditions, including the presence of continuous IL-2 stimulation. Evidence for direct up-regulation of LILRB1 in HCMV infected cells has also been reported. More specifically, HCMV immediate early proteins, IE1 and IE2, can activate transcription of LILRB1 and LILRB2 (32).

1.4 Mechanisms of transcriptional regulation

In our initial study investigating differential expression of LILRB1 on NK cells, we determined that the difference in expression is apparent at the level of mRNA transcript (29). There are multiple mechanisms possible for this differential transcript level. Some of these mechanisms will be examined in the context of LILRB1 expression in chapter 4 of this thesis. Therefore, here I will introduce more generally some of the ways that expression of a gene can be impacted at the level of transcription. There are also mechanisms that impact expression at the level of RNA and protein but these will not be discussed in this thesis. In the following sections I will discuss how SNPs can influence expression in related genes such as KIR for comparison with my studies of LILRB1.

1.4.1 Genetic regulation

Every gene has regulatory regions responsible for activating or repressing transcription by binding nuclear transcription factors. The particular DNA sequence allows for specific factors to bind, therefore polymorphisms within these binding sequences can greatly impact the overall expression of a gene, either negatively or positively. In the related KIR genes, a SNP in the promoter of the KIR2DL1 gene allows binding of the inhibitory ZEB1 transcription factor and substantially decreases surface expression of 2DL1 in these individuals (42). Conversely, a SNP in the promoter of interleukin 10 (IL-10) allows for binding of the Sp1 transcriptional activator, leading to increased expression of the cytokine in these individuals (43). SNPs can play a similarly significant role in enhancers or other regulatory sequences. In fact, many disease-associated SNPs actually regulate expression of a gene rather than altering a protein sequence (reviewed in (44)). In the case of LILRB1, in order to examine which expression-correlated SNPs may be mechanistically responsible for the variable NK cell specific expression, we had to first characterize the core promoter region for this previously minimally studied gene.

1.4.1.1 Availability of transcriptional modulators

Similarly to DNA sequence playing a role in transcription, so too does the availability of potential binding factors. The nuclear environment in each cell lineage is made up of a different collection of transcription factors. Furthermore, transcription factors themselves can exhibit variable expression levels due to genetic differences within their own regulatory elements. The difference in transcription factor availability

between cell lineages is often largely responsible for the difference in expression of a particular gene between cell lineages. When a SNP is present in the regulatory region of a gene, this can impact expression in only certain lineages where the SNP influences the binding site of a factor active specifically expressed in that cell type. This is one potential explanation for our observation that haplotype of LILRB1 is correlated with expression on NK cells but not obviously with expression on other cell types such as B cells (29). Another major factor in regulating the availability of transcription factors is signals received by the cell to produce more of that transcription factor or to convert it to its active form.

1.4.1.2 Epigenetic modifications

NK cells can either have LILRB1 on or off and this type of expression is often associated with epigenetic control. Expression of a gene is impacted significantly by epigenetic modifications of the chromatin. Epigenetic modifications impact the structure and openness of the chromatin and also directly alter the ability of regulatory factors to bind the DNA. Two broad types of epigenetic modifications will be introduced here, those that affect the DNA directly and those that affect the histones that pack the DNA.

1.4.1.2.1 DNA Methylation

DNA is methylated at CpG sites throughout the genome, meaning sites where a cytosine sits 5' to a guanine. More specifically, cytosines that are 5' to guanines can be methylated and in fact, are largely methylated within the human genome (reviewed

in (45)). Genes that are hypomethylated at CpGs are often termed "open" and this open state is often observed in cells where the gene is expressed. The frequency of CpGs in the genome is lower than what would be expected with random distribution of the four base pairs. Regions with a higher than expected CpG content are termed CpG islands and the status of these islands is often indicative of the expression of the associated gene. SNPs can impact presence of methylation sites. In fact, in our studies, 28 out of 90 SNPs we identified alter a CpG site. Methylation differences due to a loss/gain of a CpG site can directly influence chromatin organization or alternatively, can influence the ability of a transcription factor to bind at that location as some factors are methylation sensitive, for example YY1 (46).

To examine the potential impact of DNA methylation in gene regulation, I will introduce its role in regulating expression of the related KIR genes. Expression of specific KIR genes in an individual NK cell has been termed "stochastic". Stochastic expression indicates a random expression pattern, which is not in fact the case for the KIR genes as genetic variability is the main determinant of expression frequency for each gene (30, 31). The term variegated, which is also used, is perhaps more accurate. Allelic differences in a specific KIR gene greatly influence the frequency of its expression on NK cells. It has also been observed that NK cell clones lacking expression of a particular KIR gene, 3DL1, have heavy methylation around the transcription start site, while in those NK cells that express it, the gene is hypomethylated at one or both alleles (47). Additionally, treatment of a KIR negative NK cell line with a DNA methylase inhibitor induces expression of KIR on the

surface (47). These data indicate a role for methylation in regulating KIR expression on NK cells.

KIR expression is regulated by a bidirectional promoter system (Figure 1.7). It has been observed that strength of the reverse promoter, as determined by allelic variation, is inversely correlated with expression frequency (30). As mentioned above, the region around the transcriptional start site is hypermethylated in NK cell clones that do not express that particular KIR. Interestingly, a mechanism for this methylation based silencing of particular KIR genes has been uncovered recently. In NK cells with a strong reverse promoter activity as well as some level of transcription from the distal promoter observed in all developing NK cells, it was noted that a double stranded RNA (dsRNA) is produced. This dsRNA is in fact detected in cells not expressing the given KIR and a 28bp small RNA is processed from it (48). It is proposed to be a piwi-interacting RNA with a role in methylating and thus silencing the KIR gene in subsequent NK clones (48).

1.4.1.2.2 Histone Modifications

Histones are the proteins that pack DNA into chromatin and they can have a variety of post-translational modification. Modifications of these DNA packaging proteins can greatly impact their organization and thus the organization and accessibility of the DNA. Some modifications can indicate that a region of chromatin is tightly packed and inaccessible to transcriptional machinery. Other modifications indicate that DNA is loosely packed or "open", indicating the potential for transcription from that region. Histones can be methylated, acetylated, phosphorylated, ubiquitinated, or

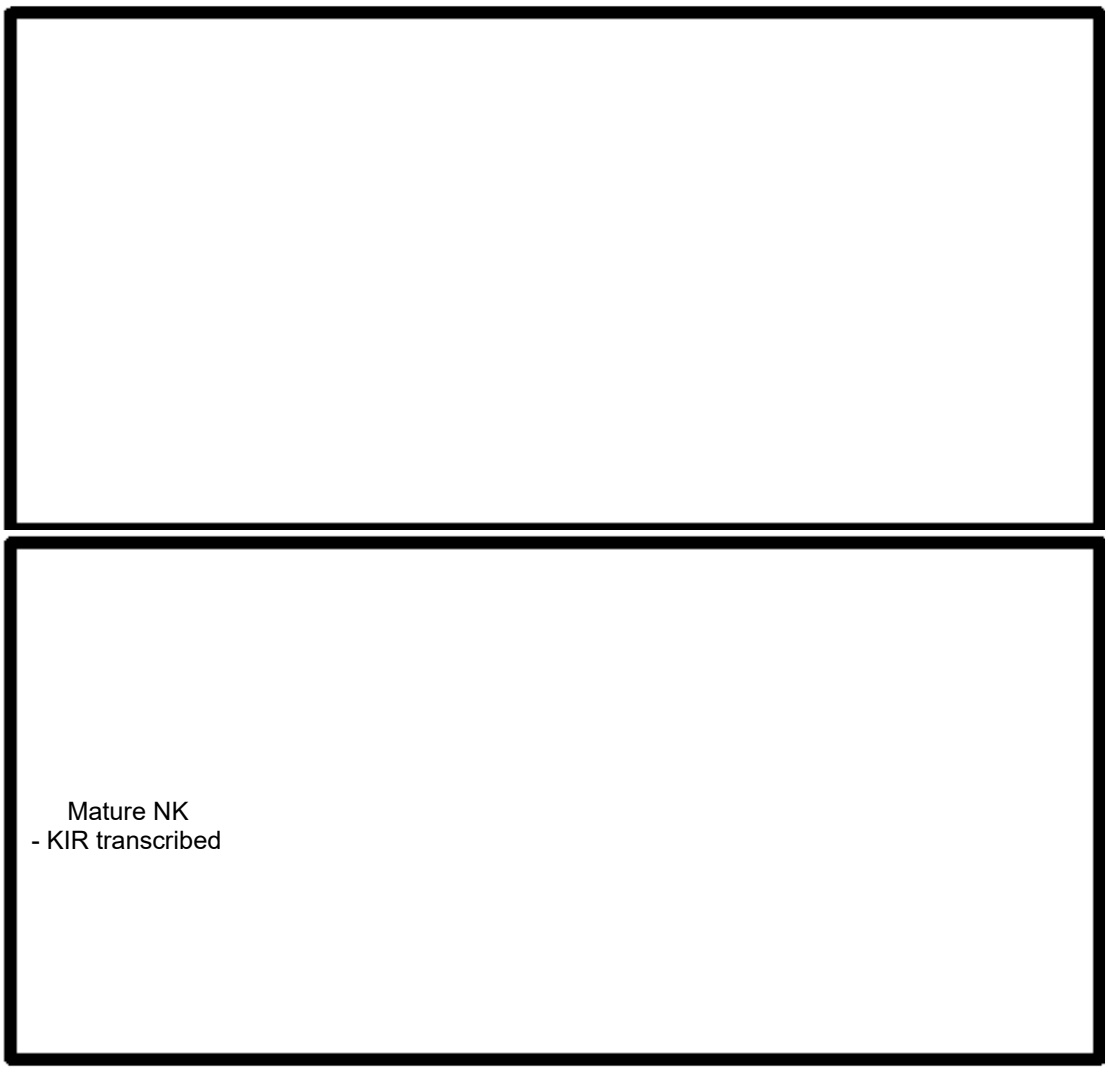


Figure 1.7. Model of a KIR bidirectional promoter and its role in turning KIR off in development (adapted from Pascal et. al (49)).

In the top box, the result of having strong reverse activity from the proximal promoter is shown. The reverse transcript from the proximal promoter and the forward transcript from the distal promoter can form a dsRNA intermediate as shown above the gene. The intermediate causes methylation over the proximal promoter preventing expression. The bottom box depicts the situation where the proximal promoter has a strong forward activity. In this case, there is no dsRNA intermediate and no methylation and therefore the gene is transcribed in the resulting mature NK cells.

sumoylated. For example, in NK cells, histones within the KIR2DL4 gene are marked with several indicators of active transcription, including acetylation of histones 3 and 4, dimethylation of histone 3 at lysine 4 and low levels of methylation of histone 3 at lysine 9 (50).

1.5 Ligands of LILRB1

LILRB1 was originally discovered due to its ability to bind UL18, an MHC-I mimic produced by Human Cytomegalovirus (HCMV) (10). UL18, like MHC-I, dimerizes with cellular β_2-microglobulin and binds endogenous peptides. The current model is that the production of UL18, coupled with mechanisms of MHC-I down-regulation, allow HCMV to evade anti-viral mechanisms of the adaptive immune system, such as cytotoxic T cells, as well as the innate immune system, through inhibition of NK cells. Other HCMV genes, such as US3 and US11, encode proteins that inhibit expression of MHC-I on the cell surface, and thus the recognition of self-MHC/viral peptide combinations by cytotoxic T cells (51, 52). It was hypothesized that UL18 was there to send inhibitory signals to NK cells through binding to receptors on their surface such as KIRs. In studying this idea, Cosman and colleagues discovered LILRB1 as the inhibitory receptor capable of binding UL18 (10).

After discovering LILRB1 and its role in binding the viral ligand, UL18, the same researchers set out to identify a cellular ligand for LILRB1 and, not surprisingly, found that ligand to be MHC-I, specifically HLA-A and B (10). Further studies determined that LILRB1 binds to all types of classical and some non-classical MHC-I

molecules. In humans, the Major histocompatibility complex (MHC) molecules are called Human Leukocyte Antigens (HLAs). The class one MHC family is made up of 3 classical (A, B and C) and 4 non-classical genes (E, F, G, and H). LILRB1 has been shown to bind to HLA –A, -B, -C, –F, and –G (10, 53-55).

1.5.1 UL18

Among many immune evasion genes encoded by HCMV, UL18 is within a group of genes that are known for interrupting the antigen presentation pathway (reviewed in (56). While UL18 varies greatly from the HLA genes in sequence, it is extremely similar structurally. Interestingly, UL18 is bound by LILRB1 with a 1000-fold higher affinity than the HLA molecules (14, 57). Also of interest, the highly similar LILRB2 receptor has comparable binding to HLA molecules as LILRB1 but has more than 1000-fold lower affinity for UL18 than LILRB1 (12).

1.5.2 Classical MHC-I – HLA A, B and C

The major endogenous ligands of LILRB1 are the classical HLA molecules, which are expressed on all nucleated cells. Their role is to present endogenous peptides from the cell to allow the immune system, particularly the cytotoxic T cells, to survey the cells of the body for things such as viral infection or tumour transformation. These molecules are used in surveillance such that cells infected with viruses will present viral peptides on MHC-I molecules, which alerts the cytotoxic T cells of the infected state. Viruses have in turn developed elaborate systems of manipulating this antigen presentation pathway, including down-regulating MHC-I on the cell surface. This down-regulation of MHC-I leaves them open to killing by NK cells due to the lack of inhibitory signal received.

MHC-I molecules are made up of three domains, alpha 1, 2, and 3 and they associate with another molecule called β_2-microglobulin as well as endogenous peptide. In humans, there are three classical MHC-I genes, HLA –A, -B, and –C. The HLA genes are extremely polymorphic, with HLA B having the most diversity in the population. Currently there are greater than 3000 HLA B alleles known (58). The diversity is focussed largely within the alpha 1 and 2 domains, which are responsible for binding the peptide being presented. The alpha 3 domain is more conserved. Each of the three HLA molecules has two copies, due to the nature of human chromosomes, resulting in each cell expressing six different HLA class I molecules. The KIR receptors mentioned previously bind to the polymorphic region of HLA molecules, α1 and α2, while the MHC-binding LILR receptors bind to the conserved α3 domain as well as the associated β_2-microglobulin molecule (13, 59, 60). This explains the difference in binding specificity between the KIRs and the LILRs in terms of HLA molecules.

1.5.3 Non-classical MHC-I – HLA E, F, and G

HLA-G is a ligand of LILRB1 and it is bound with greater affinity than the classical HLA molecules, A, B and C. HLA-G has a very limited expression profile, unlike the classical MHC molecules. HLA-G is expressed on trophoblast and thus plays a positive role in pregnancy (reviewed in (61)). The current theory is that binding of HLA-G prevents NK cells from reacting to the non-self fetus during pregnancy. Conversely, HLA-G can play an unwanted inhibitory role in cancer, with expression on tumor cells allowing them to evade detection.

1.5.4 Other ligands

In addition to the more well-characterized ligands of LILRB1, there are a few more novel ligands that have been discovered over the last 5-10 years. One report demonstrated binding of LILRB1 to several bacterial species (62), implicating this receptor in an additional role. In brief, they found that LILRB1 is able to bind Staphylococcus aureus and Escherichia coli (62). More recently, a protein designated S100A9 has been identified as a ligand of LILRB1 (63). Finally, LILRB1 has also been shown to bind to Dengue virus (64). All of these ligands, in addition to the previously mentioned ones, are potential driving forces in the evolution of LILRB1.

1.6 NK Cells

NK cells function by direct lysis of cells as well as by releasing cytokines into the environment, which influence the activity of surrounding immune cells. These functional activities are regulated largely by interactions with other cells by signals received through an array of activating and inhibitory cell surface receptors. Activating receptors start a signaling cascade that results in cellular changes at several levels that lead to activation of the degranulation response. Engagement of an inhibitory receptor begins a cascade of signals that work to impede the activating cascade. As has been discussed in detail, LILRB1 is among the inhibitory receptors on the surface of NK cells. NK cells were considered strictly innate lymphocytes until recently. This classification was due to the fact that NK cells can react immediately to a new threat with no priming period. An interesting shift in this idea occurred at

approximately the time this thesis work began. Several groups at that time were able to show evidence of adaptive qualities of NK cells (65-68). The research providing this evidence will be examined and discussed in section (1.6.4).

1.6.1 Development and Education of NK cells

Natural Killer cells develop from hematopoietic stem cells through a common lymphoid progenitor, which also gives rise to T and B cells (69). This development occurs in the bone marrow. NK cells differ from T and B cells in that they do not use recombinatory mechanisms for generation of receptor diversity. Instead, all receptors and therefore all diversity, is directly encoded within the genome. NK cells express several receptor families, some of which have already been discussed in detail. The level of diversity in each receptor family varies and impacts specificity for ligands. The KIR family is highly diverse in terms of which genes are encoded as well as alleles of those genes (70). This is logical as KIR receptors are responsible for recognizing the variable region of the highly polymorphic MHC molecules.

It was previously thought that NK cells were able to kill a target regardless of previous exposure or priming. It has now become clear that NK cells, while still innate in that they can act immediately, do undergo education prior to functional recognition of target cells (71-73). A lot of the concrete knowledge of NK education is from studies in mice where models such as knock-outs and transgenics can be utilized. The KIR equivalent in mice are the Ly49 genes. The Ly49 receptors are evolutionarily unrelated but serve the same functional role as the KIR receptors. Ly49 receptors are part of the C-type lectin family of receptors, not the Ig-super

family, which the KIR and LILR families belong to. Like KIR, Ly49 are expressed in a variegated pattern on NK cells (reviewed in (74)). One of the major observations that indicated a need for education of NK cells was made by examining human NK cells that lack inhibitory MHC-I receptors, NK cells are mature and present but they are much less responsive and their capacity to kill targets is greatly decreased (71). Similarly, mice lacking MHC-I on their cells have the same NK phenotype of hyporesponsiveness (75, 76). Interestingly, it has been determined that the number of inhibitory receptors on an NK cell positively correlates with the responsiveness of that cell to kill targets (71). Studies in mice indicate that NK cells are present without any inhibitory receptors (77), which one would expect to have a result of unregulated killing and potentially auto-immunity. This idea that cells lacking inhibitory receptors are not responsive and are unable to kill targets normally likely explains why this is not the case.

As reviewed in (78, 79), there are two extreme models of NK education as well as a more intermediate model. The first is the arming model, wherein it is thought that NK cells are inherently unresponsive and contacting inhibitory ligands allows them to become responsive. Alternatively, the disarming model indicates that NK cells are inherently responsive but a lack of signaling through inhibitory receptors coupled with presence of a stimulatory signal induces a tolerant or hyporesponsive state in the NK cell. Finally, there is the tuning or rheostat model (80), which indicates that the balance of activating and inhibitory receptors on an individual NK cell in

combination with the repertoire of MHC-I ligands in the environment directs the cytotoxic capacity of each NK cell.

1.6.2 Repertoire

While NK cells do not have the diversity in receptors of their fellow lymphocytes, they do have another method of diversifying the overall population of NK cells. This diversity between different NK cells comes from different combinations of NK receptors from the various receptor families. There is a great deal of diversity in receptor expression between different NK cells from the same individual, leading to different responses to threats such as viral infection. The variegated expression of the KIR receptors has been discussed above, as well as the highly diverse LILRB1 expression between different people.

The NKG2 family of receptors has not yet been mentioned in detail but they play an important role in some of the studies of adaptive immune qualities of NK cells and virus-induced NK cell expansions as will be discussed below. This family is made up of activating and inhibitory members (81), following the common theme in NK cell receptor biology. The majority of the family members heterodimerize with CD94 and the dimer binds to non-classical MHC molecules (81, 82). The expression of the NKG2 family members, like the others mentioned, is variable. However, expression of this family of receptors on NK cells is largely impacted by environmental cues such as cytokines, as opposed to being determined more strictly by genetics as with the KIR family (83). NKG2A and NKG2C both recognize HLA-E, which presents the signal peptide of the other HLA molecules (84). This functions as a mechanism

for monitoring the expression of the classical HLA molecules on a target cell. NKG2A is an inhibitory receptor while NKG2C is stimulatory. There is another activating family member called NKG2D that recognizes stress-induced molecules such as MIC-A and B (85, 86). NKG2D does not dimerize with CD94, unlike the other NKG2 family members and instead forms homodimers (87).

1.6.3 CMV-Induced Expansions

A relationship between CD94/NKG2C positive NK cells and HCMV infection has been studied quite extensively (83, 88). The subset of NKG2C positive NK and T cells is expanded in donors who are positive for HCMV (83). This subset of NK cells have cytotoxic and cytokine producing ability, express predominantly inhibitory KIR receptors, lack expression of NKG2A, and express high levels of Bcl-2, an anti-apoptotic molecule (reviewed in (88)). The mechanism of this expansion and the role the HCMV virus plays is still not fully understood. A more direct relationship has been studied in a mouse model of CMV. Mice that express the activating receptor, Ly49H, are much less susceptible to Murine Cytomegalovirus (MCMV) (89). NK cells expressing Ly49H expand in response to MCMV infection (68). Ly49H is a receptor for a protein produced by MCMV called m157 (90). Studies of this MCMV-induced expansion of NK cells led to the discovery of NK memory in response to a viral infection, which will be discussed in the following section.

1.6.4 NK Memory

As mentioned, until quite recently, NK cells were considered to be a component of the innate immune system. They were thought to have a strong, immediate response without any priming needed. In addition to the evidence that some education is

needed during development to make NK cells functional, there has also now been research indicating that NK cells have immunological memory. Immune memory is something that until recently was attributed exclusively to the adaptive lymphocytes, T and B cells. These cells take much longer to mount a response but once they have done so, are able to be recalled any time the same pathogen is encountered for a much more robust and rapid response.

1.6.4.1 Mouse NK Memory

Von Andrian's group identified a memory response of NK cells in response to a hapten (66). They observed persistent, hapten-specific contact hypersensitivity in mice without T and B cells but this was lost in mice that lacked T and B cells as well as NK cells. In the context of a viral infection, NK cell memory was first observed by Lewis Lanier's group, using the Ly49H/MCMV model previously mentioned (68). It was observed that a subset of Ly49H positive NK cells undergo a massive expansion when they encounter the Ly49H ligand, m157. This subset of expanded cells proved to be persistent and mediate an enhanced response and better survival upon re-exposure to MCMV.

1.6.4.2 Human NK Memory

Studying NK memory in humans has the limitation that all studies will be retrospective. As previously mentioned, there is an expanded population of NKG2C positive NK cells in people infected with HCMV. This implies a possible memory subset, however, it is difficult to determine with a persistent virus that can continue to reactivate. A more recent study of an outbreak of hantavirus in Sweden by Ljunggren's research group has shed light on the topic of human NK expansions by

allowing examination of an acute viral infection (91). In a retrospective study of the people infected, a rapid expansion of long-lived NK cells was observed. A large proportion of these NK cells expressed CD94/NKG2C. The expanded cells were capable of degranulation and therefore appear to be functional.

1.7 Project Objectives and Rationale:

Previous work in our lab has uncovered a role for both genetics and extracellular factors in determining the expression pattern of LILRB1 on NK cells. There is very little published regarding the genetic regulation of LILRB1 and even less known about LILRB1 regulation in NK cells. After determining a role for polymorphism in LILRB1 expression in NK cells, we wanted to further understand this association. We focused our studies on the 5' region of the gene and specifically the regulation of transcription. This choice was made as a result of the strong correlation with expression being present at the 5' end of the gene. Additionally, we observed a correlation between transcript level and surface expression, leading us to focus on mechanisms of regulation at the RNA level. In order to examine the association of genotype with expression, we first needed to gain a basic understanding of the transcriptional regulation of the LILRB1 gene. This part of my thesis work is discussed in Chapter 3 as well as being published in the journal *International Immunology* (20). Following the basic characterization of the distal promoter, I focused on potential mechanisms by which the SNPs could impact the NK-specific expression of LILRB1. This research is discussed in Chapter 4 of this thesis. Finally, I focused on implications of the genetic diversity of LILRB1 in the context of HCMV infection. This research is the focus of Chapter 5 of this thesis.

The work described in this thesis was aimed at better understanding the role of genetic diversity in regulating NK-specific LILRB1 expression and further, the implications of the variable expression in immune-compromised patients exposed to HCMV. In a more general sense, with LILRB1 being a broad receptor for MHC-I molecules, one can imagine the implications of the variable levels of expression on NK cells. Low LILRB1 expression could lead to minimal inhibition and potential autoimmunity. High LILRB1 expression could indicate high inhibition and therefore could potentially lead to under reacting to infections with pathogens. As mentioned, the details of how LILRB1 expression variability impacts overall health are difficult to study due to the very different gene family members in animal models. Studies such as this that investigate the genetic regulation of LILRB1 are one way to begin to understand the implications of genetic diversity in this receptor.

CHAPTER 2: Materials and Methods

Parts of this chapter have been published:

Davidson C.L., Cameron L.E. and Burshtyn D.N. (2014) The AP-1 transcription factor JunD activates the leukocyte immunoglobulin-like receptor 1 distal promoter. International Immunology. 26(1): 21-33.

2.1 Cells, human donors and antibodies

2.1.1 Cell lines

All cell lines and media used in this research are listed in Table 2.1. All cells were cultured at 37°C and 5% CO_2.

2.1.2 Primary cells

Studies performed with primary human cells were approved by the University of Alberta Health Research Ethics Board and blood was collected with informed consent from normal healthy donors as previously described (37). PBMC were isolated from whole blood using Lympholyte H (Cedarlane, Burlington, ON, CA). B cells were isolated from PBMC using the EasySep kit for human CD19 positive selection kit (StemCell Technologies, Vancouver, BC, CA). NK cells were isolated from the B cell depleted fraction or from PBMCs using an EasySep human NK cell enrichment kit (StemCell Technologies). Cells were cultured in Iscoves media supplemented with 10% human serum and 2mM L-glutamine.

2.1.2.1 In vitro expansion of NK cells

For *in vitro* expansion, NK cells were isolated from PBMCs using the StemSep human NK cell enrichment kit (StemCell Technologies) and initially plated in a 96 well flat bottom plate with irradiated 721.221 cells, 200 units/mL recombinant IL-2 (rIL-2) (Tecin, obtained from the NCI cytokine repository) and 0.5µg/mL phytohaemagglutinin (Sigma) in Iscoves medium with 10% human serum (Sigma).

Table 2.1 Cell lines

CELLS	SOURCE	CELL TYPE	SPECIES	CULTURE MEDIA
293T	ATCC	Epithelial	Human	DMEM, 10% FBS, 2mM L-Glutamine
YTS	Strominger Lab	NK	Human	Iscoves, 15% FBS, 50 uM B-mercaptoethanol, 2mM L-Glutamine
NKL	Lanier Lab	NK	Human	Iscoves, 10% FBS, 2mM L-Glutamine
NK92	Long Lab	NK	Human	AlphaMEM, 12.5% FBS, 12.5% horse serum, 0.2mM Inositol, 0.02mM Folic Acid, 2mM L-Glutamine and 50 uM B-mercaptoethanol
LNK	Anderson Lab	NK	Mouse	RPMI, 10% FBS, 2mM L-Glutamine
THP-1	Elliot Lab	Monocyte	Human	RPMI, 10% FBS, 2mM L-Glutamine, 50uM B-mercaptoethanol
U937	Ostergaard Lab	Monocyte	Human	RPMI, 10% FBS, 2mM L-Glutamine
DG75	Ingham Lab	B lymphocyte	Human	RPMI, 10% FBS, 2mM L-Glutamine
221	Long Lab	B lymphocyte	Human	Iscoves, 10% FBS, Glutamine
RBL	Stafford Lab	Basophil	Rat	MEM, 10% FBS, 2mM L-Glutamine (+/- 800ug/mL G418)

NK cells were propagated thereafter in Iscoves medium supplemented with 10% human serum, 2 mM glutamine and 200 U/mL of rIL-2. Cell purity and LILRB1 expression was examined at Day 1, prior to plating, by flow cytometry, as well as before use in further experiments.

2.1.3 CMV patient samples

2.1.3.1 CMV patient samples – Canadian Cohort

The CMV study samples were collected by Dr. Atul Humar and the details of the study cohort can be found in a previous report by this group (92) and will also be described in brief here. Ethics approval for this study was obtained as an addendum to the initial study by the Humar group. Within two Canadian transplant centres, a group of transplant patients were followed for a year following organ transplantation to monitor for CMV viremia and disease. Blood samples were taken from each patient and serum was stored for future analysis. There were initially 127 patients included in the study, however, only 67 samples from donor+/recipient- patients were still available for inclusion in the current LILRB1 genotyping study. Patients in this study were all scheduled to receive antiviral prophylaxis with ganciclovir or valganciclovir for 3 to 6 months. All patients also received immunosuppression with a variety of medications. The study coordinators obtained approval from institutional review boards before initiation of enrollment and additionally received written consent from patients involved in the study. Enrollment was between April 2008 and March 2011.

2.1.4 Antibodies

All antibodies used in this research are listed in table 2.2

2.2 Transcription reporter Assays

Fragments of the LILRB1 promoter regions were PCR amplified, with the addition of NheI and KpnI sites, from plasmids containing a larger region of the gene that was cloned from genomic DNA from donors with promoter sequences matching a reference sequence in Genbank (NC000019.9) (20). The products were cloned into pCRII using the TOPO system (Invitrogen) and the entire sequence verified (MCLab, San Francisco, CA, USA) prior to sub-cloning into the vector, pGL3 basic (Promega, Madison, WI, USA). Deletions and point mutations were introduced using the Quikchange site-directed mutagenesis kit from Agilent Technologies (Santa Clara, CA, USA). For cloning of potential enhancer regions, the region was amplified with the addition of SalI sites on each side to insert into pGL3 downstream of the firefly luciferase gene with the other steps remaining the same. The primers used to amplify regions of the LILRB1 gene for cloning into pGL3 reporter vector are listed in table 2.3. The products were cloned into pCRII using the TOPO system (Invitrogen) and the entire sequence verified (MCLab, San Francisco, CA, USA) prior to sub-cloning into the luciferase vector, pGL3 basic (Promega, Madison, WI, USA). Deletions and point mutations were introduced using the Quikchange site directed mutagenesis kit from Agilent Technologies (Santa Clara, CA, USA). Plasmids were prepared for transfection with endotoxin-free column purification (Qiagen, Mississauga, ON, CA) and the concentration was quantified using a Nanodrop (Thermo Fisher Scientific).

Table 2.2 Antibodies

ANTIBODY	ISOTYPE	SPECIES	SUPPLIER	CAT. #	NOTES:
PE Anti-human CD56	IgG1, k	Mouse	eBioscience	12-0567	
PE Isotype Control	IgG1, k	Mouse	eBioscience	12-4714	
FITC Anti-human CD14	IgG2a	Mouse	abcam	ab82434	
FITC Isotype Control	IgG2a	Mouse	abcam	ab91362	
APC Anti-human CD19	IgG1, k	Mouse	eBioscience	17-0199	
APC Isotype Control	IgG1, k	Mouse	eBioscience	17-4714	
PECy5 Anti-human LILRB1	IgG2b, k	Mouse	BD	551054	
PECy5 Isotype Control	IgG2b, k	Mouse	BD	555744	
Anti-human AML-1	Polyclonal	Rabbit	Santa Cruz	sc28679	
Anti-human Fos	IgG2a	Mouse	Santa Cruz	sc271243	Detects cFos, FosB, Fra1 and Fra2
Anti-human Fra2	Polyclonal	Rabbit	Santa Cruz	sc604	
Anti-human cJun	Polyclonal	Rabbit	Active Motif	39309	
Anti-human JunB	IgGa	Mouse	abcam	ab53543	
Anti-human JunD	Polyclonal	Rabbit	Santa Cruz	sc74	
Isotype control			Cedarlane	CLAS10-916	
Anti-HA Tag	IgG3	Mouse	Cedarlane	CLH104AP	
Isotype Control	IgG3	Mouse	Cedarlane		
PE Goat anti-Mouse		Goat	Cedarlane	CLCC30004	

Table 2.3 Primers for reporter construct cloning. The primer number indicates the number within the Burshtyn lab inventory.

NAME	SEQUENCE (5' to 3')	PRIMER NUMBER
-14570 KpnI	GGTACC AGCAGGGGCCCACTGAAAG	480
-14247 KpnI	GGTACC GCTCCTCCCAGCCAAAATGT	482
-14086 KpnI	GGTACC GCATTTTGGGCCCTCCTG	520
-13793 NheI	GCTAGC AAACCAATGGAGTGCCCACAA	485
-13920 NheI	GCTAGC TAAAGGAGGAAGTGAGCTGTG	634
-1287 KpnI	GGTACC ATCTCACCTGTGGCCTCTGTTC	438
-1 NheI	GCTAGC GGCGTCTCCTCCCACTGC	441
-1137 KpnI	GGTACC AGATGTGAGATCCAGAGTGGACAC	436
-1137 NheI	GCTAGC AGATGTGAGATCCAGAGTGGACAC	435
-589 KpnI	GGTACC CCACACGCAGCTCAGCCTG	440
-589 NheI	GCTAGC CCACACGCAGCTCAGCCTG	439
-1287 NheI	GCTAGC ATCTCACCTGTGGCCTCTGTTC	437
-1 KpnI	GGTACC GGCGTCTCCTCCCACTGC	442
-1287 SalI	GTCGAC ATCTCACCTGTGGCCTCTGT	662
-1 SalI	GTCGAC GGCGTCTCCTCCCACTGC	663

Cells were transfected with pGL3 firefly luciferase reporter vector using matched molar equivalents for each reporter plasmid and pRL-TK renilla luciferase vector to normalize for transfection efficiency (amount of DNA is indicated for each cell type in section 2.8 below). Generally, a parallel sample was transfected with a plasmid encoding EGFP and analyzed by flow cytometry to ensure transfection was at a successful level for each cell line. Cells were incubated for 16 - 24 hours before lysis in passive lysis buffer and analysis using the Dual luciferase reporter assay kit (Promega). Samples were read in a 20/20n luminometer (Turner Biosystems, Sunnyvale, CA, USA). The transcriptional activity was normalized in each sample to the amount of renilla luciferase expression from the pRL-TK plasmid and expressed relative to a control sample transfected with an empty pGL3 basic vector.

2.2.1 Transactivation reporter assays

For transactivation assays, luciferase assays were performed as above with the addition of 2.5ug of pcDNA or pcDNA-JunD vector. These luciferase assays were harvested at 24 hours post-transfection. The JunD expression plasmid (pcDNA-JunD) and the empty control pcDNA vector were produced and generously provided by Dr. Wilding (University of Wisconsin, School of Medicine and Public Health) (93).

2.3 PCR, Cloning and sequencing

2.3.1 Quantitative PCR

Total RNA was purified using the RNeasy kit and DNase treated on the column to remove genomic DNA (Qiagen). cDNA was synthesized using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Primers for quantitative PCR are listed in table 2.4. Quantitative PCR methods used to amplify the long, total, and RPL24 transcripts were described previously (20, 22, 29). All samples were run in an Eppendorf realplex2 PCR machine using SYBR green as a detection reagent (Quanta, Gaithersburg, MD), normalized to RPL24 and quantified using the $2^{-\Delta\Delta Ct}$ method (94).

2.3.2 Non-quantitative PCR

2.3.2.1 Touchdown PCR to detect proximal promoter transcript

The PCR for the proximal promoter derived transcript was performed using a touchdown program as follows: 95°C 3 min, 15x (30 sec 95°C, 30 sec t, 60 sec 72°C) where in the first cycle t was 72°C and decreased by 1°C each cycle, 31x(30 sec 95°C, 30 sec 57°C, 60 sec 72°C), 4 minutes 72°C. The specificity of the PCR was determined by sequencing of the PCR products. Products were run on a 2% agarose gel and visualized using Ethidium Bromide. The primers used are listed in Table 2.4.

2.3.2.2 Genomic DNA amplification of distal region of LILRB1

The 1.8Kb distal region was amplified from genomic DNA, extracted using the illustra kit (GE Healthcare Life Sciences) using primers “distal region forward and distal region reverse” (Table 2.4). The program used for amplification was 95 for 3 minutes, followed by 31 cycles of 95 for 30 seconds, 56 for 30 seconds, and 68 for 1

Table 2.4 LILRB1 amplification primers

NAME	SEQUENCE (5' to 3')	PRIMER NUMBER
Long/Distal Forward	CACATTTACATCAAGCTCAGCC	376
Long/Distal Reverse	CCTGCTCTGTGGATGGATG	377
Short/Proximal Forward	CTGTGCTCGCTGCCACACGCAGCTCAG	619
Short/Proximal Reverse	GGGTGGGCTTGGGGAGGTGCC	618
Total Forward	ATCCTGATCGCAGGACAGTT	378
Total Reverse	GGAAAGTTTGCATCCATCCCTG	400
RPL24 Forward	GGACCGACGGGAAGGTTTTCCAG	537
RPL24 Reverse	GGAATTTGACTGCTCGGCGGGT	538
Distal Region Forward	TGCTGGGATAACTGGCTATCCATATGCA GACAATTG	499
Distal Region Reverse	CACACAGACATCCTGTAGCTTTTACTTCA A	497

minute, and finally a 4 minute hold at 68. The PCR reaction was performed using PFU Ultra II HS DNA polymerase and its accompanying buffer. Additionally, the reactions contained 1.5uL of genomic DNA (around 100ng), 0.4mM dNTPs, and each primer at a concentration of 0.4uM. The amplified DNA segment was cloned into the pCRII-TOPO vector (Invitrogen) and sequenced with vector primers T7 and SP6 (MCLab, San Francisco, CA). Sequences were analyzed using the Geneious software.

2.3.3 Genotyping

2.3.3.1 TaqMan Genotyping

TaqMan genotyping assays for five individual SNPs within LILRB1 were designed. Four of the five SNPs, rs10416697 (-14894), rs1004443 (-1026), rs2781771 (-225), and rs1061680 (+927) were analyzed by TaqMan genotyping assays. The TaqMan assay was performed using approximately 10ng of DNA per reaction and each was done in duplicate. Within each assay, a positive control was performed using DNA from a donor with each of the three known genotypes and a negative control performed by not including DNA in the reaction. Each reaction contains 12.5uL of 2x universal master mix (Catalogue number 4304437, Applied Biosystems, Waltham, MA), 1.25uL of working stock of primer probe mix, 1uL DNA and up to 25uL with sterile water. The PCR was run on the StepOne PCR Machine (Catalogue number 4304437, Applied Biosystems, Waltham, MA) using the program: 10 minutes at 95, followed by 50 cycles of 92°C followed by 60°C for 15 and 60 seconds, respectively.

2.3.3.1.2 Genotyping - Direct sequencing of PCR samples

Patient genotype was determined at position rs16985478 using conventional PCR followed by direct sequencing of amplified product. The primers used for this amplification are +5724 Genotype forward (5'- GTGACGTATGCCGAGGTGAA-3') and +5724 Genotype Reverse (5'- CTGCAGAATTGAGTGACCCCT-3'), primer numbers 647 and 648, respectively. The touchdown PCR program used was as follows: 95°C for 2 minutes followed by 15 cycles of: 94°C for 15 seconds, 62°C for 30 seconds with a decrease of 0.8°C/cycle, and 72°C for 1 minute, finally followed by 29 cycles of 94°C for 15 seconds, 50°C for 30 seconds and 72°C for 1 minute and a final hold of 72°C for 3 minutes. Sequencing was done by MCLab.

2.4 Electrophoretic Mobility Shift Assays (EMSAs)

Double stranded DNA probes were purchased from IDT Technologies (San Diego, CA, USA) and end-labeled with γ-[^{32}P] ATP using T4 polynucleotide kinase (New England Biolabs, Pickering, ON, CA). To prepare nuclear extracts, cells were washed with PBS, resuspended in 400 µl of freshly made cold Buffer A (10 mM HEPES pH 7.6, 10 mM KCl, 0.2% NP40, 1 mM DTT, 1 mM PMSF, 2 µg/mL aprotinin, 2 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM NaF, and 1 mM NaV) and incubated on ice for 10 minutes. The nuclei were pelleted, washed with 1 mL of cold Buffer A and resuspended in 20 to 80 µl of freshly made, cold Buffer C (20 mM HEPES pH 7.9, 400 mM NaCl, 25% Glycerol, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 µg/mL aprotinin, 2 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM NaF, and 1 mM NaV) and rotated for 30 minutes at 4°C. Nuclear debris was

removed by centrifugation at 12,000xg for 5 minutes and resulting supernatants aliquoted for single use to avoid freezing and thawing, snap-frozen and stored at -80°C. The protein concentration of the extracts was determined by BCA assay (Peirce, Rockford, IL, USA). For each EMSA reaction, nuclear extract corresponding to 5 µg of protein was incubated with ~2 ng of labeled probe in 4% glycerol, 50 ng/µL Poly(dIdC), 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 1 mM $MgCl_2$ at room temperature for 20 minutes (adapted from (95)). Where indicated, competitor probes were added at a 100-fold excess or antibodies at 1-2 µg per reaction (20 µl reaction volume) for 15 minutes at 4°C prior to incubation with the probe. The complexes were electrophoresed on a 5% native acrylamide gel at 20 mAmps for ~4 hours in 0.5x TBE buffer at 4°C, the gel was dried and subjected to autoradiography. EMSA probe sequences are listed in Table 2.5 (with the sense strand of the double stranded DNA probe shown). The AP-1 consensus probe sequence was based on Asangani et al. (95) and the AML-1 consensus probe sequence on Meyers et. al (96).

2.5 Chromatin Immunoprecipitation (ChIP)

NKL cells were resuspended in fresh medium at $9x10^6$ cells/ml in a total volume of 10 ml and fixed by addition of 270 µl of 37% formaldehyde (Fisher Scientific, Ottawa, ON, CA) for 10 minutes at room temperature and then stopped by addition of 1 ml of 1.375M glycine for 5 minutes. The cells were centrifuged at 600xg for 5 minutes at 4°C, washed twice with 50 ml of cold PBS and lysed in 5 mL of cell lysis buffer (5 mM PIPES (pH8.0), 85 mM KCl, and 0.5% NP40) with protease inhibitor

Table 2.5 EMSA Probes

NAME	SEQUENCE	NOTES
-14086 to -14055 (Probe 1/A)	GCATTTTGGGCCCTCCTGGAGGTGT TTAGACC	
-14054 to -14022 (Probe 2/B)	TTCCGAGAGAGAAACTGAGACACA TGAGAGGG	
-14027 to -13996 (Probe 3/C)	GAGGGAAGAAATGACTCAGTGGTG AGACCCTG	
-13995 to -13966 (Probe 4/D)	TGTGGAGTCCCACCCACAACCAGC ACACTGT	
WT 20bp	AGAAATGACTCAGTGGTGAG	Probe 3 - Truncated to 20bp
AP-1/NFE2 Mutation	AGAAATGCAGCAGTGGTGAG	WT 20bp with AP-1/NFE2 site mutated
AML-1 Mutation	GAGGGAAGAAATGACTCAGTTTTG AGACCCTG	WT 20bp with AML-1 site mutated
AP-1 Consensus*	CCCGAGAGTTGCGGCCTGAGTCAC CGGCCC	
AML-1 Consensus*	AATTCGAGTATTGTGGTTAATACG	

cocktail (PIC) (Sigma) on ice for 10 minutes. Nuclei were collected by centrifugation at 2,500xg for 5 minutes at 4°C, resuspended in 500 µl of 1x micrococcal nuclease (MNase) buffer and digested with 4 µL of micrococcal nuclease (New England Biolabs) for 15 minutes at 37°C and the reaction stopped with 50 µl of 0.5 M EDTA. The nuclei were pelleted again (as above), resuspended in 1mL of nuclear lysis buffer (50 mM Tris-HCl (pH8), 10 mM EDTA, and 1% SDS) with PIC and lysed on ice for 10 minutes followed by a brief sonication step (2 ten second pulses, one minute apart at power 5) using the 60 sonic dismembrator (Fisher Scientific). The debris was removed by centrifugation. A sample was removed to ensure the average size range of the sheared DNA was between 200 and 1000bp by heat-treating to reverse crosslinking, purifying the DNA and analyzing on an agarose gel. The samples were precleared by addition of 50 µl of "ChIP blocked" protein G agarose beads from the EZ ChIP kit from Millipore (Billerica, MA, USA) and rotated for 1 hour at 4°C. A 25 µl aliquot of sample was set aside as the "input" control. The remaining supernatant was diluted with 2 volumes of ChIP dilution buffer (16.7 mM Tris-HCl (pH 8), 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, and 1.1% Triton-X) and divided into equal aliquots before addition of 5 µg of rabbit IgG control or anti-JunD and incubation overnight at 4°C with rotation. The complexes were collected with 25 µl "ChIP-blocked" protein G agarose beads for 2 hours, washed 3 times with 1 ml of ChIP wash buffer (20 mM Tris-HCl (pH 8)/150 mM NaCl/2 mM EDTA/1% Triton X-100/0.1% SDS) for 5 minutes with agitation. A final wash was done with high salt ChIP wash buffer (20 mM Tris-HCl (pH8)/500 mM NaCl/2 mM EDTA/1% Triton X-100/0.1% SDS). The beads and the "input DNA" sample were resuspended in 300 µl

of elution buffer (100 mM $NaHCO_3$/1% SDS) for 15 minutes at room temperature. The supernatants were treated with 100 µg of proteinase K at 45° for 1 hour, followed by 65° for 5 hours to reverse the formaldehyde crosslinking. The DNA was purified from the reaction using a PCR purification kit (Qiagen) and eluted in 40 µl of water. PCR was performed using 5 µl of purified DNA with 0.4 µM primers using Taq polymerase (Invitrogen). The primers used to detect DNA quantity in each precipitation are listed in Table 2.6. The PCR program included 34 cycles and an annealing temperature of 55°C. The PCR products were run on a 2% agarose gel and stained with Ethidium bromide. For quantitative ChIP PCR, the same primers were used as above, however, a SYBR green master mix was used (Quanta Biosciences) with 1 µl of purified DNA. The calculation used was: Fold change = $2^{\Delta\Delta CT}$ where: $\Delta\Delta CT = \Delta CT_{JunD\ IP} - \Delta CT_{IgG\ control\ IP}$ and $\Delta CT = CT_{Input} - CT_{IP}$.

2.6 siRNA Assays

NKL cells were transfected using the 4D nucleofector (AMAXA Biosystems, Gaithersburg, MD, USA). The nucelofections were performed with 2.5 $x10^6$ cells/sample using program CM-150 and Kit L with the siRNA at a concentration of 100nM. Cells were nucleofected with either a pool of four siRNAs targeting JunD or a pool of four control siRNAs (Thermo Fisher Scientific (Dharmacon)). JunD and LILRB1 transcripts were then quantified using quantitative PCR methods as described in the previous methods section on PCR, including an internal control transcript, RPL24. JunD was amplified using primers: Forward 5'-TCC AGT CCA ACG GGC TGG TCA-3' and Reverse 5'-GAC GAA GCC CTC GGC GAA CTC-3'.

Table 2.6 ChIP Primers

NAME	SEQUENCE (5' to 3')	PRIMER NUMBER
ChIP Intron 1 Forward	GCTTTAGGAATTACATAGTTTCAGGT	568
ChIP Intron 1 Reverse	ACATACTGAGCTTTGCTAAAGTCAA	569
ChIP AP-1 site Forward	GCATTTTGGGCCCTCCTGGAG	562
ChIP AP-1 site Reverse	AGCTGTGGGCTAGAGGTTTGTGCA	563

2.7 Unbiased transcription factor identification assay

In this assay, a biotinylated dsDNA probe representative of a 20bp region of the LILRB1 distal promoter containing the known AP-1 binding site was used (Table 2.5, probe "WT 20bp"). Approximately 1mg of streptavidin agarose beads were washed three times in Buffer A (5mM Tris (pH 8), 0.5mM EDTA, 1M NaCl) by rocking in buffer for 5 minutes followed by a 1 minute spin to collect the beads and resuspension in 100uL of Buffer A. 200pmoles of biotinylated probe were added to the beads and rocked for 15 minutes at room temperature. The beads were then washed twice with 500uL of Buffer C (10% glycerol, 50 ng/µL Poly(dIdC), 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5mM dithiothreitol and 1 mM $MgCl_2$) before adding 300ug nuclear extract from NKL cells and adding buffer C up to 200uL volume with 10uL poly dI:dC (500ng) for 15 minutes. The beads were then again washed three times with buffer C before spinning down and adding 50uL of reducing sample buffer (62.5mM Tris (pH 6.8), 10% glycerol, 2% SDS, 50mM beta-mercaptoethanol, and bromophenol blue) and boiling for 5 minutes. The beads were then spun down and the supernatant loaded into a 10% acrylamide gel and run. The gel was silver stained with the following protocol. The gel was soaked for 1 hour in 40% ethanol/10% acetic acid followed by two 15 minute washes in 30% ethanol and one 15 minute wash in sterile water. The gel was then sensitized for one minute in 0.02% Sodium thiosulfate followed by three 20 second washes in sterile water. The gel was then moved to a new clean tray and soaked one minute in sterile water and then developed in 3% Na2CO3 with 0.05% formaldehyde. The gel was removed from the solution as soon as bands started to appear and not exceeding 5 minutes, as

is optimal for samples that will be sent for mass spectrometry analysis. Finally, the gel was rinsed in sterile water and placed in 5% acetic acid for 30 minutes to stop the staining. At this point, the gel was photographed and the visible bands were cut out and placed in 1.5mL tubes with enough sterile water to cover them and placed at -20 until being brought to the IBD facility for mass spectrometry analysis.

2.8 DNA Methylation Analysis

PBMC were isolated from whole blood as described in section 2.1 followed by sequential CD14 positive selection, CD19 positive selection and NK cell negative selection based enrichment, all using StemCell Easy sep kits and following manufacturer protocols. Each cell subset was tested for purity (CD19 for B cells, CD14 for monocytes and CD56 for NK cells) and LILRB1 expression using flow cytometry (antibodies listed in table 2.2). Genomic DNA was then isolated using the illustra kit from GE Healthcare and was then bisulfite converted using the EZ DNA Methylation kit (Zymo research) following the manufacturers protocol. The amount of cells used for genomic extraction was dependent on how many were available after cells were collected for FACS but was generally approximately $5x10^6$ cells. Each conversion reaction was done on 500ng of genomic DNA and half of one reaction was generally used in each PCR reaction. Primers were designed to amplify the DNA regions of interest (Table 2.7), avoiding inclusion of CpG sites in the primer binding regions as this can create a site where two nucleotides are possible. The regions were amplified using the bisulfite primers listed in Table 2.7 using a program of 94°C for 2 minutes, followed by 36 cycles of 94°C for 20 seconds, 49°C for 30

seconds and 68°C for 1 minute, followed by a 4 minute hold at 68°C. The PCR reaction used HiFi DNA polymerase (Quantas) and its accompanying PCR buffer and Magnesium sulphate (used at 2mM). Additionally, the reaction contained 0.4mM dNTPs, 4uL of bisulfite converted DNA (approximately 200ng not considering loss during conversion) and 0.4uM of each primer (forward and reverse). PCR products were run on an agarose gel and extracted according to manufacturer's protocol using the Qiaquick gel extraction kit (Qiagen). An A-overhang reaction was performed on purified PCR products before performing TA cloning into the pCR-II vector following the TOPO cloning kit protocol (Invitrogen). The A-overhang reaction includes 6.6uL of purified PCR product, 1uL of Taq polymerase and 1X accompanying buffer (Invitrogen), 0.2mM dATP, and 2mM Magnesium chloride and an incubation at 70°C for 20 minutes. The cloned PCR products in pCRII were transformed into TOP10 E. coli as per manufacturer's protocol. Ten to twenty clones were selected and cultured in LB with ampicillin for 16 hours for each PCR product before using the QiaPrep Spin Miniprep kit (Qiagen) to isolate plasmid DNA. The plasmid DNA was sent for sequencing at MCLab (San Francisco, CA) using T7 and SP6 vector primers. Each CpG site was analyzed in all clones to determine a "percent methylation" score for each CpG cytosine.

2.9 Cell Transfections

2.9.1. Nucleofection

Cell lines were transfected using the Amaxa nucleofection system (AMAXABiosystems, Gaithersburg, MD,USA) with various protocols dependent on

Table 2.7. Primers used for amplification from bisulfite converted DNA. The * on the exon 6 primers indicates that cloning and sequencing this product was unsuccessful.

NAME	SEQUENCE (5' to 3')	PRIMER NUMBER
Distal - BS Forward	GGG GTT TAT TGA AAG TTT TTA GGA T	656
Distal - BS Reverse	CCC ACA AAA AAA TCA CTC TTC TTA C	657
Proximal - BS Forward	GGT TGT TTG GGG TTT ATT TTA GTT T	658
Proximal - BS Reverse	AAA CAA ATC TCT AAA TCT TTC CTA CCT C	659
Exon 2 - BS Forward	GGG GAT TAT TTG GTT GAA AGA AAA TTT ATA	641
Exon 2 - BS Reverse	ACT ACC CTC CCC AAA ACC CTT ACT C	642
Exon 6 - BS Forward*	AGG GTT TAG GGT TTT TGG GGT TAG A	645
Exon 6 - BS Reverse*	TAA ACT AAC ACC TCC CCA AAT CTC C	646

the cell line. In all cases, small scale nucleofections were done in wells the size of 96 well plates using the 4D nucleofector.

2.9.1.1 Nucleofection of NK92 and NKL

1 x 10^6 cells were pelleted per reaction and resuspended at 1 x 10^6 cells in 20uL of nucleofection solution from the SF Cell line kit S. For each reaction, the cells were mixed with 2µg of pGL3 vector with or without insert and 10ng of pRL-TK vector and run on program CM-150. Post-nucleofection, 30µL of recovery media (RPMI supplemented with non-essential amino acids and 100U/mL IL-2) was added and cells were incubated at 37°C for 10 minutes before transferring to a well of a 96 well flat-bottom plate with 150µL complete medium (NK92 or NKL) and incubating for 16 to 24 hours.

2.9.1.2 Nucleofection of U937

For U937 nucleofections, 3 x 10^5 cells were pelleted per reaction and resuspended at 3 x 10^5 cells in 20µL of nucleofection solution from the SF Cell line kit S. For each reaction, the cells were mixed with 1µg of pGL3 vector with or without insert and 5ng of pRL-TK vector and run on program "U937 new" on the Amaxa 4D Nucleofector. Post-nucleofection, 30µL of recovery media was added and cells were incubated at 37°C for 10 minutes before transferring to 96 well flat-bottom plate with 150uL U937 medium and incubating for 16 hours.

2.9.1.3 Nucleofection of THP-1

3 x 10^5 cells were pelleted per reaction and resuspended at 3 x 10^5 cells in 20µL of nucleofection solution from the SF Cell line kit S. For each reaction, the cells were mixed with 0.5µg of pGL3 vector with or without insert and 5ng of pRL-TK vector and run on program "THP-1" on the amaxa 4D nucleofector (program FF100). Post-nucleofection, 30µL of recovery media was added and cells were incubated at 37°C for 10 minutes before transferring to 96 well flat-bottom plate with 150uL THP-1 medium and incubating for 16 hours.

2.9.1.4 Nucleofection of YTS

YTS cell nucleofections were performed with 5×10^6 cells per sample were then transfected with 2.5µg of pGL3 with or without the insert, and 10ng of the pRL-TK plasmid using the Amaxa nucleofection system (AMAXABiosystems,Gaithersburg, MD,USA) on program T-020 using KitR. Transfected cells were then cultured for 16 hours before collection and analysis. For IL-2 experiments, YTS cells were cultured for 22 hours in the presence or absence of IL-2 (1000U/ml) and for 16 hours post-nucleofection, again with or without IL-2 in the media, before collection and analysis.

2.9.2 Calcium phosphate transfection

293T cells were seeded the evening prior to transfection at 2 x 10^5 cells in 2mL of media per well in a 6 well plate. For each transfection reaction, 2.5ug of JunD or control pCDNA vector DNA was used along with 2ug pGL3 vector with or without insert and 50ng pRL-TK. DNA was combined and brought to 90uL with sterile water

and 250mM Calcium chloride, to which 100uL of 2X HBS was added slowly before incubating for 15 minutes at room temperature. Finally, 3.6uL of chloroquine was added and the solution mixed before adding dropwise to the cells. After 5 hours at 37°C and 5% CO_2, the media was changed to fresh 293T media and incubated another 43 hours at 37°C and 5% CO_2 before collection for luciferase analysis.

2.9.3 Xfect Transfection

RBL cells were seeded in the evening at 1.5 x 10^5 per well in 1mL of RBL media. For each well, two tubes are prepared. Tube one contains 1ug of DNA and Xfect reagent up to 25uL and tube two contains 0.3uL of Xfect polymer and Xfect reagent up to 25uL. The two tubes are combined and left for 10 minutes at room temperature. RBL media is removed from the cells and replaced with 250uL of OPTI-MEM before adding the Xfect mixture dropwise to the cells. The cells are left for 4 hours at 37°C and 5% CO_2 before adding 750uL of RBL media. After 48 hours, the medium was replaced with RBL medium containing 800ug/mL of G418 for selection. After growing up in this selection media, the cells were sorted by flow cytometry based on HA expression and continued to be cultured in G418 selection media to maintain receptor expression.

2.9.4 Electroporation

2.9.4.1 LNK electroporation

LNK cells were collected and resuspended at 1 x 10^6 cells/100uL RPMI media. For each reaction, 300uL (3 x 10^6 cells) was mixed with 10ug pGL3 with or without

insert and 100ng pRL-TK in a 1.5mL tube before pipetting into a 4mm BTX cuvette. Electroporation was performed at 250mV with three 7ms pulses with 100ms intervals. Cells were placed on ice and pipetted into 6 well plates with 5mL pre-warmed LNK media with 500u/mL IL-2 in each well. Cells were incubated for 48 hours before collection and analysis of luciferase activity.

2.9.4.2 DG75 electroporation

DG75 cells were collected and resuspended at 1 x 10^7 cells per mL in DG75 media. For each reaction, 500uL of cells were combined with 10ug of pGL3 DNA and 100ng pRL-TK DNA before placing into a cuvette on ice. Electroporation was performed at 225mV with three 8ms pulses with 1s intervals. Cells were placed on ice and pipetted into 6 well plates with 5mL pre-warmed DG75 media in each well. Cells were incubated for 24 hours before collection and analysis of luciferase.

2.10 UL18 binding assays

2.10.1 Production of LILRB1 and LILRB2 constructs

LILRB1 was amplified from cDNA of a donor with known LAIS homozygous genotype. The coding sequences of LILRB1 and LILRB2 were amplified without the signal peptide using primers with restriction site additions (XmaI at the 5' end and SalI at the 3' end – see Table 2.8). The amplified genes were cloned into the TOPO pCR-II vector using TOPO-TA cloning. The LILRB1 and LILRB2 genes were digested out of the pCR-II vector and were cloned into the pDisplay expression vector, in frame with the HA tag. The PTTI variant of LILRB1 was produced using sequential site-directed mutagenesis of the LAIS variant in pCR-II. Site-directed

Table 2.8 Primers used for LILRB1 and LILRB2 cloning and mutagenesis.

NAME	SEQUENCE (5' to 3')	PRIMER NUMBER
XmaILIR1 no SP	CCCGGG GCAGGGCACCTCCCCA	488
SalILIR1 stop	GTCGAC CTAGTGGATGGCCAGAGTGGCGTA	418
XmaILIR2 no SP	CCCGGG ACAGGGACCATTCCCAAGCC	489
SalILIR2 stop	GTCGAC CTAGTGGATGGCAGGGTG	490
L68P - FOR	GAAAACAGCACCCTGGATTACACGGATCCCAC	403
L68P - REV	GTGGGATCCGTGTAATCCAGGGTGCTGTTTTC	404
A93T - FOR	CACCTGGGAACATACAGGGCGGTATCGC	405
A93T - REV	GCGATACCGCCCTGTATGTTCCCAGGTG	406
I142T - FOR	GGAGGGAATGTAACCCTCCAGTGTGACTCACAGG	407
I142T - REV	CCTGTGAGTCACACTGGAGGGTTACATTCCCTCC	408
S155I - FOR	GGTGGCATTTGATGGCTTCATTCTGTGTAAGGAAGG	409
S155I - REV	CCTTCCTTACACAGAATGAAGCCATCAAATGCCACC	410

mutagenesis was done following manufacturer instructions for the Quikchange Lightning Mutagenesis kit (Agilent Technologies) and primers listed in Table 2.8. Each mutation was confirmed by sequencing the insert. The variant was again digested out of the pCRII vector and cloned into the pDisplay vector.

2.10.2 Flow cytometry-based binding assays

Two HA-tagged variants of LILRB1 and a single LILRB2 variant were stably expressed on the surface of RBL cells by X-fect transfection followed by selection in G418 (see 2.9.3). UL18 Fc-fusion protein was generously provided by Adnane Achour (Karolinska Institute). For each binding reaction, 1 x 10^5 RBL cells were resuspended in 20uL of FACS buffer (PBS, 1mM EDTA and 1% FBS). To each tube of cells, antibody or Fc-fusion protein was prepared at the desired concentration in a total volume of 100uL before being added to the cells. The binding was done for 1 hour on ice. The cells were then washed with cold FACS buffer and pelleted before addition of the secondary antibody for 20 minutes at 4°C. The cells were finally washed once again with cold FACS buffer before fixation and analysis by flow cytometry. In each assay, the cells were stained separately using anti-HA and an isotype control antibody as well as a range of concentrations of the UL18 Fc-fusion protein. The anti-HA and isotype antibodies were detected using a PE labelled anti-mouse secondary while the UL18 Fc-fusion protein was detected with a PE labelled anti-human IgG (Table 2.2). For each assay, the binding of UL18 to the transfected cells was normalized to the receptor expression, as detected by the anti-HA antibody.

2.11 Statistical Analysis for CMV studies – Canadian cohort

Results comparing demographic data and the SNP frequencies between patients with/without CMV disease were analyzed using (i) chi-square or Fisher's Exact Test for categorical variables or (ii) Mann-Whitney U Test for continuous variables, with a significance level of 0.05 and a confidence interval of 95%. Each SNP was examined using a Log-rank (Mantel-Cox) test for difference or Logrank test for trend, also with a significance level of 0.05. This analysis was done by Luiz Lisboa in the Humar lab.

CHAPTER 3: Characterization of the LILRB1 distal promoter

A version of this chapter has been published:

Davidson C.L., Cameron L.E. and Burshtyn D.N. (2014) The AP-1 transcription factor JunD activates the leukocyte immunoglobulin-like receptor 1 distal promoter. International Immunology. 26(1): 21-33.

3.1 Background

In spite of the interesting pattern of expression and potential importance for tight regulation of LILRB1's expression to maintain a balance in signals, there is little known regarding transcriptional regulation of the LILRB1 gene. The first study of the LILRB1 promoter characterized a region that is active in monocytes and is now known as the proximal promoter. This study defined a 160bp core promoter ~500bp upstream of the translational start codon that can be transactivated by the transcription factors PU.1 and Sp1 (32). More recently, lymphoid cells were shown to use a distal promoter region located more than 13 kb upstream of the proximal promoter. The resulting longer transcript includes an additional exon that lacks coding sequence (21). However, the additional exon does contains multiple ATG sites as well as an ARE site and together these elements decrease the translational efficiency of the longer transcript relative to transcripts produced from the proximal promoter (21). The difference in translational efficiency likely explains why monocytes, which preferentially use the proximal promoter, produce more protein than B cells while producing a similar amount of total transcript (21). However, there remain many questions regarding LILRB1 transcription such as how expression is regulated in different lymphoid lineages, why NK and T cells tend to have lower amounts of receptor on the cell surface than B cells, as well as what initiates expression in only subsets of NK and T cells leading to the variegated expression pattern seen in these cell types? In order to understand the differential regulation of LILRB1 within lymphoid cell subsets and between individuals we require a better knowledge of the factors involved in LILRB1 transcription.

In this chapter, I characterize regulatory regions and identified transcription factors involved in LILRB1 transcription, focusing on the distal promoter and expression in NK cells. We identified overlapping AP-1 sites within the core distal promoter that are required for maximal transcription in NK cells. We identified JunD as an AP-1 factor that not only occupies the region in the context of the native promoter, but also enhances activity of this promoter when added exogenously. Additionally, I observed decreased expression of endogenous LILRB1 distal transcript when cellular JunD levels were decreased. Together, our results provide the first insight into the factors involved in transcription from the LILRB1 distal promoter as well as, to our knowledge, the first report of JunD regulating a gene in NK cells.

3.2 Results

3.2.1 Characterization of active regulatory regions in NK cells

We have previously found activity from both the distal and proximal promoter regions using reporter assays in an NK-like cell line, YTS (29, 37). Others have suggested transcription from the distal promoter is more prevalent in NK cells (21). Therefore, I tested the activity of these promoter constructs in a representative monocyte line as well as YTS and the LILRB1 expressing NK line, NKL. We again observed activity of both promoters in YTS cells, as well as in NKL and THP-1 cells (Figure 3.1a). The comparable activity of the two promoter constructs in all cells tested despite reports of lineage specific promoter activity suggests that cell type specific regulation of endogenous promoter usage is likely subject to a level of epigenetic control.

Although I observed transcriptional activity of the proximal promoter in NK cell lines using reporter assays (Fig 3.1a and (37)), there is no evidence to date that transcription from this promoter occurs in NK cells. To test this, I developed a PCR assay for the shorter transcript, using touchdown PCR to provide specificity due to the minimal amount of unique sequence in the short transcript (see Figure 3.1b). As expected, for sorted primary monocytes I observed a substantial amount of product corresponding to the short transcript and the total message, but not the long transcript (Figure 3.1c). Again as expected, I detected the long transcript in purified NK cells, as well as cultured NK cells (Figure 3.1c). Although the true amounts of long and short transcript relative to one another cannot be discerned from this non-quantitative analysis, these results indicate the proximal promoter is used only minimally, if at all, by *ex vivo* NK cells (Figure 3.1c). However, these data do suggest a potential increase in transcription from the proximal promoter under culture conditions.

Given that I detect transcription from the distal promoter in *ex vivo* and cultured NK cells as well as NK cell lines, I wanted to further characterize which regions of this promoter are involved. Figure 3.2a illustrates the mapping of an active region between -14,086 and -13,965 using luciferase assays in YTS cells. This initial mapping was done prior to identification of the transcription start site for the distal promoter transcript of LILRB1 and the realization that sequences in the exon lead to less efficient translation of the transcript (21). In view of this, I also tested luciferase constructs using the transcriptional start as the 3' end of the promoter fragments (Figure 3.2b). In agreement with Lamar et. al (21), these constructs are more active in YTS cells than those with the first portion of exon one present, however, the core

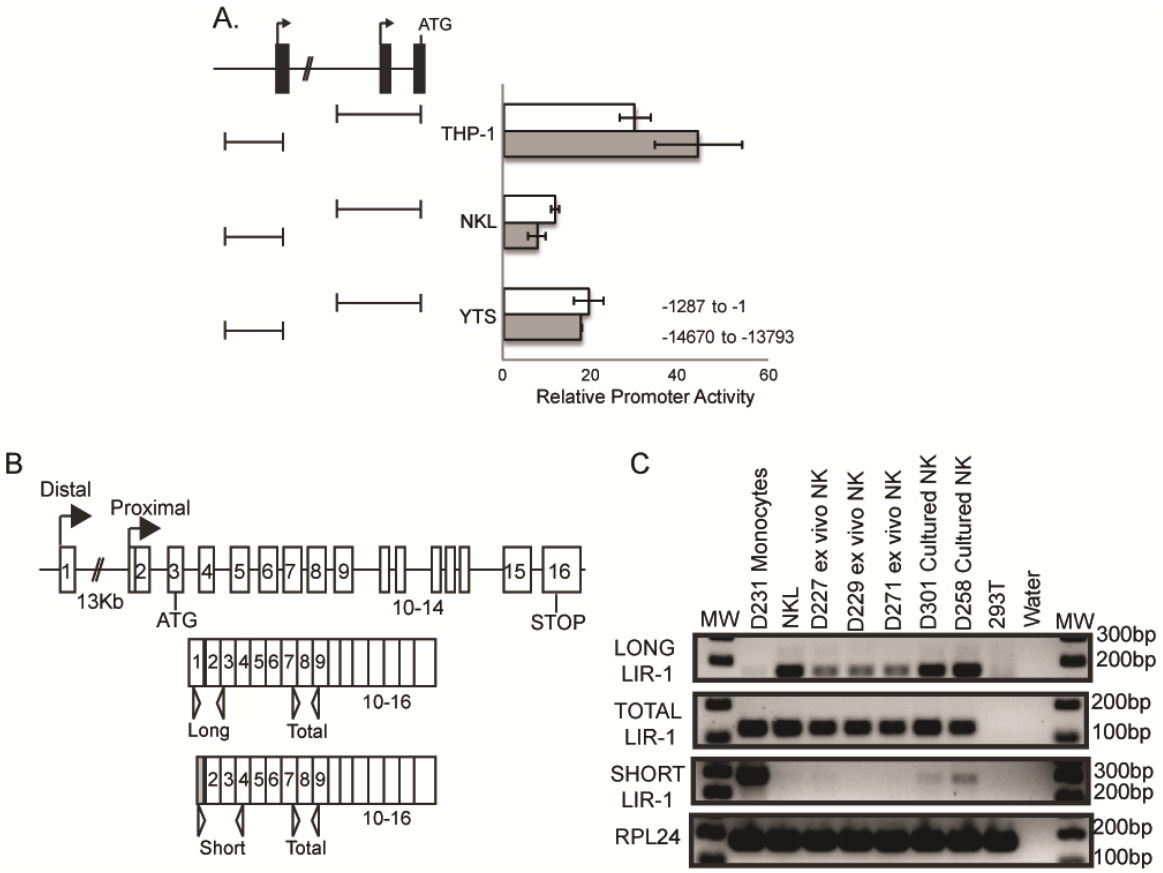


Figure 3.1 Activity of the LILRB1 promoters in NK cells versus monocytes.

A. The arrows indicate the two transcriptional start sites. The relative activities of the two promoter fragments were determined in monocyte (THP-1) and NK (YTS and NKL) cell lines transfected with pGL3 vectors containing the indicated fragments upstream of the firefly luciferase gene. The fragments are numbered relative to the translational start site in exon 3. The average of three or more experiments is shown and the error bars indicate standard error. **B.** The location of the primers used in C are shown for the transcripts generated from the distal (long) and proximal (short) promoter regions as well as primers used to amplify the total message. **C.** RNA was extracted from the cell types indicated above each lane. The transcripts were amplified using a touchdown PCR method as described in the materials and methods and visualized in the gel by ethidium bromide. RPL24 was used as the control for RNA integrity between samples and the 293T cDNA was used as a negative control for amplification of LILRB1.

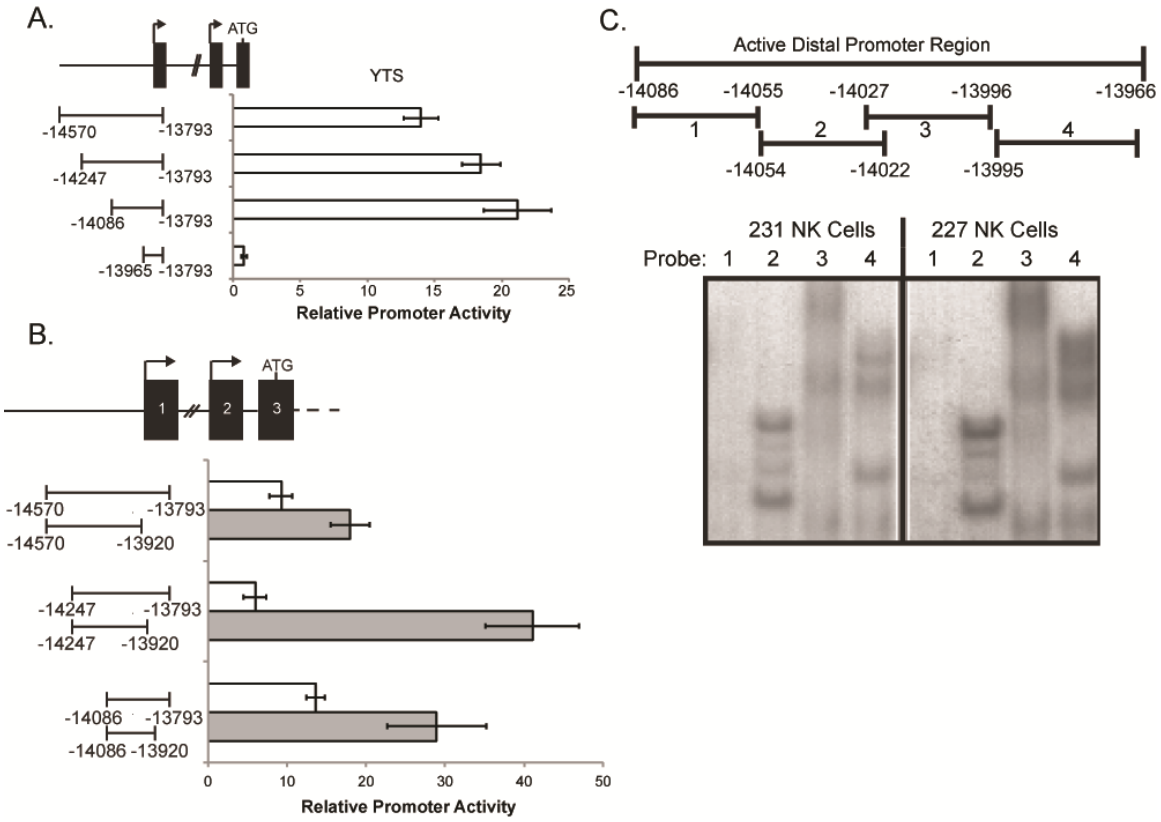


Figure 3.2 Characterization of the distal promoter region.

A and B. Mapping of the core distal promoter. The series of constructs with subregions of the distal promoter inserted into pGL3 were transfected into YTS cells and the luciferase activity measured as described in Materials and Methods. The average of three or more experiments for each construct is shown with error bars representing the standard error. C**.** The schematic at the top illustrates the probes used for analysis of sub-regions of the distal promoter by EMSA. The cell source of the nuclear extracts is indicated above and the number at the top of each lane corresponds to the probe. The NK cell extracts are from *ex vivo* purified cells. The input was normalized for the protein concentration in each extract. The results are representative of NK cells from two separate donors.

region of activity remains between -14086 and -13965 (Figure 3.2b).

To assess the ability of transcription factors to bind to the active region of the distal promoter, I performed EMSA using nuclear extracts from donor NK cells and probes that correspond to ~30bp sections of the 120bp active region (Figure 3.2c). Upon incubation of nuclear proteins from primary NK cells with probe 1, no complexes were evident (Figure 3.2c). Probe 2 produced two distinct complexes in NK cells while probe 3 and 4 produced multiple distinct bands (Figure 3.2c).

3.2.2 A predicted AP-1 site is involved in transcriptional activity of the distal promoter

In the region corresponding to probe 3, there are multiple highly predicted transcription factor binding sites, including several overlapping AP-1/NF-E2 sites and an AML-1 site (Figure 3.3a). Both AP-1 and AML-1 are expressed in lymphoid cells and thus are good candidates for involvement in regulation of transcription in NK cells. Therefore, I tested the effect of competitor oligos corresponding to optimal AP-1/NFE2 and AML-1 binding sites on factor binding to probe 3 by EMSA using nuclear extracts from the NKL cell line as well as primary NK cells. AP-1 and NFE2 have the same consensus binding sequence such that an AP-1 consensus sequence is also an NFE2 consensus sequence. The AP-1 consensus probe completely competed binding of both major species to probe 3, while competition with the AML-1 consensus probe shows only a slight decrease in the binding of factors to probe 3 and this decrease was inconsistent between experiments (Figure 3.3b, left panel). However, the presence of AML-1 in our extracts was confirmed using the AML-1

consensus oligo as a probe and an anti-AML-1 antibody (Figure 3.3b, middle panel). While I were able to see a slight supershifted complex bound by probe 3 in the presence of the AML-1 antibody, mutation of the AML-1 site in probe 3 did not change the pattern of transcription factor binding.

A smaller 20bp probe that contains the AP-1, NFE2 and AML-1 sites retained the ability to produce the complex with higher mobility seen with probe 3 (Figure 3.3c, left panel). The complex produced with the 20bp fragment was competed by a "self" competitor oligomer but not when the AP-1 site was mutated in that oligomer (mutation shown in Figure 3.3a), indicating the importance of this sequence for factor binding (Figure 3.3c, right panel). Also of note, the mutation used to eliminate the predicted AP-1 site also disrupts the less highly predicted MZF-1 and C/EBP sites. Additionally, the AP-1/NFE2 mutated probe did not form any complexes when it was labeled and used as the probe with the same extracts. The supershifted complex observed with the addition of AML-1 antibody is not present when the shorter 20bp probe is used. Together, these results indicate that AP-1 (and/or NFE2), but not AML-1, is likely bound to the probe corresponding to the 20bp region of the distal promoter.

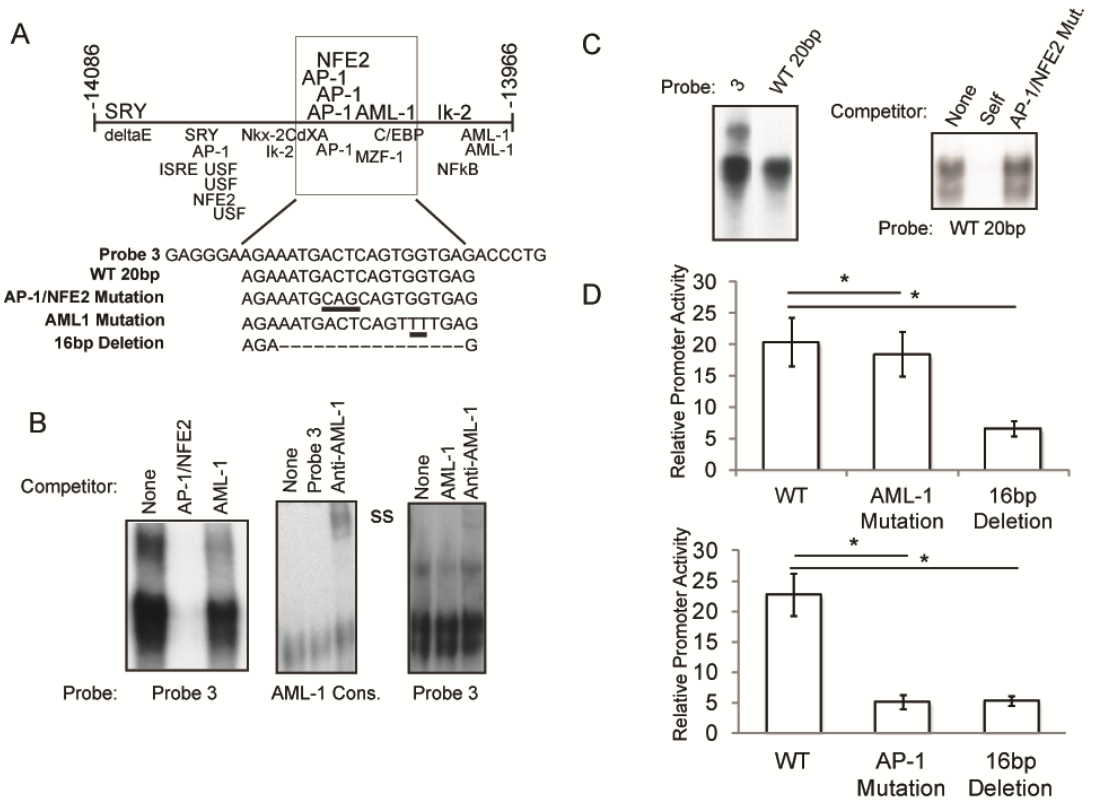


Figure 3.3 Functional analysis of the AP-1/NFE2 site in the distal promoter.

A. Detailed analysis of the sites predicted within the active region of the promoter. Sites predicted with a score of greater than 85 are shown above the line in a larger font and sites with prediction scores between 80 and 85 are shown below. The boxed region indicates an area containing the most highly predicted sites and the corresponding sequence of Probe 3 is indicated below. Shown underneath the Probe 3 sequence is the region corresponding to the 20 base pair probe (WT 20bp) as well as the mutations made in this probe and/or the reporter constructs to negate the predicted AP-1 and AML-1 sites. **B.** The specificity of the Probe 3 interaction is shown with nuclear extracts from NKL cells. The left hand panel shows incubation of the wildtype Probe 3 with NKL nuclear extract alone or with the AP-1 or AML-1 consensus sequences as competitors. The middle panel shows an AML-1 consensus probe with unlabeled probe 3 as a competitor (middle lane) and with an antibody to AML-1 (right lane). The right panel shows probe 3 with the AML-1 consensus probe as a competitor or an antibody against AML-1. Competitors were used at 100x molar excess. Results are representative of 3 experiments. **C.** NKL nuclear extracts were used to compare the pattern of complexes formed with Probe 3 and the 20bp core (left hand panel). The right hand panel shows the complexes formed from primary NK cell nuclear extracts with the WT 20bp probe in the presence of unlabeled competitor oligo or the same region with the AP-1 site mutated. Competitors were used at 100x molar excess. Results are representative of 3 experiments**. D.** The involvement of the predicted AP-1 and AML-1 sites were tested using reporter assays in YTS cells.

The constructs consist of the sequence -14086 to -13793 with the mutations illustrated in A indicated on the X axis. The results are the average of three experiments.

To address the importance of the region containing the predicted AP-1/NFE2 sites for activity of the distal promoter, I generated luciferase constructs of the -14086 to -13793 region with the same mutations of the AP-1/NFE2 and AML-1 sites as were used for EMSA oligomers, as well as a 16bp deletion that removes predicted binding sites for all three factors (see mutations and deletions in Figure 3.3a). While mutation of the AML-1 site reduced promoter activity by ~10%, both deletion of the whole region and point mutation of the AP-1/NFE2 sites led to an ~80% decrease compared to the activity of the wildtype construct (Figure 3.3d). These observations suggest that AP-1 likely mediates activity of the distal promoter, as there are no reports on expression of NFE2 in lymphocytes. The slight decrease in activity observed for the AML-1 mutation is likely due to a contribution of AML-1, as a small amount of complex is shifted by the AML-1 antibody with probe 3 (Figure 3.3b).

Since several AP-1 factors are activated by phosphorylation by JNK, I examined the impact of inhibition of JNK on LILRB1 transcript levels. Treatment of NKL cells with an inhibitor of JNK, SP600125, led to a decrease in LILRB1 transcript as compared to NKL cells treated with DMSO alone (Figure 3.4), supporting our observation that AP-1 factors may regulate LILRB1 transcription from the distal promoter.

3.2.3 Association of AP-1 factors with the distal promoter sequence

The AP-1 family of transcription factors includes cJun, JunB, JunD, cFos, FosB, Fra1 and Fra2, which can form homodimers or heterodimerize with other members of the family. To determine which, if any, of these factors associate with the 20bp distal

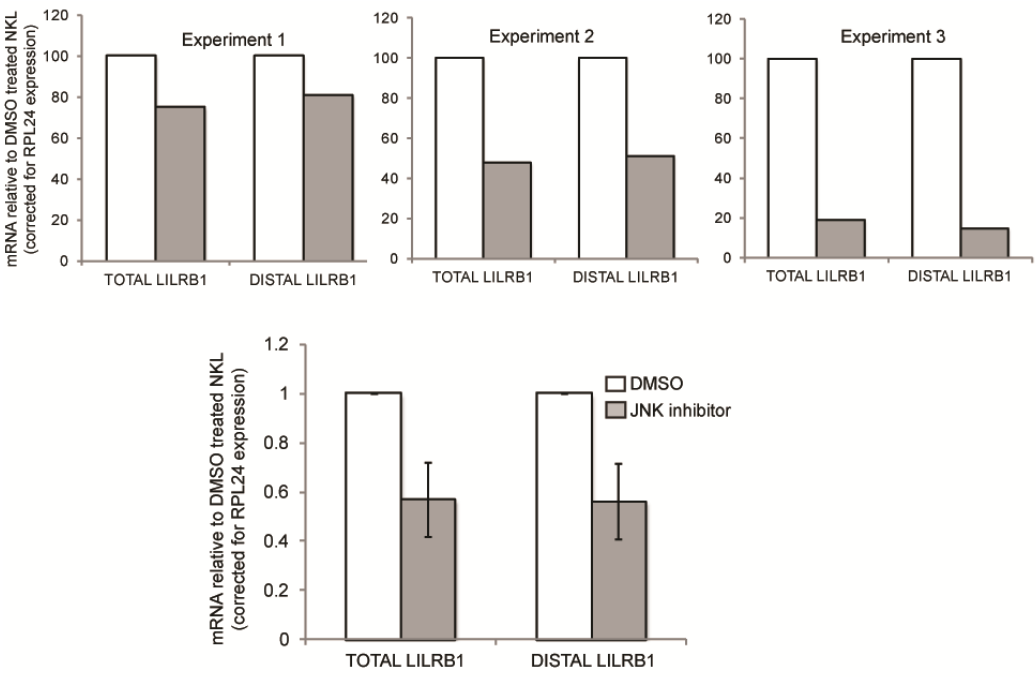


Figure 3.4 Inhibition of JNK leads to a decrease in LILRB1 transcript.

NKL cells were incubated with 25uM concentration of JNK inhibitor (SP600125) for 16 hours before RNA was extracted. Total and distal LILRB1 transcript were measured by quantitative PCR with RPL24 as an internal control. The top three panels are the results for each of the three individual experiments and the bottom is a graph averaging the results of the three.

promoter probe I tested binding by EMSA in the presence of anti-AP-1 antibodies. We observed a supershift of the complexes with four of the five AP-1 antibodies tested, although the relative amounts that shifted varied somewhat for extracts from cultured primary NK cells, NKL and YTS cell lines (Figure 3.5a). Supershift with anti-Fra2 in NKL and cultured NK cells varies from faint to undetectable in different experiments and different donors. Despite using two antibodies against c-Jun, one of which has been validated for ChIP, I observed no c-Jun binding (Figure 3.5). This may be due to a lack of high levels of active c-Jun in NKL cells or could alternatively be due to a problem with the antibodies used. Also of note, the supershift observed in the presence of the JunD antibody was more complete and produced a larger sized complex (Figure 3.5a).

This size difference could potentially be explained by the binding of more than one antibody molecule to the complex if it were composed of a JunD homodimer. The presence of at least three AP-1 family members in the shifted complex indicates there are likely several AP-1 complexes present in NK nuclei able to bind this DNA sequence.

3.2.4 JunD-containing complexes in NK and B cells bind the distal promoter sequence

The presence of JunD in the binding complexes was particularly interesting in that, to our knowledge, there are no previous reports of JunD function in NK cells. To test if JunD is present in resting *ex vivo* NK cells and/or *ex vivo* B cells, I produced nuclear extracts from *ex vivo* NK and B cells isolated from PBMCs. For both extracts, the 20bp probe formed a complex detected by EMSA that unlabeled self-competitor

competed, while a competitor lacking the AP-1 site did not (Figure 3.5b). Addition of an anti-JunD antibody, but not an anti-cJun antibody, produced a supershift of the complex for both the NK and B cell extracts, as well as cultured NK cell extracts (Figure 3.5b and 3.5c). Interestingly, the binding of factors, including JunD, to the promoter sequence appears enhanced with extracts from cultured versus *ex vivo* NK cells from the same donor (Figure 3.5c). These results indicate that JunD is present in the extracts of *ex vivo* and cultured NK cells, as well as B cells, and binds the promoter sequence *in vitro*.

To determine if JunD binds the native LILRB1 promoter, I performed a ChIP assay. We used NKL cells, in which the distal promoter is active (Figure 3.1c). We observed specific amplification of the region surrounding the predicted AP-1 site from the samples immunoprecipitated with anti-JunD compared to the control IgG and relative to primers for a region within intron 1 (Figure 3.6). These results indicate that JunD is bound to the LILRB1 distal promoter in the context of chromatin in cells where it is transcriptionally active.

3.2.5 JunD enhances LILRB1 distal promoter activity

JunD has been shown to have both activating and repressive effects on transcription. While the overlapping AP-1 sites are required for maximal activity of the promoter (see Figure 3.3), implying an activating role for one or more AP-1 factors, this does not indicate the specific activity of JunD. Therefore, I employed transactivation assays using a JunD expression plasmid in conjunction with the distal LILRB1

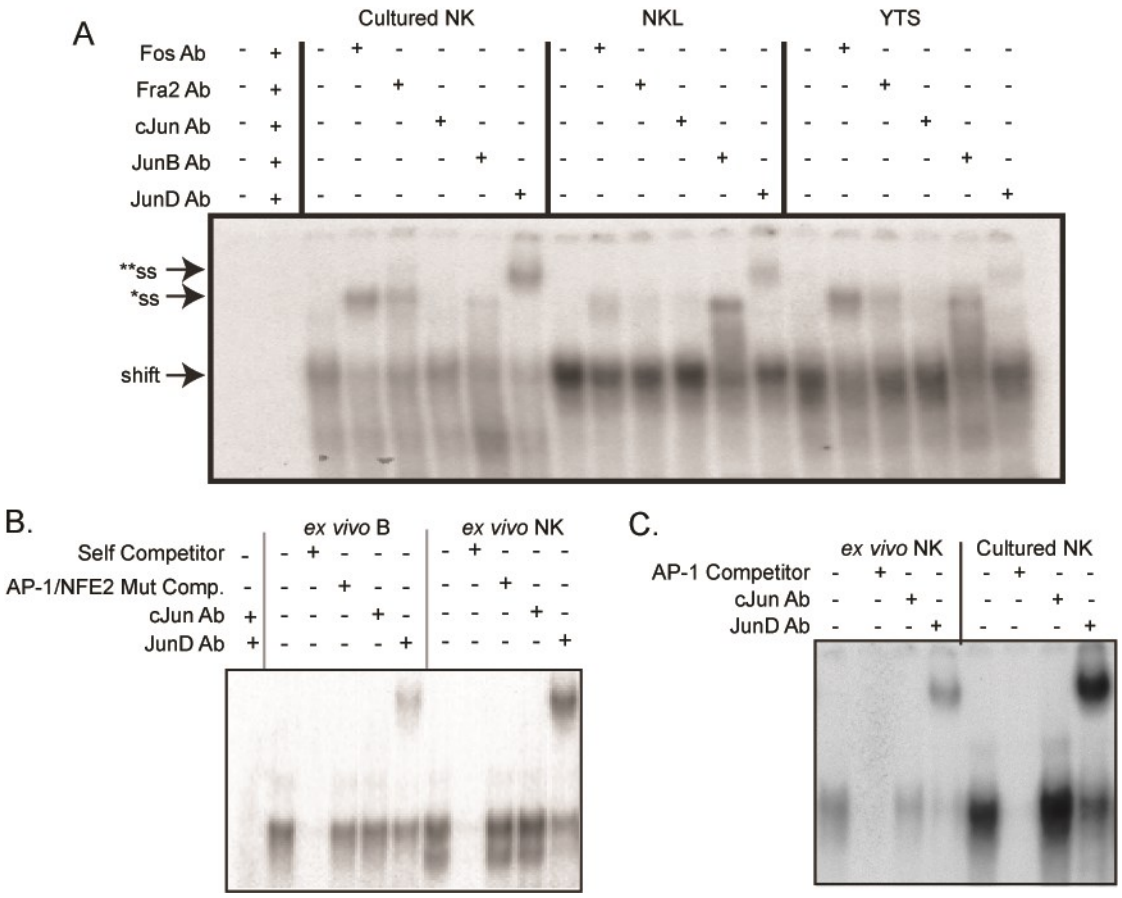


Figure 3.5 The 20bp probe binds multiple AP-1 factors.

A. Nuclear extracts were incubated with the 20bp probe with or without antibodies against specific AP-1 factors as listed on the left. The left hand lane has probe alone and the sample with all 5 antibodies has probe but no extract. The cell source of the nuclear extracts is indicated on the top. The supershifted bands for the various antibodies are indicated with *SS and **SS. The results are representative of two or more experiments done with extracts from different days and/or donors. Note: The Fos Antibody recognizes several Fra and Fos family members **B.** Nuclear extracts from *ex vivo* NK and B cells were incubated with the WT 20bp probe with or without unlabeled competitors and with or without antibodies to cJun or JunD. The results are representative of two experiments, using extracts from different donors. **C.** EMSAs were performed with nuclear extracts with equivalent protein content from *ex vivo* or cultured NK cells incubated with the 20bp probe with or without competitor probe and with or without antibodies against cJun and JunD. The results are representative of 2 experiments using extracts from different donors.

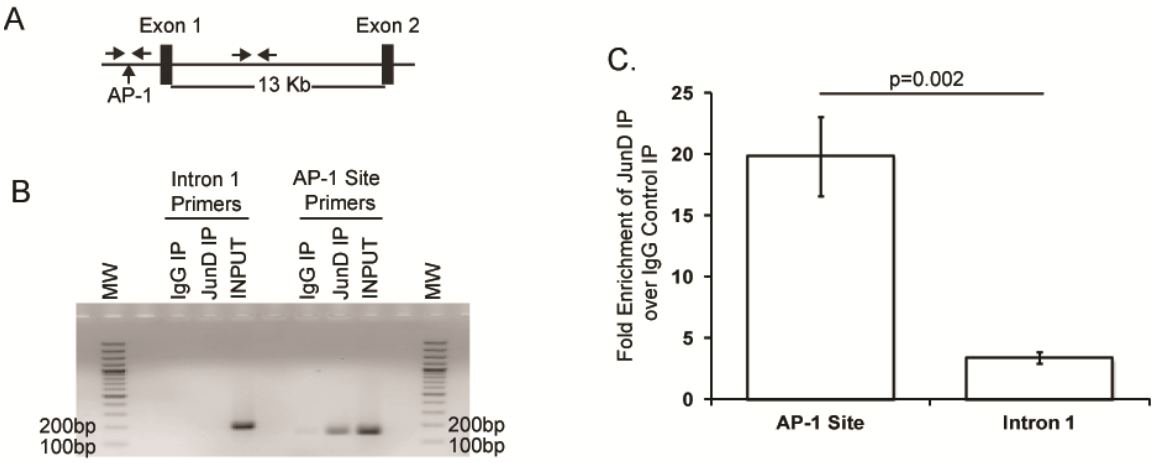


Figure 3.6 JunD association with the LILRB1 promoter.

ChIP was performed as described in the Materials and Methods using NKL cells **A.** Schematic illustrating the location of the primers used to amplify the region surrounding the AP-1 site and a control region in intron 1. **B.** The PCR amplified products are shown for the Input DNA as a positive control for the PCR and for each ChIP sample for the reactions indicated at the top. The bands were visualized with Ethidium Bromide. The results are representative of 4 independent experiments. C. The PCR amplified products are shown quantitatively for each amplified region as fold enrichment of product in the JunD IP versus the IgG control IP. Amplification from input was used as an internal control using the following formula: Fold change $= 2^{\Delta\Delta CT}$ where: $\Delta\Delta CT = \Delta CT_{\text{JunD IP}} - \Delta CT_{\text{IgG control IP}}$ and $\Delta CT = CT_{\text{Input}} - CT_{\text{IP}}$. Results of four experiments are shown with error bars indicating standard error between experiments.

promoter luciferase constructs in both a non-hematopoietic cell line (293T) and an NK cell line (YTS). Activity of the promoter was enhanced by addition of JunD in 293T (Figure 3.7a). In YTS cells, I observed an increase in promoter activity with the addition of JunD in each of the three assays included in Figure 3.7b. However this did not reach statistical significance when all assays were combined despite the trend being maintained within each experiment (empty pcDNA control:pcDNA JunD = 16.7:20.4, 18.5:24.4, and 19.7:36.6). In contrast to the enhanced activity of the wildtype promoter construct, activity of the promoter with the AP-1 site mutated was unaffected or very minimally altered in 293T and YTS, respectively.

In order to test the role of JunD on the endogenous LILRB1 distal promoter, I targeted JunD expression in NKL cells using siRNA. LILRB1 distal promoter transcript was decreased by an average of more than 35% in the presence of JunD siRNA compared to control siRNA (Figure 3.7c). Moreover, the decrease in LILRB1 transcript was well correlated with JunD knockdown efficiency as determined by quantitative PCR (Figure 3.7c).

3.2.6 Unbiased assay for transcription factor binding analysis

There are methods to identify all locations in the genome where a particular transcription factor is bound, but there is not a well-developed method to identify all transcription factors that can bind a particular region of the genome. In order to address this, I developed a mass spectrometry based technique capable of identifying all transcription factors from a particular cellular environment that are able to bind a particular DNA sequence. I used a biotinylated version of the 20bp dsDNA probe

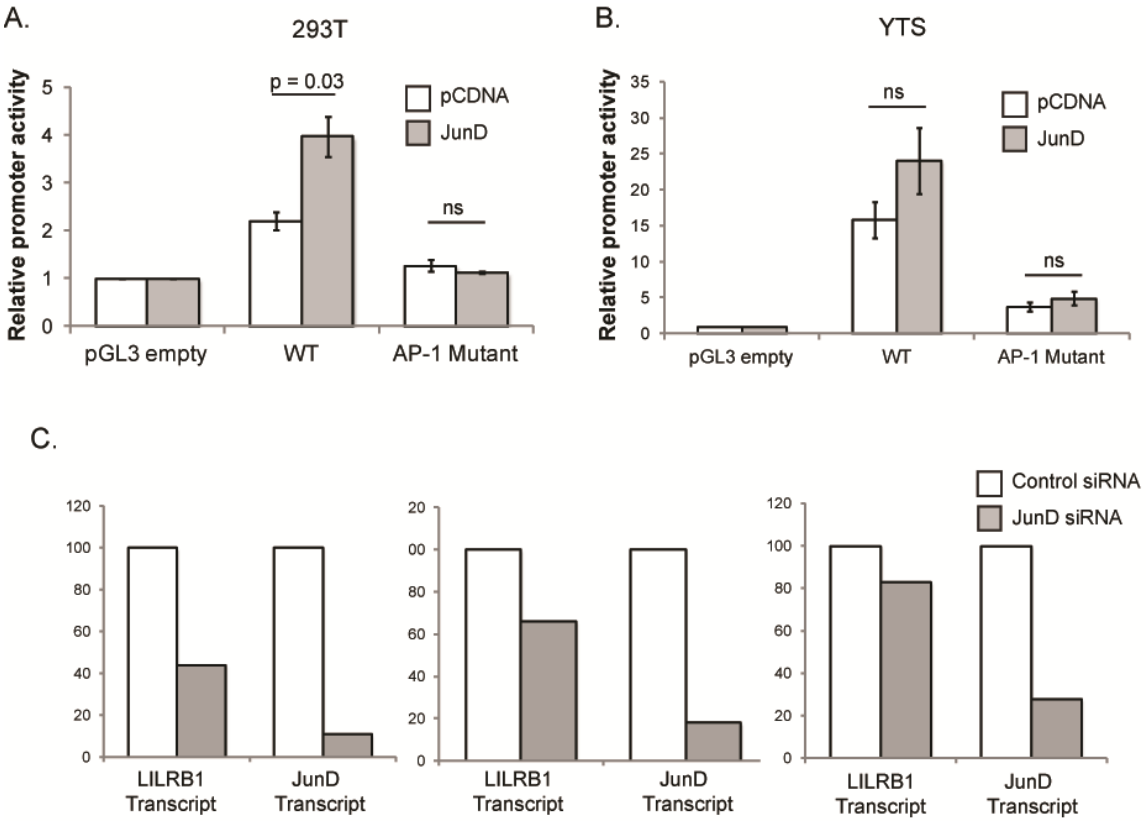


Figure 3.7 JunD enhances the core promoter activity through the predicted AP-1 sites.

The effect of JunD on core distal promoter activity was tested using luciferase reporter assays with the region of the LILRB1 from -14086 to -13793 (WT) and with the AP-1 binding sites mutated (AP-1 Mutant) as in Figure 3.3. These constructs were compared to pGL3 empty as a negative control for background luciferase activity from the vector with no LILRB1 promoter sequence. The cells were transfected also either with pCDNA vector alone or with pCDNA vector expressing JunD. Reporter assays were done in the non-hematopoietic cell line, 293T (**A**) and YTS cells (**B**). **C.** The effect of JunD knockdown was tested using a pool of four siRNAs that target JunD versus a pool of control siRNAs. LILRB1 and JunD transcript was measured by quantitative PCR and normalized to RPL24 transcript. The results from each of three experiments are shown.

used in our EMSAs and containing the confirmed AP-1 binding site. Using this probe mixed with nuclear extract from an NK cell line, I used streptavidin agarose beads to pull down the probe and any bound transcription factors. After optimization to aqcuire a gel with proteins present, I sent the material from one pull-down for analysis by mass spectrometry. I sent bands 2, 3 and 4 for analysis (Figure 3.8, Appendix A2). Among the proteins that were identified, JunB, was identified by mass spectrometry analysis of a band around 30KDa in size, labelled band 3 (Figure 3.8, Appendix A2). This was only a preliminary experiment to determine if the principle behind the assay would work in a setting where transcription factors are already known by EMSA. This assay needs further optimization and trial in order to use it as a method to determine all factors bound to a larger DNA fragment. Upon further optimization, this assay would be useful in determination of differential LILRB1 promoter binding factors between B and NK cells.

3.3 Summary

Here I have shown transcripts arise from the distal LILRB1 promoter in NK cells and B cells, but not monocytes. We delineated a 120 base pair region of the distal promoter that is required for optimal activity in NK cell lines and contains many predicted binding sites for transcription factors. We also found that the region binds several factors expressed by NK cells. The identity and relative importance of most of these factors for expression remains to be determined. Mutation of the predicted AP-1 sites reduced activity by 80%, indicating this site is highly important for the activity of the region. On the other hand, mutation of a predicted AML-1 site had

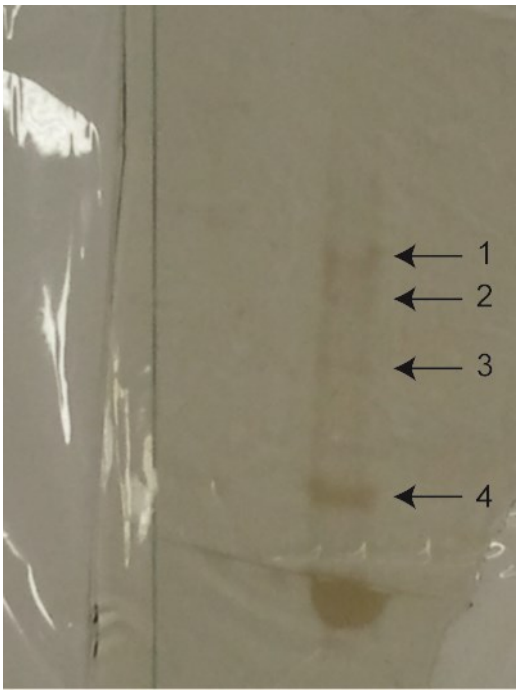


Figure 3.8 DNA binding proteins pulled down by 20bp AP-1 binding site.

Proteins within the nuclei of NKL cells that are able to bind a 20bp DNA sequence, including the AP-1 binding site, were collected by pull down with a biotinylated DNA probe. The pulled down proteins were run on an acrylamide gel and detected with silver staining. The four bands sent for mass spectrometry analysis are indicated by arrows labeled one through four. See appendix A2 for detailed results of the mass spectrometry analysis.

only a modest effect. While I found evidence for only a slight AML-1 association with the sequence, several members of the AP-1 family of transcription factors did form complexes with the 20bp probe containing the predicted AP-1 site. Together these results suggest AP-1 is a major family of transcription factors involved in the activity of the core region of the distal promoter of LILRB1.

Interestingly, JunD appears as a major component of the bound AP-1 complexes from NK and B cells as most of the complexes are shifted with anti-JunD antibodies. Additionally, I found JunD associated with the endogenous distal promoter in LILRB1+ NKL cells. Moreover, ectopic expression of JunD enhanced activity of the core promoter, while reduction of JunD levels led to a decrease in LILRB1 transcript from the endogenous promoter, suggesting that JunD-containing complexes stimulate, rather than repress, transcription.

In conclusion, I have characterized some of the DNA elements required for LILRB1 transcription in NK cells. I determined that the family of AP-1 transcription factors plays an activating role in transcription from the distal promoter and that the atypical AP-1 family member JunD is involved in this activation. In addition to cytokines, stimulation of NK cells through the natural cytotoxicity receptors also regulates expression of AP-1 family members (97) suggesting the net effects on a gene such as LILRB1 would be influenced by integration of many pathways during NK cell activation. Therefore, while questions remain such as whether other AP-1 family members are involved in transcription of LILRB1 and what events are involved in initiation of LILRB1 expression from the distal promoter in NK cells, these studies

suggest a mechanism for how LILRB1 expression can be dynamically modulated in lymphocytes through the MAPK/AP-1 pathway.

CHAPTER 4: NK-specific regulation of LILRB1 and the role of SNPs in expression in NK cells

4.1 Background

Having characterized the distal promoter in the previous chapter, in this chapter I return to my initial questions: What is involved in NK specific regulation of LILRB1 and how do the SNPs impact this lineage specific expression pattern? LILRB1 has an interesting pattern of expression between different immune cell lineages. In Peripheral Blood Mononuclear Cells (PBMCs), LILRB1 is expressed on all monocytes and B cells, and subsets of T and NK cells. However, the amount of cell surface receptor detected per cell is much higher on monocytes than B cells (21). LILRB1 expression on T cells is associated with terminally differentiated memory effector cells, being highly expressed within the CD56+ T cell subset (98). LILRB1 expression on NK cells varies between individuals in both its detection per cell and the number of cells that stain positive (9, 10, 29, 37, 55, 99). In all likelihood, expression of LILRB1 in NK cells is regulated in a manner somewhat similar to the variegated expression of KIRs (30), as not all NK clones express LILRB1 and we recently reported a correlation of LILRB1 genotype with expression on NK cells (29). However, Nick Li in our lab has also found some changes in the expression on NK and T cells over time in normal healthy subjects (37) and perturbations in LILRB1 expression profiles on NK and T cells have been reported in several situations including post transplant HCMV disease, HIV infection and first trimester pregnancy (38-41). In addition, Nick also observed that IL-2 and/or IL-15 cause some enhancement of surface expression of LILRB1 on NK and T cells *in vitro* as well as a potential role for IL-2 in regulating promoter activity in one NK-like cell line (37).

Expression of LILRB1 on NK cells differs from expression on B cells and monocytes. Monocytes transcribe LILRB1 from the proximal promoter, which is more translationally efficient (21). Lymphocytes primarily use the distal promoter, which produces a transcript that stutters during translation, making it less efficient in producing LILRB1 protein (21). This describes an inherent difference in regulatory mechanisms between NK cells and monocytes. The difference in regulation of LILRB1 expression between NK and B cells is less apparent, despite their expression patterns being distinct from one another. As previously described, there is little variability in expression of LILRB1 on B cells regardless of donor genotype. However, LILRB1 expression on NK cells varies greatly between people and correlates with genotype of the proximal promoter and 5' end of the LILRB1 gene. LILRB1 expression on NK cells varies between donors in a genotype-correlated manner. The NK cell population can be between 20 – 80% positive for LILRB1 surface expression, depending on the donor. Both B cells and monocytes, however, have relatively uniform expression between donors. LILRB1 is generally expressed on all monocytes and B cells. The NK-specific role for genotype indicates that different regulatory regions or mechanisms are at play in NK cells versus the other two lineages.

Our previous genotype-phenotype correlation study included only the proximal promoter and the region of the gene downstream from it. To fully understand the role of genotype in NK expression, I expanded the sequencing study to include the distal promoter and exon 1. I addressed several hypotheses relating to NK specific LILRB1 expression in this chapter. I first aimed to determine whether there were SNPs and

possibly major haplotypes in the distal promoter and exon one region. Using this analysis, I also aimed to determine the degree of linkage disequilibrium between SNPs within the distal and proximal promoter. Due to the 13Kb distance in a highly variable region of the genome, I anticipated a low level of linkage disequilibrium with any SNPs I identified in the distal promoter and those in the proximal promoter that are correlated with expression on NK cells. Second, I hypothesized that B and NK cells use different regions and factors to activate transcription from the distal promoter. This would be an explanation for the different expression patterns. In conjunction with this hypothesis, I predicted differences in DNA methylation patterns of LILRB1 in NK cells versus other LILRB1+ cell lineages and potentially between the two major haplotypes mentioned previously when examined in NK cells. I also tested the possibility that SNPs within the distal promoter have an impact on promoter activity in NK cells since this is the primary promoter used by NK cells to express LILRB1. Finally, I tested the hypothesis that the proximal promoter plays a role in expression of LILRB1 in NK cells.

4.2 Results

4.2.1 Differential transcription factor binding in B and NK cells

There is likely a role for cellular environment and availability of transcription factors in determining lineage specific LILRB1 expression patterns. Both NK cells and B cells primarily use the distal promoter (see Figure 3.1 and references (20, 21)). I hypothesized that the role for genotype in NK expression of LILRB1 and not in B cell expression may be due in part to a difference in transcription factor availability to drive expression at the distal promoter. B cells may express a transcription factor that

acts as a strong activator and is lacking in NK cells. This would allow for consistent expression, which would mask any variability between donors caused by differential factor binding elsewhere due to SNPs. To address this hypothesis, I used EMSAs to analyze potential distal promoter binding factors from both B and NK cell nuclear environments. The DNA sequence I chose to examine is the core 126bp distal promoter from -14086 to -13966, relative to the translational start site. As indicated in chapter 3, this region does not contain any SNPs in any of our donors although there are SNPs located outside of this core promoter region that will be discussed in the following section. This 126bp core was split into four approximately 30bp dsDNA probes for use in EMSAs (Figure 4.1B). Figure 3.2C shows the transcription factor binding profile for these four probes using two donors' NK cells where all factors are darker and more apparent than shown here. The binding profile in NK cells is very faint as compared to B cells, however, this is due to the B cells saturating and overpowering the NK cell results.

Transcription factors that are either specific to the B cell nuclear environment or appear enhanced in the B cell nuclear environment are indicated with red arrows in Figure 4.1c. The band found to contain AP-1 factors in chapter three is indicated with a dashed box around it and also appears darker in B cells, though this assay is not quantitative so exact amounts cannot be conclusively determined (Figure 4.1c). These results do not confirm that these factors bind *in vivo* but they do indicate that the nuclear environments differ between B and NK cells in terms of factors able to bind this region and this may, at least partially, explain the difference in NK and B

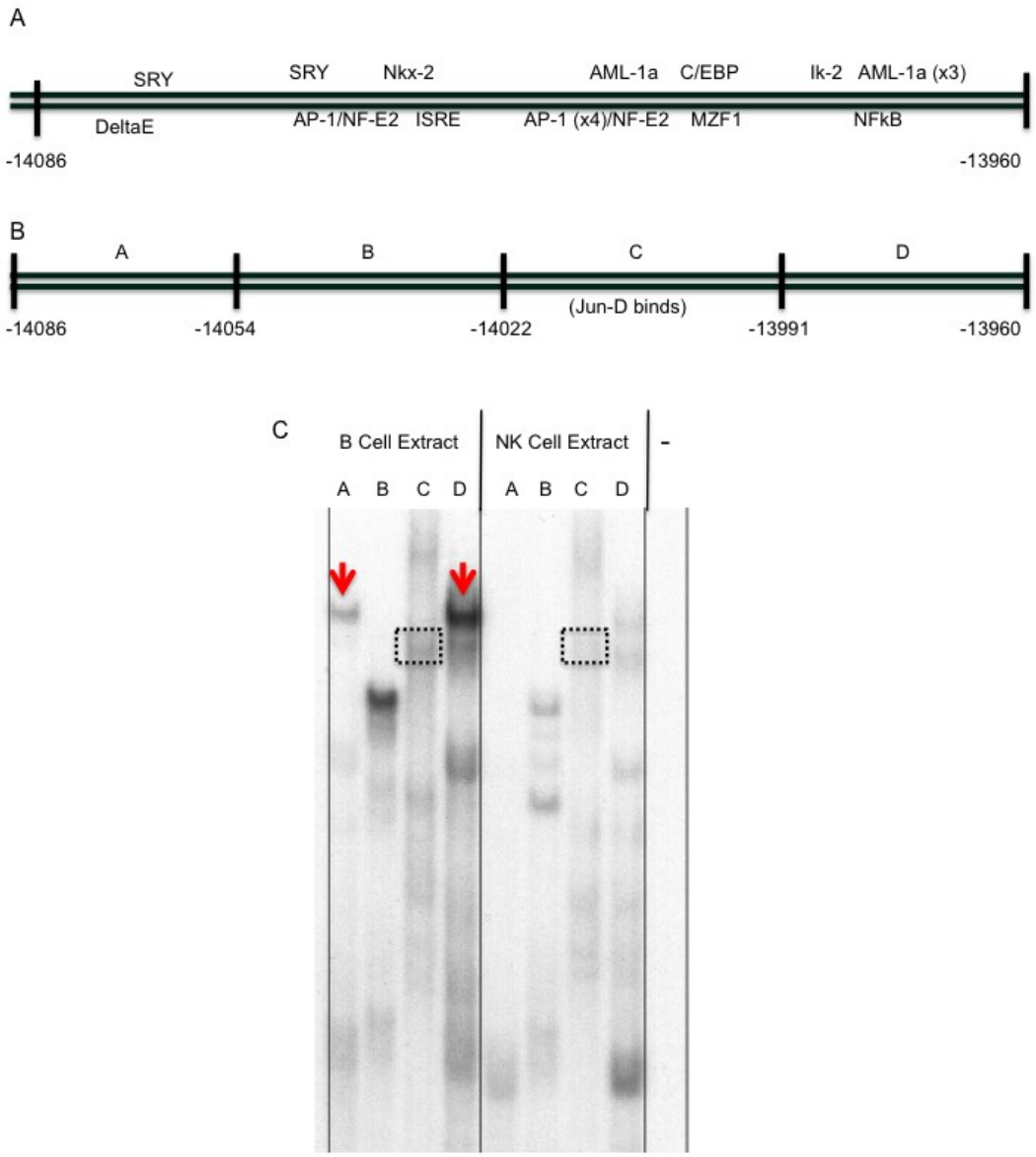


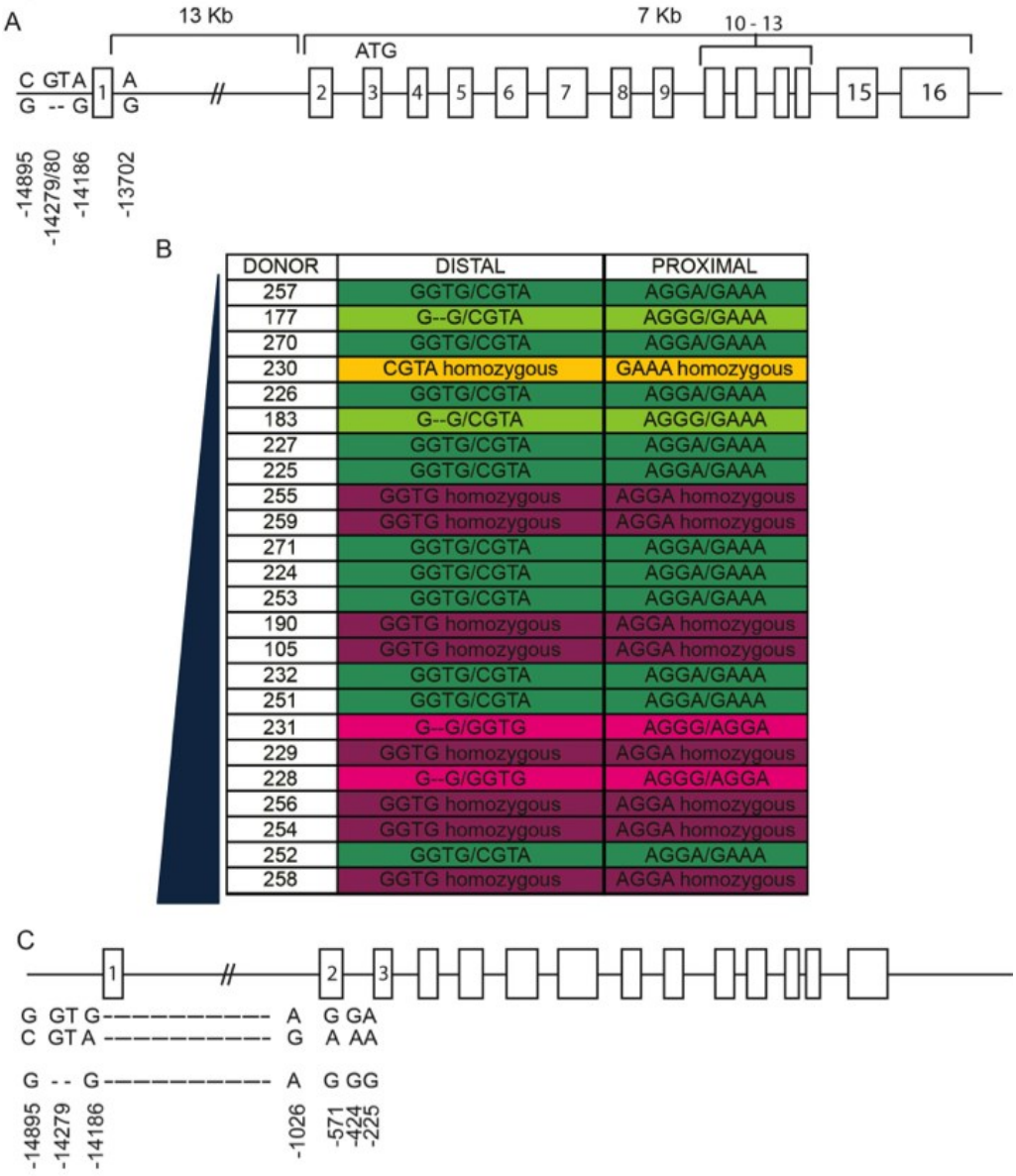
Figure 4.1 Transcription factor binding profiles of the core distal promoter differ in B versus NK cells.

We made four dsDNA probes representative of the core distal promoter and used them in EMSA assays with primary NK or B cell nuclear extracts. A) Predicted transcription factor binding sites (Using TFSearch online program) and B) location of the four dsDNA probes is shown. C) B cell and NK cell nuclear extracts from the same donor are compared. Lineage specific bound factors are indicated with arrows. The band known to contain JunD and other AP-1 factors is indicated by a dashed box around it. This is representative of results from two different donors.

cell expression of LILRB1. To determine the identity of the B cell specific factors bound to probes A and D, one can in future use similar methods as those employed in chapter 3 to identify AP-1 and specifically JunD. DeltaE, also known as YY1, is the predicted factor within probe A and NFkB, Ik-2 and AML-1 are predicted to bind probe D, so these would be the logical factors to begin with in the investigation. However, as I was focused on NK specific regulation, I did not further study these potential B cell factors. I instead employed another strategy to study the differences in NK and B cell regulation of LILRB1. I determined here that NK and B cells use different regions of the distal promoter, which led me to focus again on SNPs but to examine any SNPs present in the distal promoter as well.

4.2.2 Several SNPs in the distal and proximal promoters of LILRB1 are in strong linkage disequilibrium

Our previous work indicates a correlation between expression on NK cells and a haplotype of the region spanning the proximal promoter through exon 3 of LILRB1 (29). Since publishing this work, I and others found that LILRB1 has a second promoter 13Kb upstream of the proximal promoter that is responsible for the majority of LILRB1 transcription in lymphocytes (20-22). I therefore expanded our previous sequencing project to include a 1.8Kb region surrounding the distal promoter in the same 25 donors analyzed for genotype and phenotype in the previous study. I found 5 SNPs within this region spanning the distal promoter and exon 1 (Figure 4.2a). Four of the five consecutive SNPs form two major haplotypes in this region that appear to be in perfect linkage disequilibrium with the two major proximal promoter



DONOR	DISTAL	PROXIMAL
257	GGTG/CGTA	AGGA/GAAA
177	G--G/CGTA	AGGG/GAAA
270	GGTG/CGTA	AGGA/GAAA
230	CGTA homozygous	GAAA homozygous
226	GGTG/CGTA	AGGA/GAAA
183	G--G/CGTA	AGGG/GAAA
227	GGTG/CGTA	AGGA/GAAA
225	GGTG/CGTA	AGGA/GAAA
255	GGTG homozygous	AGGA homozygous
259	GGTG homozygous	AGGA homozygous
271	GGTG/CGTA	AGGA/GAAA
224	GGTG/CGTA	AGGA/GAAA
253	GGTG/CGTA	AGGA/GAAA
190	GGTG homozygous	AGGA homozygous
105	GGTG homozygous	AGGA homozygous
232	GGTG/CGTA	AGGA/GAAA
251	GGTG/CGTA	AGGA/GAAA
231	G--G/GGTG	AGGG/AGGA
229	GGTG homozygous	AGGA homozygous
228	G--G/GGTG	AGGG/AGGA
256	GGTG homozygous	AGGA homozygous
254	GGTG homozygous	AGGA homozygous
252	GGTG/CGTA	AGGA/GAAA
258	GGTG homozygous	AGGA homozygous

Figure 4.2 The distal and proximal promoters of LILRB1 are in linkage disequilibrium.

A) The 5 SNPs identified in sequencing of the distal promoter region are indicated and numbered relative to the translational start site B) Distal and proximal promoter regions were sequenced in 24 donors who were phenotyped for LILRB1 expression on NK cells. Each genotype is indicated by a different colour in the table. Donors are listed from low to high expression frequency of LILRB1 on NK cells, indicated by the triangle to the left of the table. C) Each of the regions, distal and proximal, has two major haplotypes and one minor/rare haplotype, made up of four SNPs each, with positions indicated.

haplotypes in this sample set (Figure 4.2b). The low or "GAAA" haplotype of the proximal promoter is linked with the "CGTA" haplotype of the distal promoter (Figure 4.2c). The high or "AGGA" proximal promoter haplotype is expressed with the "GGTG" haplotype of the distal promoter (Figure 4.2c). Additionally, the rare "AGGG" proximal promoter haplotype is paired with the "G- - G" haplotype of the distal promoter (Figure 4.2c). Therefore, the correlation between NK cell expression of LILRB1 and genotype of the proximal promoter extends to SNPs in the distal promoter as well. This is of interest because it means that the primary promoter used by NK cells is involved in the genotype-phenotype correlation as well. There is a fair bit of variation within haplotypes between the proximal promoter and the 3' end of the gene (a 7 Kb distance), which led us to predict that there would be variation from the proximal promoter to the distal promoter (a 13Kb distance). Therefore, it was a surprising discovery and differs from what I had anticipated for these two regions of the gene.

4.2.3 LILRB1 DNA methylation patterns differ in NK cells versus B cells and monocytes

In this section, I examine the possibility that there may be differences in epigenetic modifications of the LILRB1 gene between lineages.. Differences in DNA methylation at specific CpG sites may indicate differential roles for those sites between the lineages. Methylation itself can block binding of a transcription factor. Additionally, methylated DNA can indicate a "closed" genomic region. This likely indicates a region is not critical for binding of regulatory factors. In this section, I

examine the possibility that LILRB1 is differentially methylated between NK cells, monocytes and B cells.

There are no true CpG islands within the LILRB1 gene according to prediction software, however, there are regions with higher CpG content than the rest of the gene. I designed primers for four regions of the LILRB1 gene for the methylation analysis (Figure 4.3). Three of the four regions were chosen due to their relatively high CpG content compared to the rest of the gene. However, the distal region was chosen despite having a normal CpG content because it is the promoter used by NK cells. Unfortunately, PCR amplification of the region spanning intron 5 through intron 6, which has the highest CpG content was inconsistent so there is no data on this region at this time. For the remaining three regions, I used bisulfite conversion, PCR amplification and cloning and sequencing of products to determine the methylation state of the different CpG sites in each region. I analyzed at least 10 clones from each cell lineage for each region from each donor. I then used this sequencing data to calculate a "percent methylated" score for each CpG site.

To examine primary monocytes, B cells and NK cells, I immunomagnetically isolated them by positive, positive and negative selection, respectively. This allowed me to isolate all three lineages from a single blood donor. The subsets were tested by flow cytometry to determine the level of enrichment of CD14+, CD19+ and CD56+ cells (Figure 4.4). The positive selection antibody for CD19 partially blocks binding of the antibody used to test B cell purity by CD19, likely leading to under-representation of enrichment in this subset. One caveat is that the CD56+ subset does not exclude

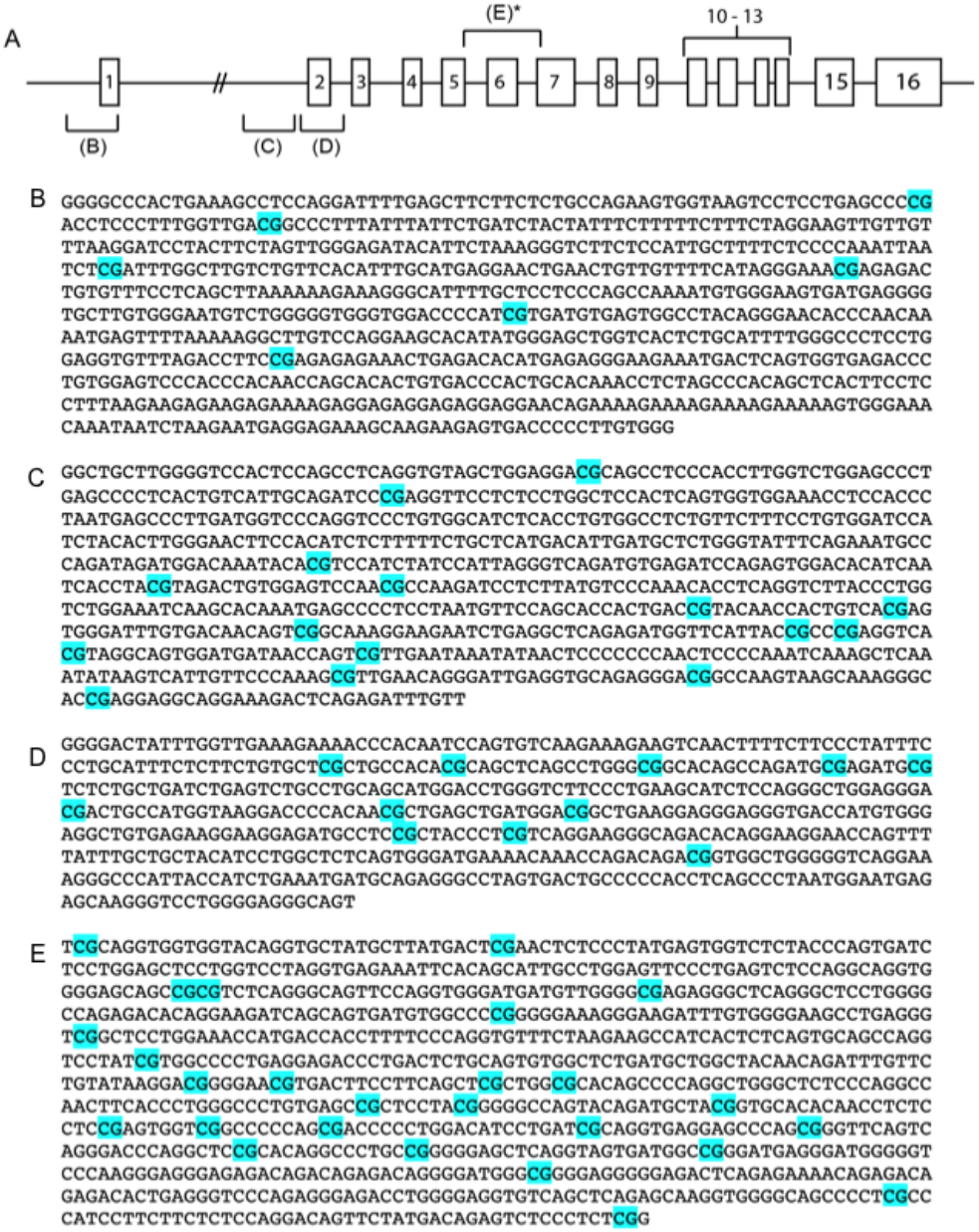


Figure 4.3 CpG sites within three regulatory regions of the LILRB1 gene.

A) A schematic of the LILRB1 gene with the three regions examined for DNA methylation indicated. The distal (B), proximal (C) exon 2 (D) and exon 6 (E) region sequences are shown with CpG sites highlighted.

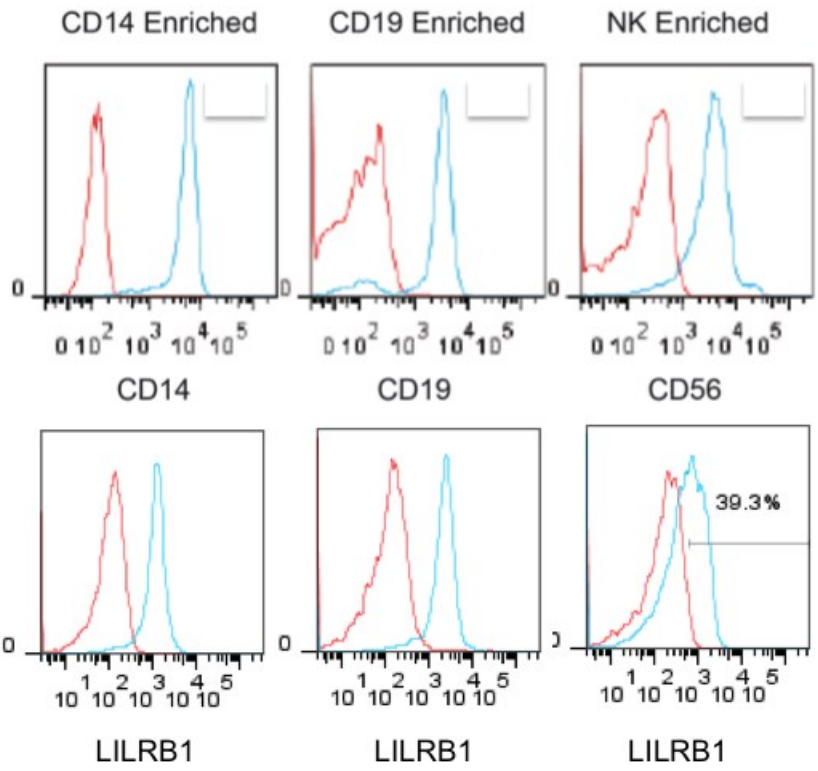


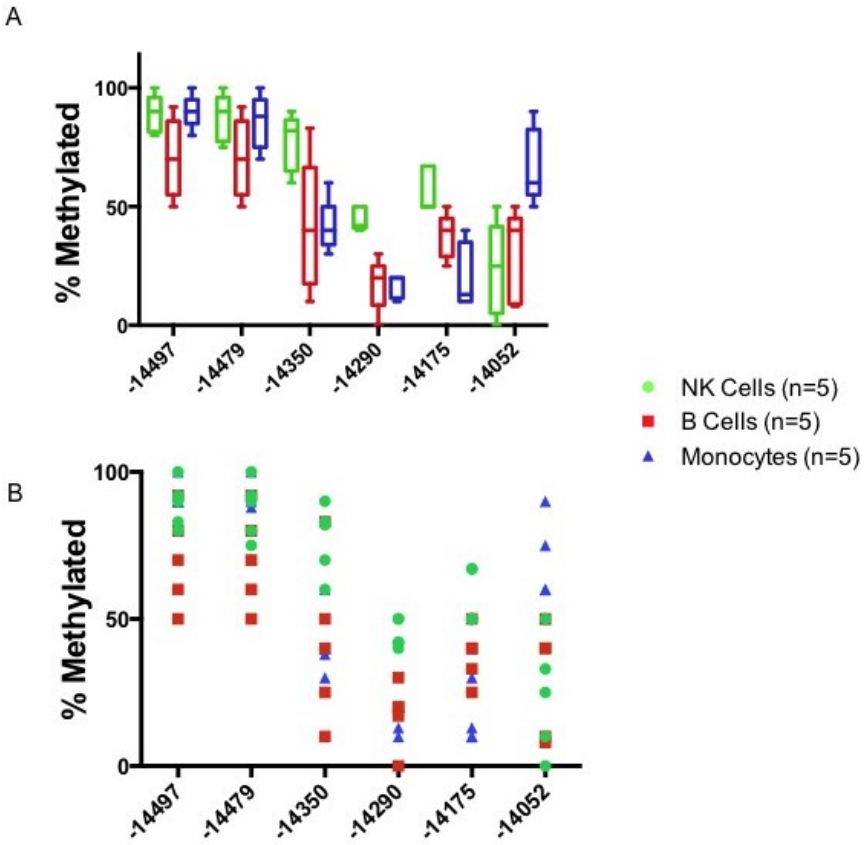
Figure 4.4 A representative flow cytometric analysis of primary monocytes, B cells, and NK cells.

From PBMCs, CD14 and CD19 positive cells were sequentially isolated by immunomagnetic positive selection. NK cells were enriched from the remaining cells using immunomagnetic negative selection. A sample of each of the three cell populations was used to stain for a marker of the cell type, CD14, CD19 and CD56, respectively, as well as LILRB1. The remaining cells were used to isolate genomic DNA for methylation analysis. The flow cytometry plots here are from a representative donor, donor 313. The y-axis represents cell count.

contaminating CD56+ T cells, however, the kit used does select against T cells so these should be minimal. I isolated all three cell types from 5 independent donors.

In all three regions examined, there are significant differences in methylation at some of the CpG sites between NK cells and the other two lineages (Figures 4.5, 4.6, 4.7). In the distal region, all three lineages are highly methylated in the 5' end of the area examined (Figure 4.5). In the middle at positions -14350 and -14290, NK cells are more highly methylated than the other two lineages (Figure 4.5). Interestingly, it is only at the most 3' CpG site, -14052, that I see B and NK cells with a more unmethylated state than monocytes (Figure 4.5). This makes sense with data from the previous chapter mapping the core distal promoter activity to a region spanning -14086 to -13966 (Figure 3.2). The -14052 CpG site is the only one that is within this core and is the one with the expected result as far as methylation in the two lineages that use the distal promoter, B and NK cells, versus that which does not, monocytes.

In the proximal region, there are three CpG sites, -907, -895 and -802, where NK cells are more methylated than the other two lineages (Figure 4.6). While there are single CpG positions where NK cells differ in methylation than the other lineages, this region is not active as a promoter in resting NK cells so it is unclear what the implications of these differences are at this point. In the exon 2 region, all three lineages have little methylation (Figure 4.7) and much less than in the other two regions I examined. In particular, in monocytes, this region is almost completely unmethylated in all five donors (Figure 4.7). From the 5' end until -524, NK cells are

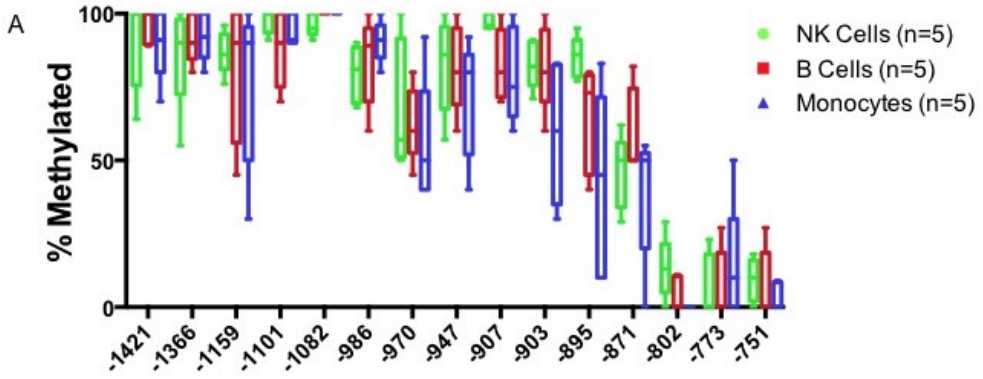


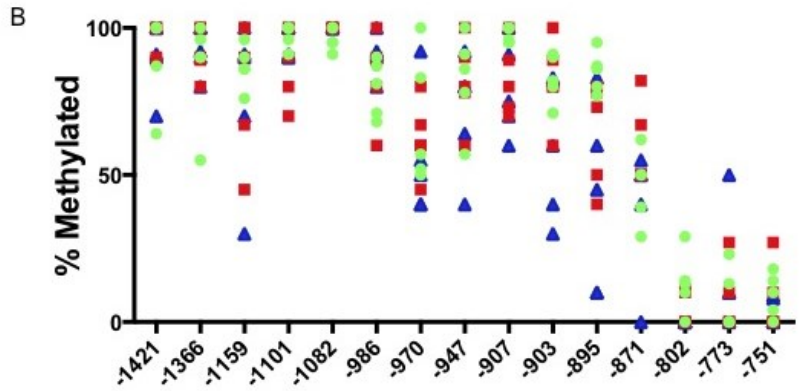
C

	-14497	-14479	-14350	-14290	-14175	-14052
Monocytes	0.84	0.8	0.001	0.00001	0.001	0.005
B cells	0.05	0.08	0.03	0.0009	0.01	0.63

Figure 4.5 Lineage specific DNA methylation patterns of the distal regulatory region of LILRB1.

Genomic DNA was isolated from populations enriched for monocytes, B cells and NK cells from five unique, healthy donors. Percent methylation was calculated at each CpG site by analyzing 10 or more cloned PCR fragments. A and B) Percent methylation is shown at each CpG site for each lineage. C) At each CpG site, a t test was used to determine whether the methylation in NK cells differs from methylation in either monocytes or B cells, as indicated in the table of p values. CpG sites with statistically significant differences in methylation as compared to methylation at that site in NK cells are indicated in yellow in the table. The x-axis lists the position of the CpG, relative to the translational start site of the gene.



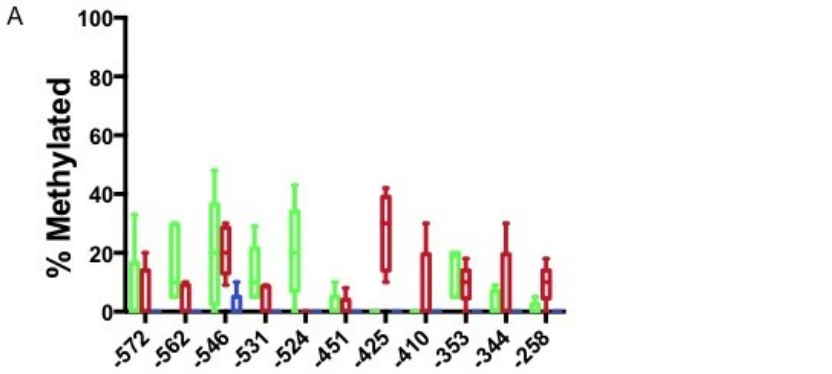


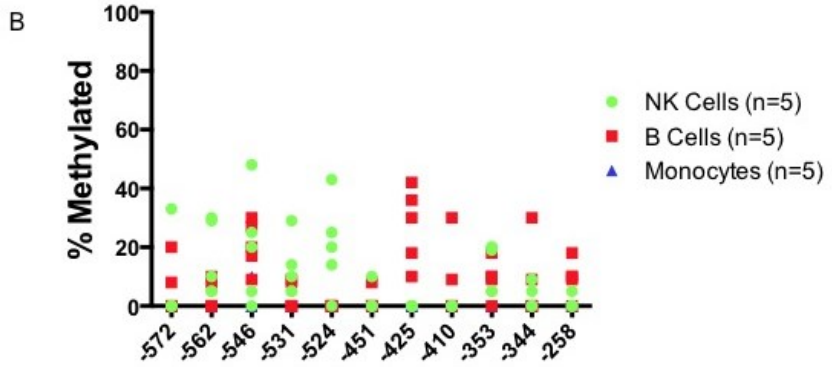
C

	-1421	-1366	-1159	-1101	-1082	-986	-970	-947	-907	-903	-895	-871	-802	-773	-751
Monocytes	1	0.5	0.44	0.31	0.06	0.07	0.37	0.36	0.04	0.07	0.02	0.56	0.02	0.53	0.17
B cells	0.48	0.55	0.58	0.16	0.06	0.6	0.61	0.94	0.03	0.9	0.04	0.14	0.13	0.98	0.78

Figure 4.6 Lineage specific DNA methylation patterns of the proximal regulatory region of LILRB1.

Genomic DNA was isolated from populations enriched for monocytes, B cells and NK cells from five unique, healthy donors. Percent methylation was calculated at each CpG site by analyzing 10 or more cloned PCR fragments. A and B) Percent methylation is shown at each CpG site for each lineage. C) At each CpG site, a t test was used to determine whether the methylation in NK cells differs from methylation in either monocytes or B cells, as indicated in the table of p values. CpG sites with statistically significant differences in methylation as compared to methylation at that site in NK cells are indicated in yellow in the table. The x-axis lists the position of the CpG, relative to the translational start site of the gene.





C

	-572	-562	-546	-531	-524	-451	-425	-410	-353	-344	-258
Monocytes	0.347	0.024	0.078	0.022	0.02	0.347	1	1	0.005	0.164	0.347
B cells	0.9	0.08	0.917	0.097	0.02	0.88	0.002	0.217	0.366	0.436	0.024

Figure 4.7 Lineage specific DNA methylation patterns of the exon 2 region of LILRB1.

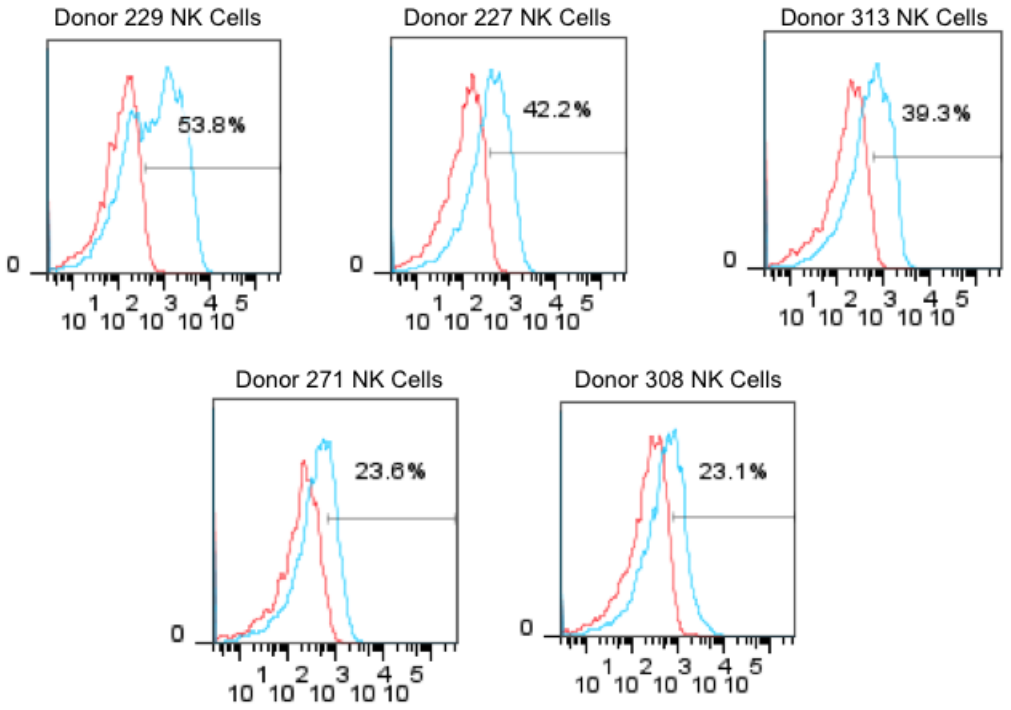
Genomic DNA was isolated from populations enriched for monocytes, B cells and NK cells from five unique, healthy donors. Percent methylation was calculated at each CpG site by analyzing 10 or more cloned PCR fragments. A and B) Percent methylation is shown at each CpG site for each lineage. C) At each CpG site, a t test was used to determine whether the methylation in NK cells differs from methylation in either monocytes or B cells, as indicated in the table of p values. CpG sites with statistically significant differences in methylation as compared to methylation at that site in NK cells are indicated in yellow in the table. The x-axis lists the position of the CpG, relative to the translational start site of the gene.

more methylated than monocytes and in most cases, B cells as well (Figure 4.7). The differences in the region are of interest because this is the portion of the proximal promoter that is required for activity in NK cells. This methylation data will be further analyzed with the data on potential roles for the proximal promoter in NK cells (to follow in section 4.2.7). This indicates that there are differences in epigenetic modifications of LILRB1 between NK cells and the two consistently LILRB1-positive cell lineages, B cells and monocytes. This may indicate a differential regulatory role for these specific regions in the cell lineages.

4.2.4 Possible difference in DNA methylation between the two major LILRB1 haplotypes in NK cells

A possible role for the SNPs implicated in regulating expression on LILRB1 is that the haplotypes may have differential DNA methylation, potentially by differences in CpG content. I was able to further analyze the previously described methylation data to examine the possibility that the two major haplotypes are differentially methylated in NK cells. The NK cells of five donors used for the methylation analysis were assessed for LILRB1 expression by flow cytometry. The NK cells from the five donors have various LILRB1 levels, as anticipated (Figure 4.8). I was able to determine the haplotype of each clone due to the presence of one or more haplotype representative SNPs in each region. This made it possible for me to examine the four heterozygous donors' low and high haplotypes separately to determine if the two are differentially methylated.

A



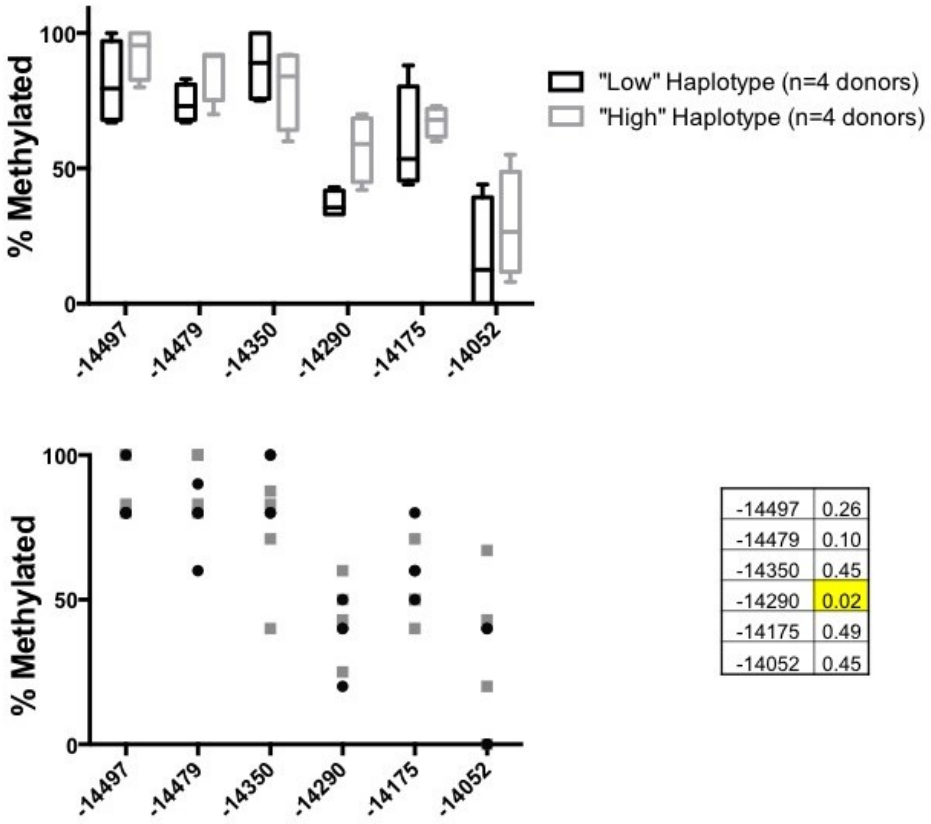
B

	Genotype	NK cell MFI Ratio	NK cell %LILRB1+
Donor 308	Heterozygous	2.22	23.1
Donor 271	Heterozygous	2.73	23.6
Donor 313	Heterozygous	3.37	39.3
Donor 227	Heterozygous	3.54	42.2
Donor 229	Homozygous High	6.8	53.8

Figure 4.8 LILRB1 expression profile on NK cells from the five donors included in the methylation analyses.

A) LILRB1 expression on NK cells with percent LILRB1 positive indicated. The red line is the isotype control while the blue is the LILRB1 antibody. The y-axis represents cell count. B) The Mean Fluorescence Intensity (MFI) ratio of LILRB1 expression and percent LILRB1 positive values are shown for each donors' NK cells.

In the distal region, the high haplotype is more methylated at position -14290 (Figure 4.9). This holds true with the data from all four donors combined, as well as within each individual donor when high and low haplotype clones are compared (Figure 4.10). This may indicate a regulatory role for the region directly surrounding this site. This is not a difference that would be detectable in luciferase assays as the LILRB1 gene's cellular methylation state is not reflected in this assay as the plasmids will all by equally undermethylated. There are multiple predicted transcription factor binding sites that are inclusive of this CpG site, including NFAT-1, Stat-4 and c-Ets. The binding ability of some transcription factors is impacted by the DNA methylation state in the region they bind. One of these factors could potentially have a repressive role and this is dampened by increase methylation in the high haplotype. Also of interest, AP-1 can dimerize with both Ets and NFAT, potentially introducing a mechanism for how this regulatory region may impact transcription from the distal promoter. In the proximal promoter region, there is a difference in methylation between the two haplotypes at position -907 that reaches significance by t test, however, the methylation is very high on both (Figure 4.11). The four individual donors are shown in order to demonstrate donor to donor variability and compare the two haplotypes from the same donor (Figure 4.12). In the exon 2 region, while there is no significant difference at any position, it could be due to the high degree of variability between donors and the relatively low number of donors examined (Figure 4.13). In the 5' end of the region, in the data from four donors combined as well as individual donors, it is clear that the high haplotype is more methylated (Figure 4.13 and 4.14). This increased methylation is of interest because it is in the region



-14497	0.26
-14479	0.10
-14350	0.45
-14290	0.02
-14175	0.49
-14052	0.45

Figure 4.9 DNA Methylation patterns on low versus high haplotype of the distal promoter on NK cells.

At least 20 clones were analyzed for each donor to calculate percent methylation at each CpG position. The haplotype of each clone was determined to group them into low and high haplotype. The table indicates the p values for each CpG site as determined by t tests. n = 4 donors. The x-axis lists the position of the CpG, relative to the translational start site of the gene.

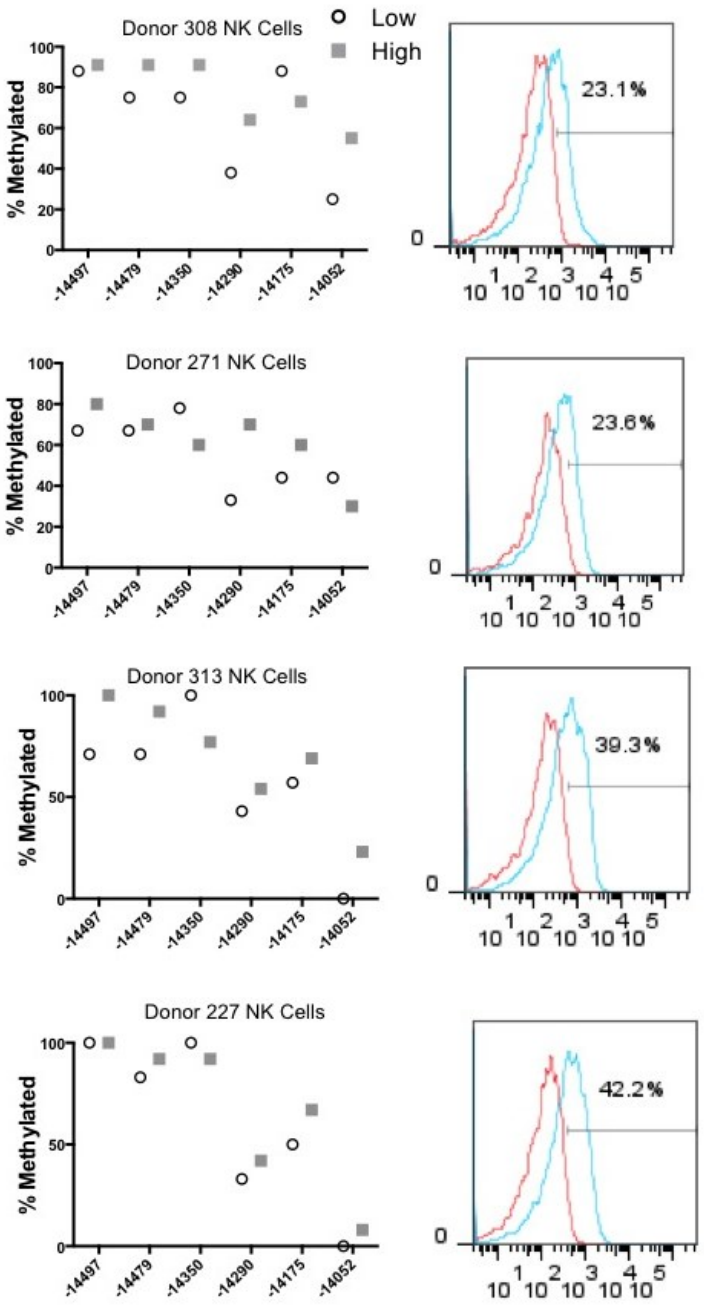
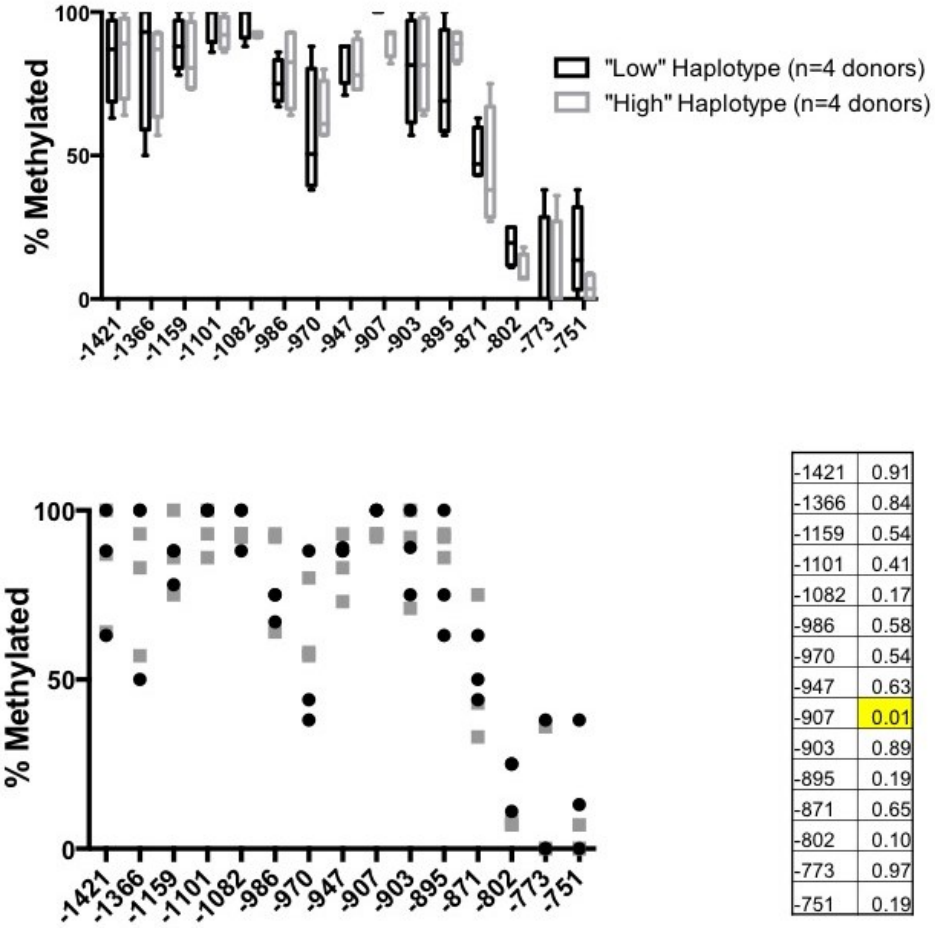


Figure 4.10 DNA Methylation patterns on low versus high haplotype of the distal promoter on NK cells from individual heterozygous donors.

The percent methylation at each CpG site and LILRB1 expression on NK cells is shown for each donor by flow cytometry with the plots to the right. Red is the isotype control and blue is staining with an antibody against LILRB1. . The x-axis lists the position of the CpG, relative to the translational start site of the gene.



-1421	0.91
-1366	0.84
-1159	0.54
-1101	0.41
-1082	0.17
-986	0.58
-970	0.54
-947	0.63
-907	0.01
-903	0.89
-895	0.19
-871	0.65
-802	0.10
-773	0.97
-751	0.19

Figure 4.11 DNA Methylation patterns on low versus high haplotype of the proximal promoter on NK cells.

At least 20 clones were analyzed for each donor to calculate percent methylation at each CpG position. The haplotype of each clone was determined to group them into low and high haplotype. The table indicates the p values for each CpG site as determined by t tests. n = 4 donors. The x-axis lists the position of the CpG, relative to the translational start site of the gene.

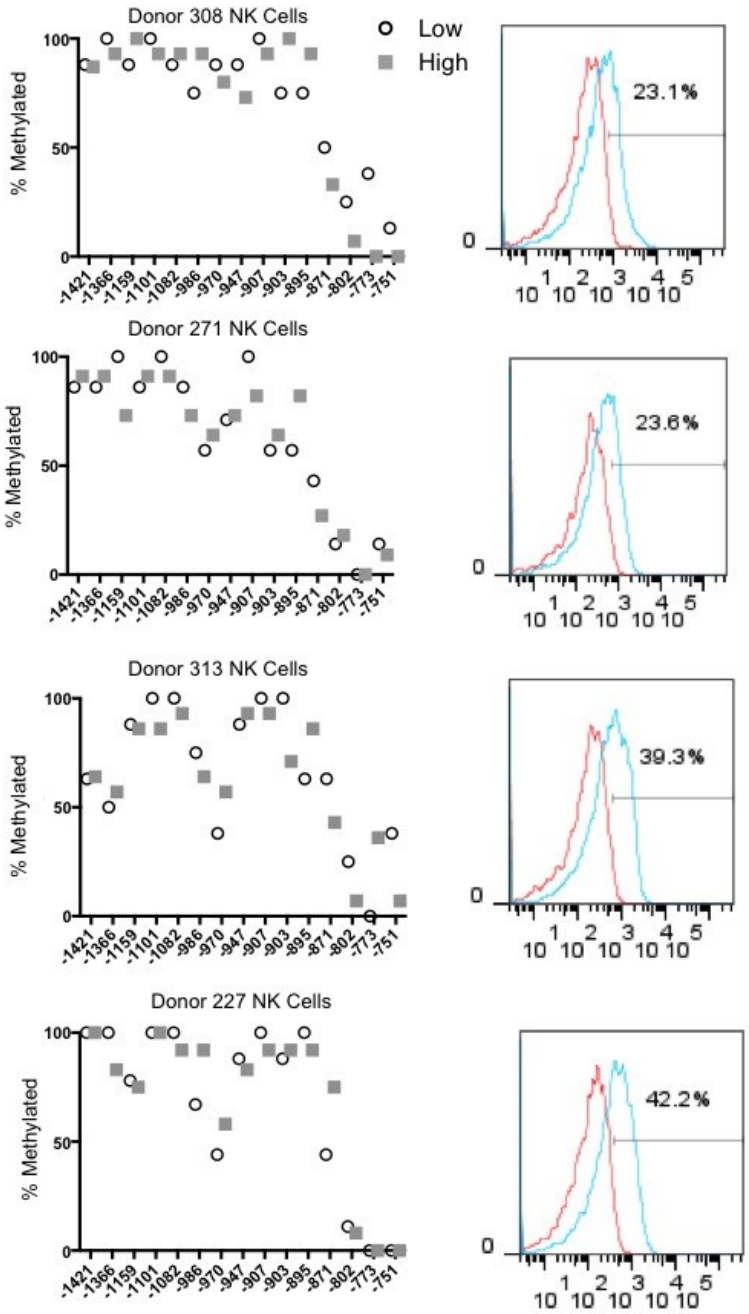
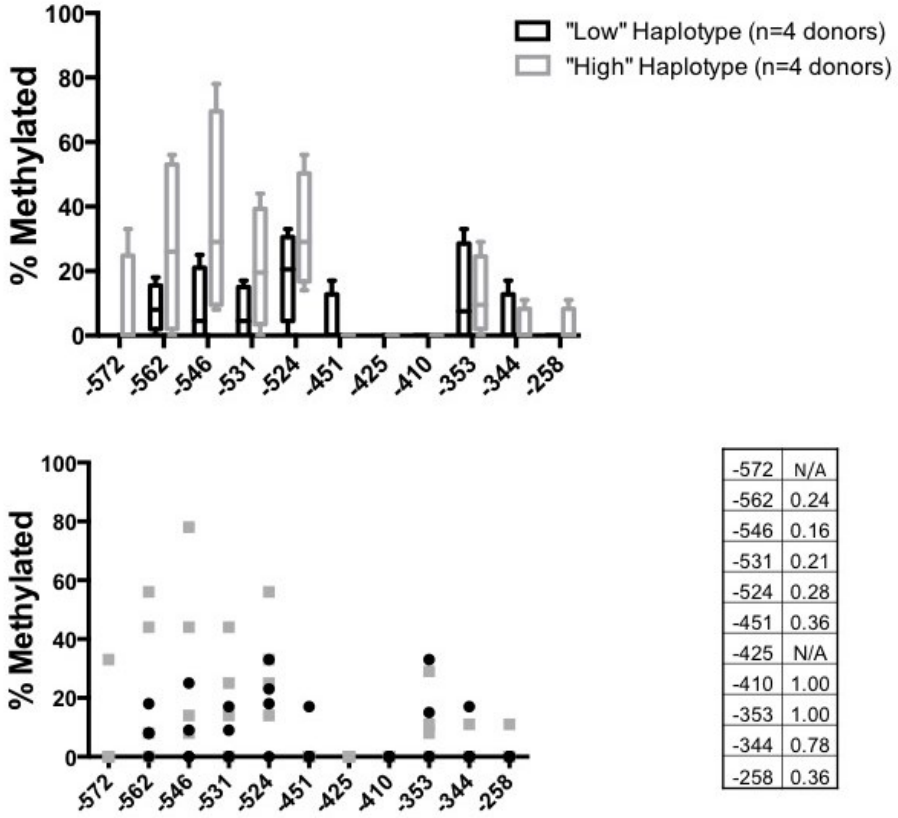


Figure 4.12 DNA Methylation patterns on low versus high haplotype of the proximal promoter on NK cells from individual heterozygous donors.

The percent methylation at each CpG site and LILRB1 expression on NK cells is shown for each donor by flow cytometry with the plots to the right. Red is the isotype control and blue is staining with an antibody against LILRB1. The x-axis lists the position of the CpG, relative to the translational start site of the gene.



-572	N/A
-562	0.24
-546	0.16
-531	0.21
-524	0.28
-451	0.36
-425	N/A
-410	1.00
-353	1.00
-344	0.78
-258	0.36

Figure 4.13 DNA Methylation patterns on low versus high haplotype of the exon 2 region on NK cells.

At least 20 clones were analyzed for each donor to calculate percent methylation at each CpG position. The haplotype of each clone was determined to group them into low and high haplotype. The table indicates the p values for each CpG site as determined by t tests. n = 4 donors. The x-axis lists the position of the CpG, relative to the translational start site of the gene.

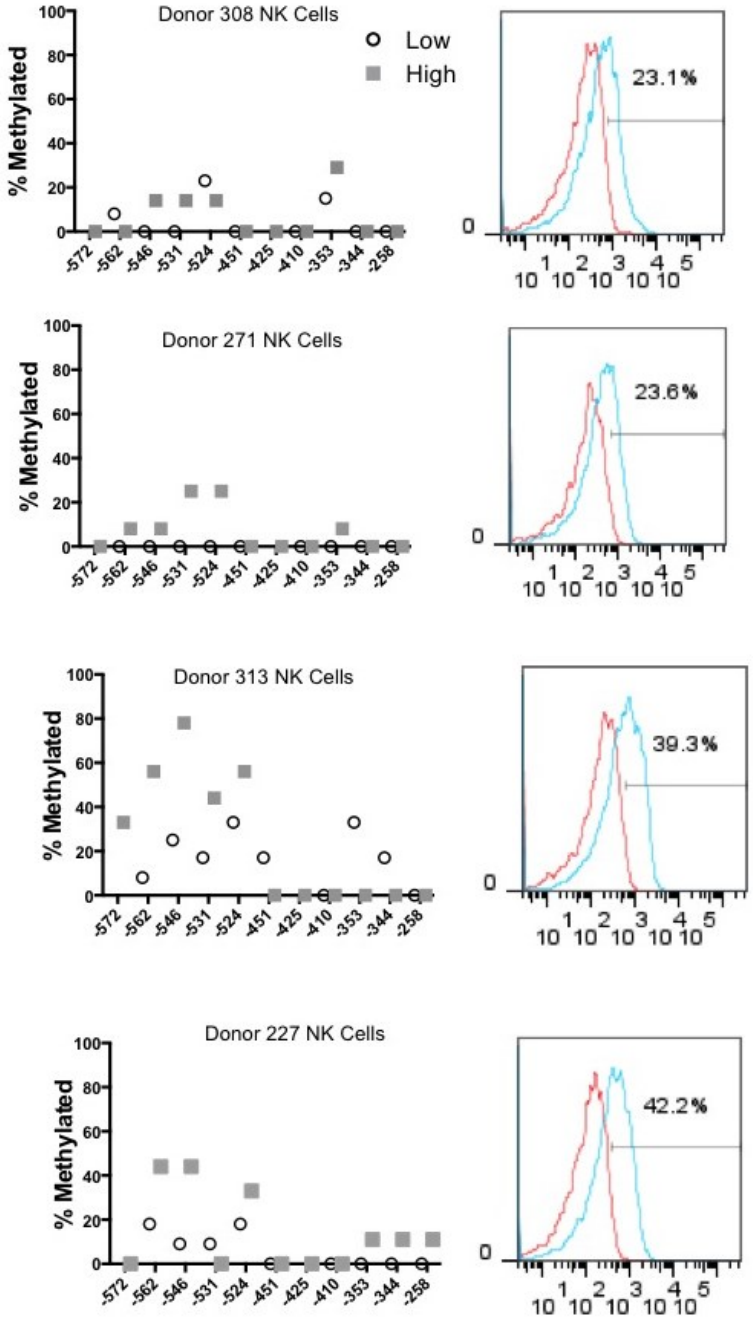


Figure 4.14 DNA Methylation patterns on low versus high haplotype of the exon 2 region on NK cells from individual heterozygous donors.

The percent methylation at each CpG site and LILRB1 expression on NK cells is shown for each donor by flow cytometry plots to the right. Red is the isotype control and blue is staining with an antibody against LILRB1. The x-axis lists the position of the CpG, relative to the translational start site of the gene.

required for proximal promoter activity in NK cells. This will be further discussed in section 4.2.7. Note that positions -572 and -425 do not have data points for the "low" haplotype because these CpG sites are absent in the low haplotype due to G to A nucleotide changes (Figure 4.13 and 4.14).

4.2.5 Investigation of the influence of the SNPs in LILRB1 expression using reporter assays

The most straightforward explanation for a correlation between LILRB1 expression and genotype is that one or more SNPs alter the DNA sequence in a transcription factor binding site, altering the ability of that factor to bind with the same efficiency. To examine this possibility, I created luciferase constructs containing each major haplotype of each of the two promoters to test the transcriptional activity of each in an NK cell line, NK92. The two major haplotypes of the distal promoter active in NK cells have the same activity as one another (Figure 4.15a). The "low/GAAA" associated proximal promoter haplotype, however, shows 27% lower transcriptional activity than the "AGGA" proximal promoter haplotype (Figure 4.15b). This indicates that one or more of the SNPs in this region influence promoter activity. This result was not what I expected. The distal promoter has a clear role in expression on LILRB1 in NK cells and I therefore predicted the SNPs would have an influence on the activity of this promoter and not the proximal promoter. This data indicates that SNP(s) within the proximal promoter likely influence factor binding, and thus, promoter activity. This discovery emphasizes the importance of studies to determine the role of the proximal promoter in NK cells. We have not completely uncovered its

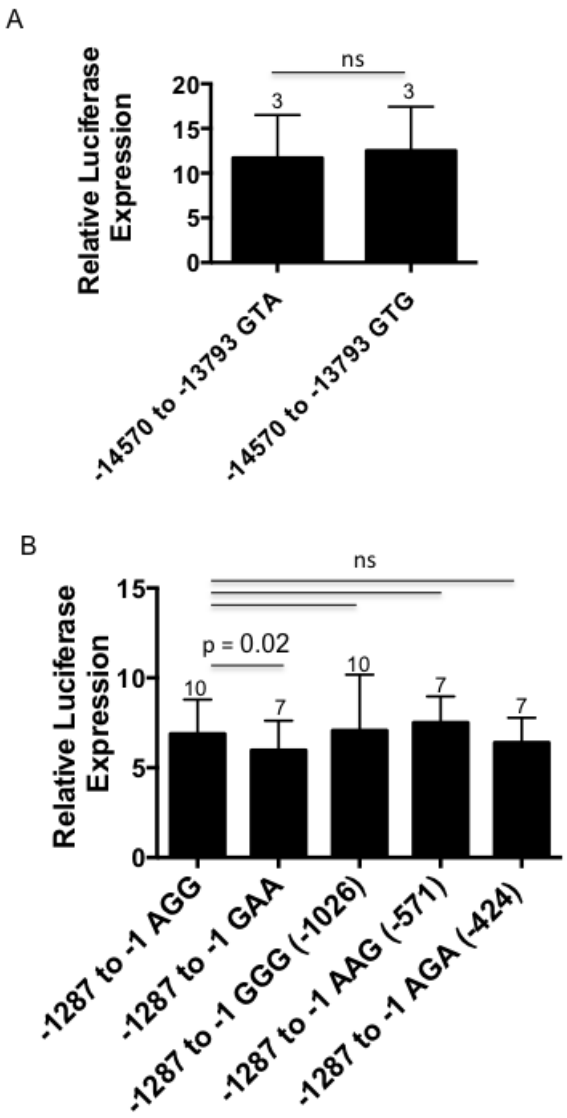


Figure 4.15 The two major proximal promoter haplotypes have different promoter activity

A) The two major distal promoter haplotypes have no difference in promoter activity when tested by luciferase assay in an NK cell line, NK92. B) The two major proximal promoter haplotypes have a significant difference when tested by luciferase assay in an NK cell line, NK92. Individual point mutations of the AGGA haplotype to the GAAA haplotype show no significant difference from the AGGA haplotype. The number of experimental points for each is indicated above the error bar. The bar indicates the average for each with the error bar showing the standard deviation.

role, however, its activity under activating conditions (see Figure 3.1C) and it producing a translationally more efficient transcript introduces a new question. When the proximal promoter is active, is the "high" proximal promoter haplotype more active under activating conditions than the "low" haplotype? This is a question that should be addressed in future experiments. I made luciferase constructs with the individual SNPs but was unable to detect an effect of a single one of the SNPs on their own (Figure 4.15b). This may be due to the small number of repeats of the experiment as the differences are quite small and often a large number of repeats is required to determine whether there is a difference.

4.2.6 Possible roles of the proximal promoter region in NK cells

The main promoter used to express LILRB1 in NK cells is the distal at determined using RT-PCR. However, there is activity from the proximal promoter in NK cells when examined by luciferase assays ((20) and Chapter 3). These instances of proximal promoter activity in combination with the difference in activity between haplotypes in reporter assays led me to address the question of what the proximal promoter does in NK cells.

4.2.6.1 Mapping of proximal promoter activity in NK cells

The proximal promoter region can function in NK cells, as evidenced by luciferase assays shown in Chapter 3, however, transcript from this promoter is either minimally produced or absent by PCR in *ex vivo* NK cells (Figure 3.1). Since the proximal promoter is capable of activity in NK cell lines, I defined the core proximal promoter

in NK cells compared to monocytes using model cell lines. I made luciferase constructs with two different fragments of the proximal promoter. Another group mapped the activity of the proximal promoter in a monocyte line and found that the region from -1137 to -589 relative the translational start site is active in this cell type (32). Therefore, I used this fragment as our smallest fragment and found that it has maximal activity in the monocyte lines that I tested, THP-1 and U937 (Figure 4.16a). This fragment has no activity in the NK cell lines that I tested, YTS and LNK (Figure 4.16b). In the B cell line I tested, DG75, the smallest fragment has reduced activity as compared to the large fragment but is still active to some extent (Figure 4.16c). However, all luciferase activity was low in the B cell line. The largest fragment, spanning -1287 to -1, is active in both NK cell lines tested (Figure 4.16b and 4.17a). A region spanning -1287 to -589 has no activity in the NK cell line, indicating that the region from -589 to -1 is required for activity in the NK cell lines but not the monocyte cell lines (Figure 4.16a and 4.17b). In fact, in monocyte lines, the -589 to -1 region limits the activity (Figure 4.16A).

4.2.6.2 Reverse activity of the LILRB1 proximal promoter

As previously mentioned, the LILR genes are related to the neighbouring KIR genes. KIR expression is regulated by a two-promoter system with a distal promoter and a bidirectional proximal promoter (100). The strength of the reverse activity of the proximal promoter is determined by SNPs within the reverse regulatory region (30). During NK development, the stronger the reverse activity, the less likely that expression of that gene will be fixed on an NK cell during development and through

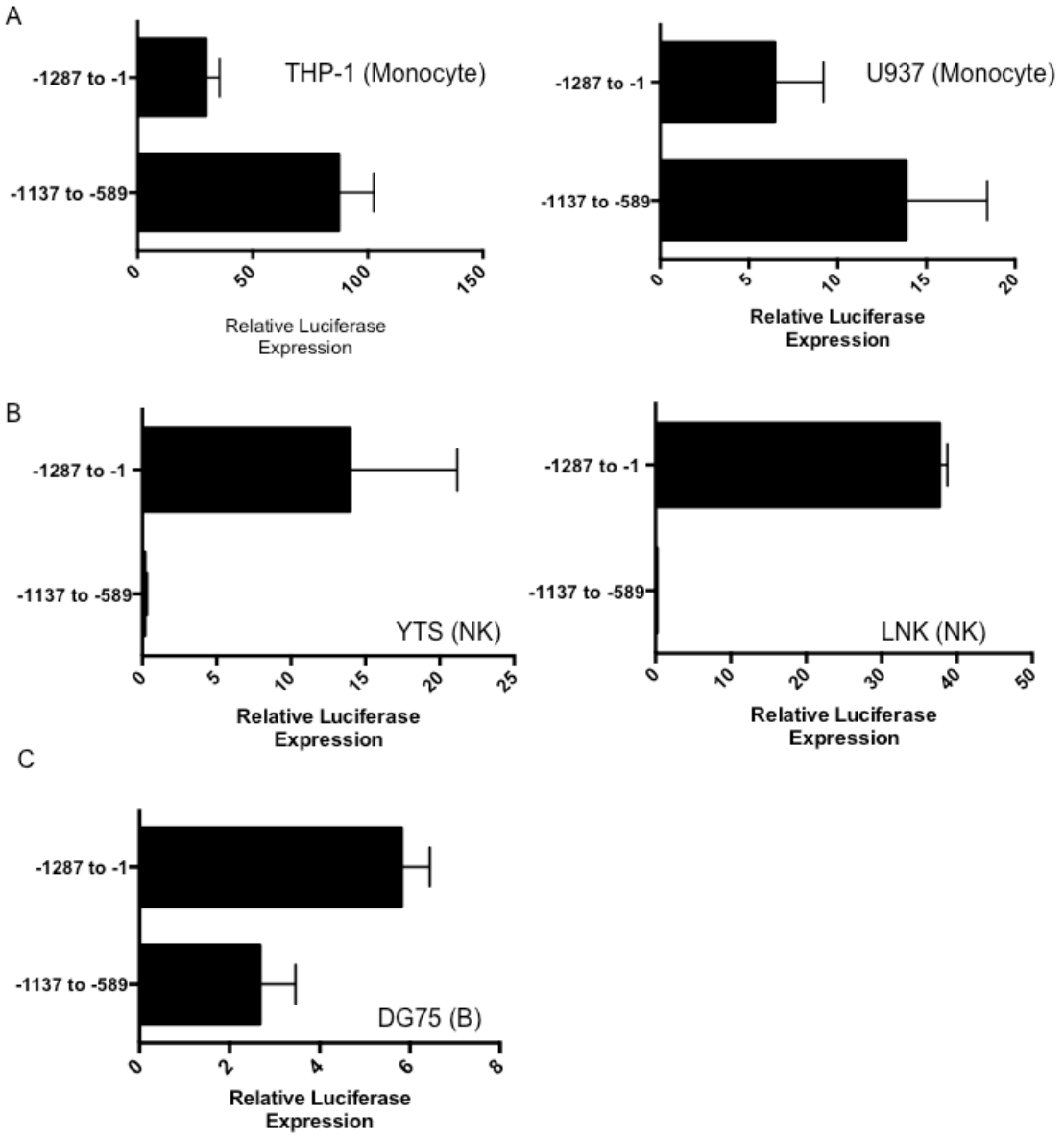


Figure 4.16 Monocyte and NK cell lines use distinct regions of the proximal promoter.

Two fragments of the LILRB1 proximal promoter were cloned upstream of the luciferase gene in the pGL3 basic plasmid. A) Luciferase plasmids containing the sequence from -1137 to -589 relative to the translational start site and the larger fragment from -1287 to -1 were transfected into A) the THP-1 and U937 monocytic cell lines B) the YTS and LNK NK cell lines and C) the DG75 B cell line. The average of three experiments are shown for all. The bar indicates the average for each with the error bar showing the standard deviation.

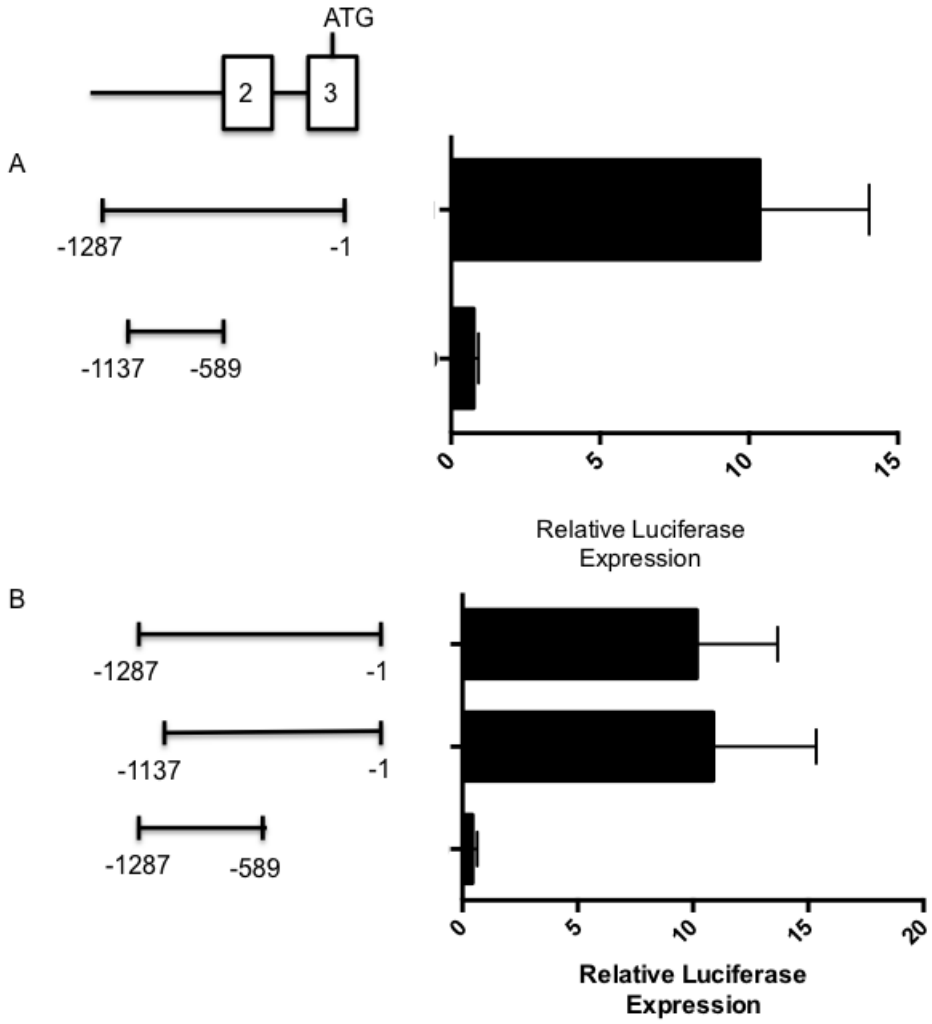


Figure 4.17 Sequence from -589 to -1 is required for NK cells to transcribe from the proximal promoter.

A) Luciferase plasmids containing the sequence from -1137 to -589 relative to the translational start site and the larger fragment from -1287 to -1 were transfected into the NKL NK cell line. B) A luciferase plasmid containing -1287 to -589 was transfected to compare to the -1137 to -589 and -1287 to -1 plasmids. The average of three experiments are shown for all. The bar indicates the average for each with the error bar showing the standard deviation.

epigenetic programming, sustained in progeny cells. I hypothesized that being a relative of KIRs, LILRB1 may use a similar method of regulation. Bioinformatic analysis of the LILRB1 promoters indicates that there may be reverse activity from the proximal promoter, which has indications that a reverse transcript could be produced (Figure 4.18a). An exon in the reverse orientation is predicted followed by repetitive polyA sites in the reverse direction (Figure 4.18a). The predicted features indicative of a coding transcript are surprising. I would have predicted a non-coding RNA would be expressed from the reverse promoter. To address the idea of a reverse promoter, I made luciferase constructs containing the largest and smallest proximal promoter fragments in the reverse orientation. Only the smallest proximal promoter fragment in the reverse orientation generated a low but detectable level of reverse promoter activity in the NK cell line, NKL (Figure 4.18b). Interestingly, the proximal promoter has relatively strong reverse activity in THP-1 cells, a monocyte cell line (Figure 4.18c). While the reverse activity appears stronger in THP-1 than NKL, it is still much lower than the activity of the proximal promoter in the forward direction in THP-1 (Figure 4.18c). In both the monocyte and the NK cell line, the largest fragment exhibits activity below background. This is likely due to the fact that in the short region spanning -1287 to -1137, there are six ATG sequences in the reverse direction, three of which are within relatively strong kozak sequences. If a transcript is produced in the reverse direction, as predicted, this would lead to multiple starts that likely disrupt production of the luciferase protein. This is supported by the activity of this promoter being below background levels. Interestingly, the reverse activity in NK cells is stronger from the “high” haplotype

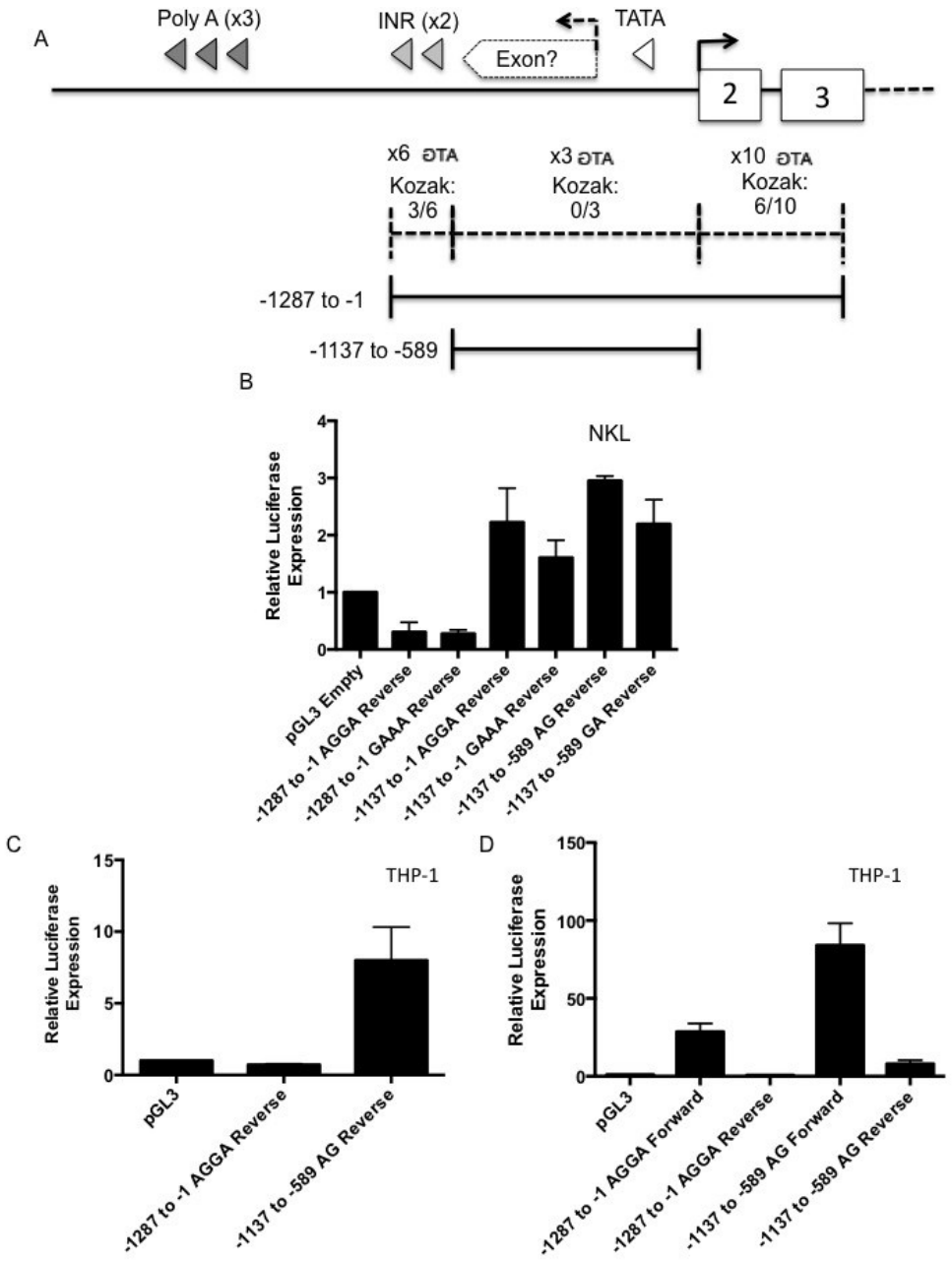


Figure 4.18 The proximal LILRB1 promoter has reverse activity in NK and monocyte cell lines.

A) Bioinformatic analysis of the proximal promoter showing indications of a likely reverse promoter and exon. ATG sequences in the reverse orientation are indicated for the largest and smallest proximal promoter fragments B) Reverse orientation proximal promoter fragments in luciferase plasmid pGL3 were transfected into the NKL cell line and C) the THP-1 monocytic cell line. D) Comparison of forward and reverse orientation proximal promoter fragments for activity in the THP-1 cell line by luciferase assay. The average of three experiments are shown for all.

(Figure 4.18b), however, further repetition of this assay is required to determine if this is significant. The minimal reverse activity in NK cells in this assay does not rule out the possibility that this activity is more predominant in developing NK cells, playing a role in whether the gene is initially turned on as is the case for the bidirectional proximal promoter in the KIR family. This could potentially explain the presence of subsets of LILRB1- and LILRB1+ NK cells.

4.2.6.3 Activity of proximal promoter under activating conditions

The presence of proximal promoter transcript in NK cells cultured under activating conditions (see Figure 3.1C) is an interesting finding in studying the potential role of the proximal promoter in NK cells. To further examine the possibility that activating conditions can enhance the activity of the proximal promoter, I transfected the large proximal promoter fragment luciferase constructs into an NK cell line that is IL-2 independent, YTS. I found that there was a trend toward increased activity in the cells treated with IL-2 (Figure 4.19). There was substantial variability in the numbers from day to day, however, in each of the three experiments the proximal promoter had higher activity in the IL-2 treated YTS cells (22). The previous data indicates that, while not active at a detectable level under normal conditions, the proximal promoter can be activated under stimulating conditions such as cytokine treatment. This experiment shows that the proximal promoter itself may be directly regulated by IL-2. This provides part of the explanation of the activity of the proximal promoter in NK cell expression of LILRB1.

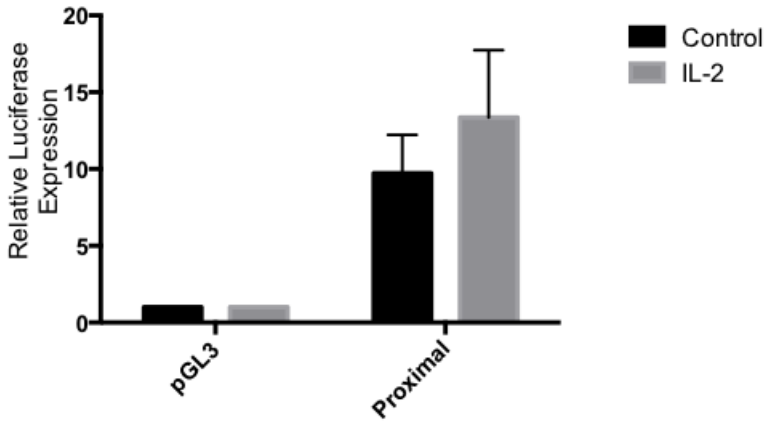


Figure 4.19 Activity of the LILRB1 proximal promoter is increased when cells are cultured in IL-2.

The proximal promoter region (-1287 to -1) was cloned into a luciferase construct and transfected into the YTS NK cell line. Transfected YTS cells were cultured with or without IL-2. The average of three experiments are shown for all. The bar indicates the average for each with the error bar showing the standard deviation.

4.2.6.4 Proximal promoter as a regulator of distal promoter activity

One other possibility for how the proximal promoter impacts LILRB1 expression on NK cells is that it acts as an enhancer of the distal promoter in NK cells. To address this possibility, I created a luciferase construct containing the distal promoter 5' of the luciferase gene and the proximal promoter 3' of the luciferase gene, in each orientation (Figure 4.20a). We originally hypothesized that the proximal promoter would enhance the distal. By this assay it seems the proximal promoter does have a regulatory effect on the distal promoter, however, it has a repressive role (Figure 4.20b). This assay does have a major limitation due to the fact that I cannot clone the whole region into a luciferase construct to study the relationship between proximal and distal because it is more than 13Kb in size. There may be a specific distance needed for the two regulatory regions to act on one another, which is different in this assay than in the context of the whole gene.

4.3 Summary

First, in this chapter I investigated NK specific regulation of LILRB1 transcription. I found significant differences between NK cells and both monocytes and B cells in DNA methylation at particular CpG sites within multiple regulatory regions. Next, I used EMSAs to examine the nuclear environment of B cells and NK cells and the presence of transcription factors in each cell type that are able to bind the core distal promoter. It is clear that there are factors unique to B cells that are able to bind this promoter sequence. I next examined the role of the proximal promoter in NK cells. Previous work by us and others (20, 21) has shown that NK cells primarily use the

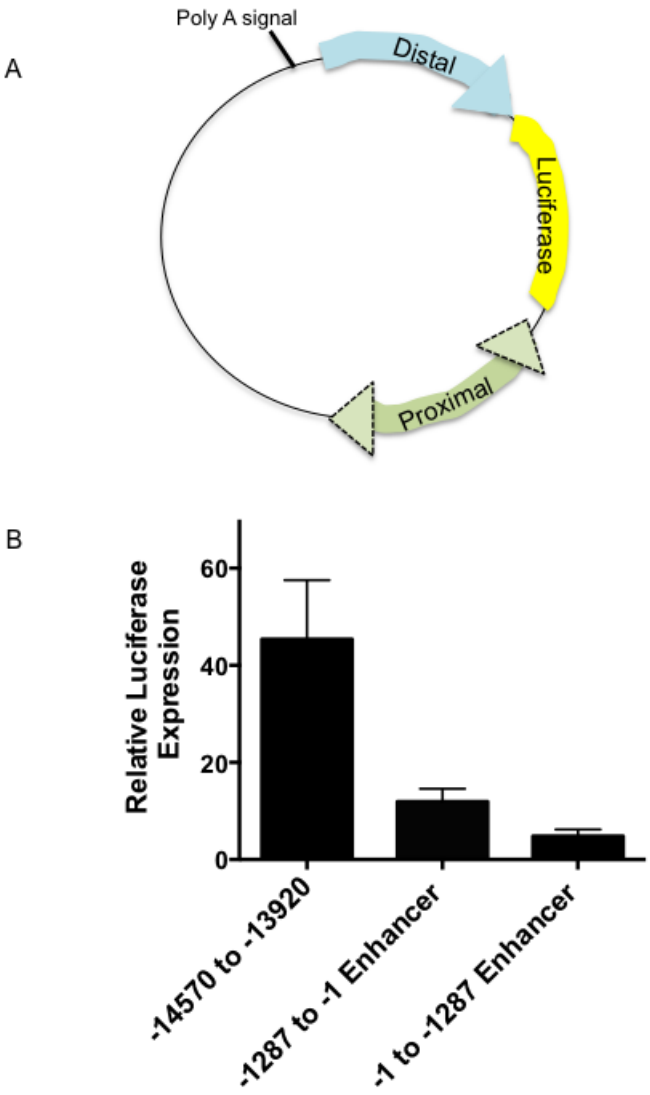


Figure 4.20 The LILRB1 proximal promoter represses the activity of the distal promoter.

A) Schematic of the LILRB1 proximal promoter cloned into the distal luciferase construct downstream of the luciferase gene in both the forward and reverse orientations. B) The distal luciferase construct is compared in a luciferase assay in NK92 cells to the same construct with the addition of the proximal promoter downstream of the luciferase gene. The average of three experiments are shown for all.

distal promoter, however, under certain activating conditions I see that the proximal promoter is active in NK cells (Figure 3.1). I also found that the proximal promoter does not act as an enhancer of the distal promoter, and in fact in the assay I used, it actually represses activity of the distal promoter. I also mapped the activity of the proximal promoter in NK cells and compared to its activity in monocytes, which primarily use this promoter to transcribe LILRB1. I found that NK cells use a distinct region of the proximal promoter from that used by monocytes. Finally, I examined the possibility of the proximal promoter having reverse activity in NK cells as this is a mechanism of KIR transcriptional regulation. I found that the short proximal promoter fragment has a small amount of reverse activity in the NKL cell line. Additionally, both promoters have some reverse activity in two monocyte lines tested. Finally, I examined the role of the expression correlative SNPs in expression of LILRB1 in NK cells. I found that the distal promoter, like the proximal promoter, has two major haplotypes and one rare haplotype and appears to be in perfect linkage disequilibrium with the proximal promoter. I did find significant differences in DNA methylation between the two major haplotypes at one position within the distal promoter and one within the proximal promoter region. Finally, to investigate the role of the correlative SNPs, I used luciferase assays and found that while there is no difference in promoter activity between the two major distal promoter haplotypes, there is a significant difference in the two major proximal promoter haplotypes, with the low haplotype exhibiting decreased promoter activity. This further highlights the importance of uncovering the role of the proximal promoter in NK cells.

CHAPTER 5: LILRB1 genetic variation: implications in the context of HCMV infection

5.1 Background

Cytomegaloviruses are Betaherpesviruses with members that infect a broad range of species (101). There are well-characterized family members specific for mice (MCMV) and humans (HCMV), among others. A large proportion of the population is infected with HCMV with minimal observable effect on health (101). However, this usually latent virus can be harmful in cases of immunosuppression such as post-organ transplant when immunosuppressive drugs are administered and during viral induced immunosuppression, such as infection with the Human Immunodeficiency Virus (HIV). An additional concern is in pregnant women who are infected for the first time during pregnancy or who's infection reactivates during pregnancy. In fact, HCMV was first observed in the population specifically in still-born infants over 100 years ago (reviewed in (102)). In non-fatal cases, HCMV infection can also lead to serious health issues and lifelong defects for the baby due to congenital CMV infection. Additionally, it has more recently been discovered that infection with CMV may be correlated with increased cardiovascular disease in the overall population (103).

HCMV and its human host have been evolving together for many years and have likely reached somewhat of a balance. While the virus has many immune evasion techniques, the host also has many immune responses directed at containing the virus (56). In a healthy adult host with a competent immune system, this leads to a balance where the infection persists in the host, and thus the population, but remains asymptomatic in the host. However, the issues arise when the host is immune-

compromised and the immune evasion techniques out weigh the immune response, skewing the usual balance.

HCMV infects many cell types and once an infection occurs, that individual will remain infected (Reviewed in (102)). The virus becomes latent but is able to reactivate intermittently throughout the life of the individual. HCMV is very host specific, however, many species that have been examined do have their own specific cytomegalovirus including mice, guinea pigs, chimpanzees, and Rhesus macaques (reviewed in (56)). HCMV requires Glycoprotein B and the Glycoprotein H-L dimer in order to enter cells and this complex has been shown to bind to several cell surface receptors, likely explaining HCMV's broad cell tropism (104). Further viral proteins are required for entry into specific cell types but are not required for all cellular entry (reviewed in (102)). HCMV infection is characterized by involvement of several organs and tissues. This is due in part to its ability to infect several cell types such as fibroblasts, endothelial cells, epithelial cells, smooth muscle cells, and monocytes (reviewed in (105)). Interestingly, HCMV can infect monocytes but the infection is not productive in this cell type. However, HCMV infection of monocytes does induce extravasation into tissues where they differentiate to macrophages, in which the virus can replicate (106). This is another explanation for the wide spread infection as the monocytes are able to transport the infection throughout the body.

HCMV infection causes significant morbidity post-transplant. Pharmacological immunosuppression given to patients after solid organ transplant to prevent rejection of the allogeneic organ leaves people susceptible to CMV disease. CMV disease includes symptoms such as fever, malaise, leukopenia and if left untreated, tissue

invasive disease which can present in many ways (92). As mentioned, the majority of people are already infected with HCMV. However, in the case where the donor is infected with HCMV but the recipient is not previously exposed to the virus results in the highest occurrence of CMV disease in recipients post-transplant (Reviewed in (107)). Prophylactic antiviral treatment is given to all patients after transplant, however, disease still occurs, particularly once the course of antiviral treatment is completed resulting in "late-onset" CMV disease (108). Antiviral medications also have side effects and this among other things often leads to non-compliance.

Previous studies have investigated a correlation between CMV susceptibility and KIR genotype with varying results (109-112). In another immune-compromised group, HIV positive patients, a correlation between CMV disease and a non-synonymous SNP in LILRB1 was observed (113).

Recently, measuring cell-mediated immunity to CMV infection as a method of predicting CMV disease was proposed (92). An alternative or complimentary approach is to investigate inherent qualities of the recipients, such as genetic predisposition to susceptibility to CMV disease. The aim of our study is to address whether patients with a specific LILRB1 genotype are more likely to develop CMV viremia or disease after solid organ transplant. The rationale is that LILRB1 binds to UL18 and suppresses NK responses. The ability to predict a patient's inherent risk of developing CMV viremia/disease could be used to design post-transplant prophylaxis and monitoring protocols. In this study we examined two different cohorts of post-transplant patients, a pilot one from Canada and a validation in collaboration with the Swiss Transplant Cohort.

5.2 Results

5.2.1 LILRB1 protein variants and their binding affinity for UL18

LILRB1 and LILRB2 are highly related inhibitory molecules from the LILR family of receptors. Our previous work as well as work by others has identified many amino acid positions altered by the polymorphisms in LILRB1 (29, 34, 35). The location of the four binding domain SNPs within the LILRB1 protein are shown in Figure 5.1. Within the two Ig-domains that make up the binding portion of LILRB1, Ig1 and Ig2, there are two major protein variants of LILRB1 made up of differences at 4 amino acid positions (Figure 5.2a). Interestingly, despite the 82.9% identity at the amino acid level between LILRB1 and LILRB2 in the binding domain (Figure 5.2b), both receptors bind MHC-I but only LILRB1 binds UL18. This indicates that one or more of the differences between the two receptors abrogate binding of LILRB2 to UL18. We found that of the four amino acid changes variants in LILRB1, three that were present in the more rare variant were the amino acids found at the corresponding position in LILRB2. This led me to hypothesize that the more "LILRB2-like" variant of LILRB1, named here as "PTTI" may have decreased binding to UL18. To address this hypothesis, I created expression vectors with each of the LILRB1 variants as well as LILRB2 tagged with HA. I expressed these tagged LILR proteins in RBL cells to use in UL18 binding assays. Using a UL18 Fc-fusion protein in a flow cytometry-based assay to detect binding with the two variants of LILRB1 as well as LILRB2, as a negative control. In each assay, the binding was normalized to receptor expression on that day by staining for expression level with an antibody against HA. In three out of five experimental repeats, I detected higher binding of the PTTI variant to UL18

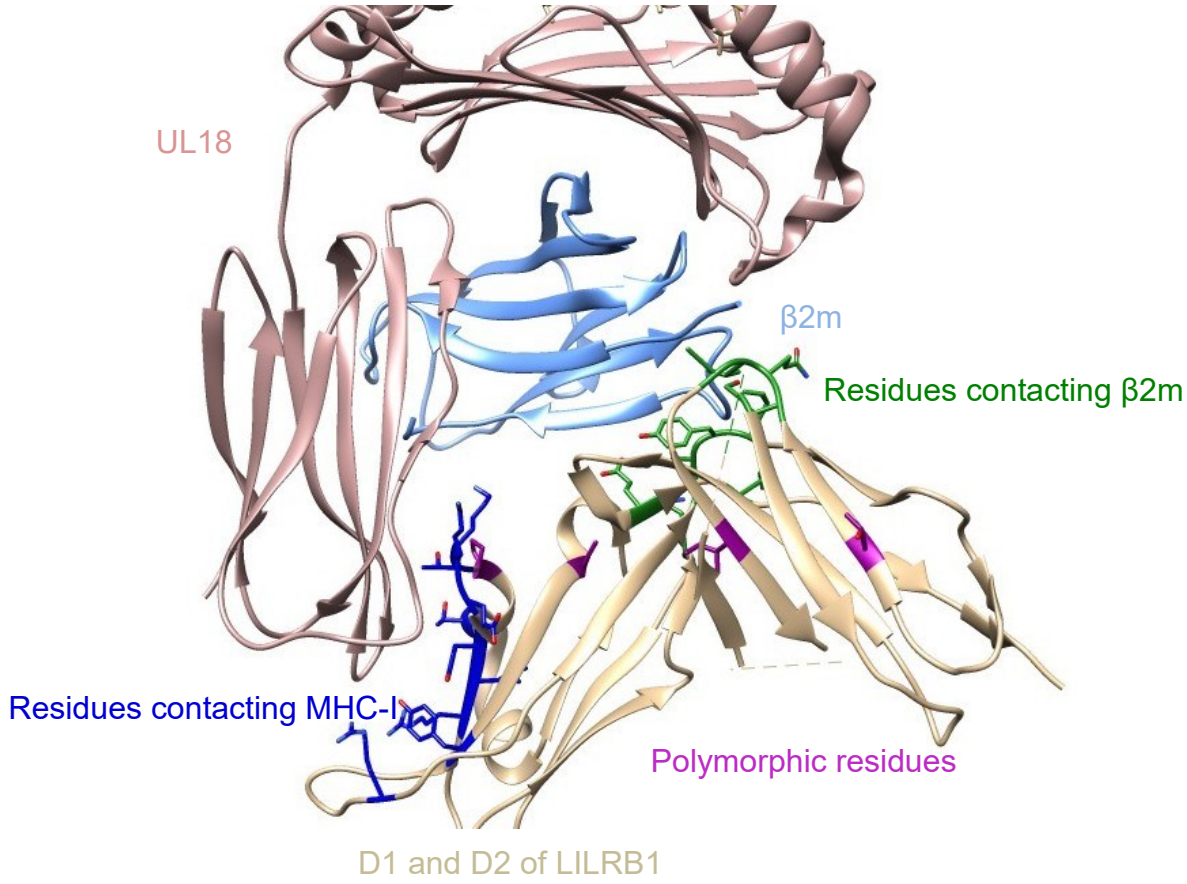


Figure 5.1 Crystal structure of LILRB1 bound to UL18 with polymorphic amino acid residues of the binding domain shown.

Made using the Chimera software from UCSF to analyze the co-crystal with PDB ID 3D2U (14).

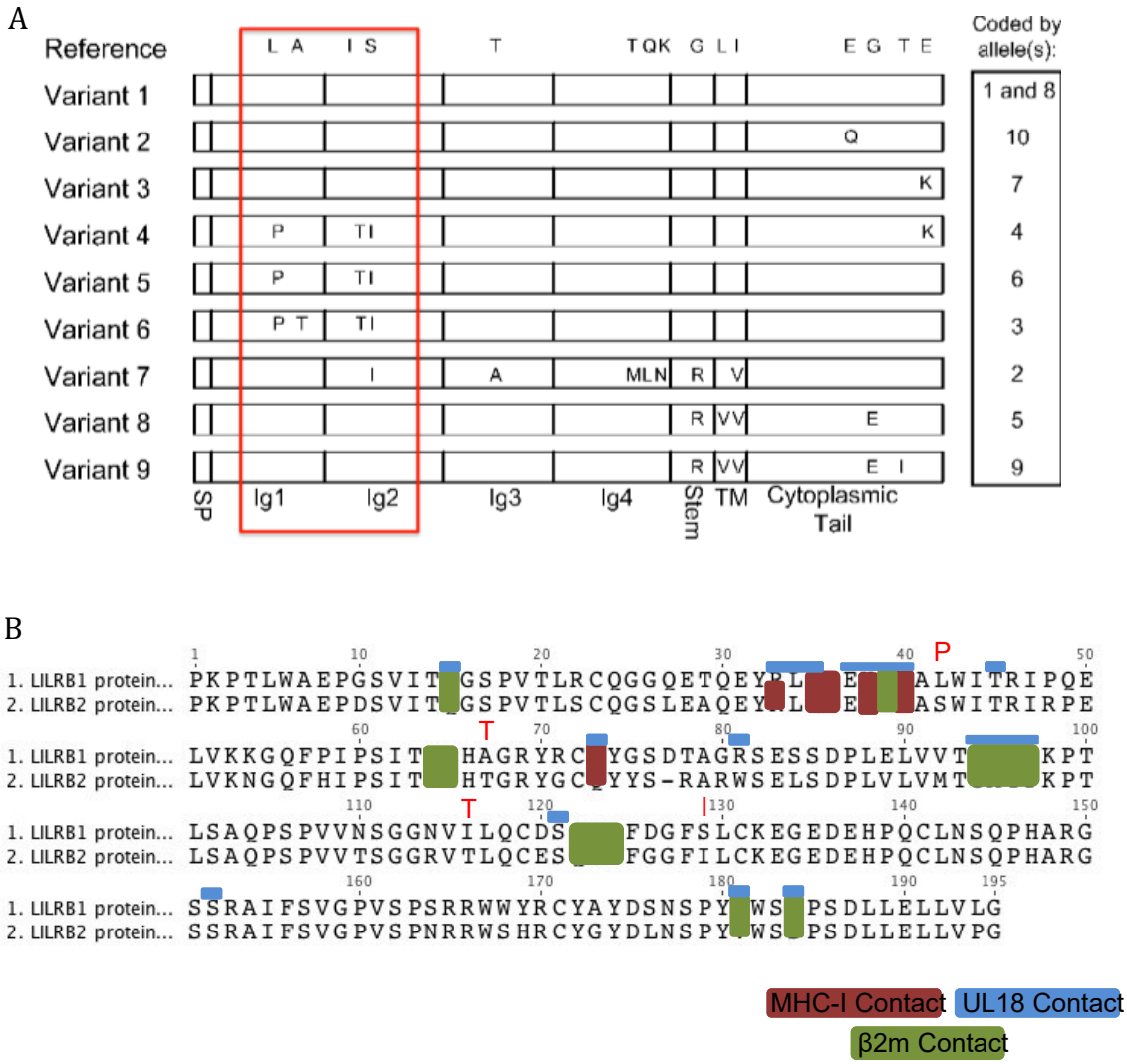


Figure 5.2 Protein variants of LILRB1 binding domains and position relative to binding residues.

A) Nine protein variants identified in a panel of 11 healthy donors (29). Four of the 15 amino acid changes are located in domains D1 and D2, which make up the binding domain of LILRB1 to UL18 and MHC-I, as indicated by the red box. B) Location of the four amino acid changes in D1 and D2 and proximity to contact residues.

(Figure 5.3a). In the remaining two, one resulted in equivalent binding of the two variants and the other resulted in higher binding of the LAIS variant. In all experiments, the LILRB2 receptor did not bind UL18 (Figure 5.3a). Further assays have since been done by Kang Yu and have confirmed higher binding of the PTTI variant to UL18 (Figure 5.3b). In these follow-up assays, the UL18 protein was expressed on 221 cells and the D1 and D2 domains of the LILRB1 protein variants were generated as Fc proteins. While the differences we detect in binding might be quite subtle, following these studies done in *2012* there are published results with I142T LILRB1 and CMV in HIV (113), and the result is consistent with the model that PTTI would be more readily inhibited by UL18 (113).

5.2.2 Three LILRB1 polymorphisms correlate with occurrence of CMV disease post-transplant: Canadian cohort

We examined 67 previously collected samples from a Canadian transplant cohort for LILRB1 genotype at five polymorphic residues (Figure 5.4a). All patients enrolled in this study were CMV seronegative pre-transplant but received a CMV+ donor organ (Donor+/Recipient- patients). Information on the 67 patients included in the analysis is displayed in Table 5.1. Patients included a mixture of organ types (Table 5.1). In the year following transplant, 25% of patients developed CMV disease and approximately 50% developed CMV viremia.

The positions of the five polymorphisms studied are spread throughout the gene: rs10416697 (-14894), rs1004443 (-1026), rs2781771 (-225), rs1061680 (+927), and rs16985478 (+5724) (Figure 5.4a). No substantial difference was observed for CMV

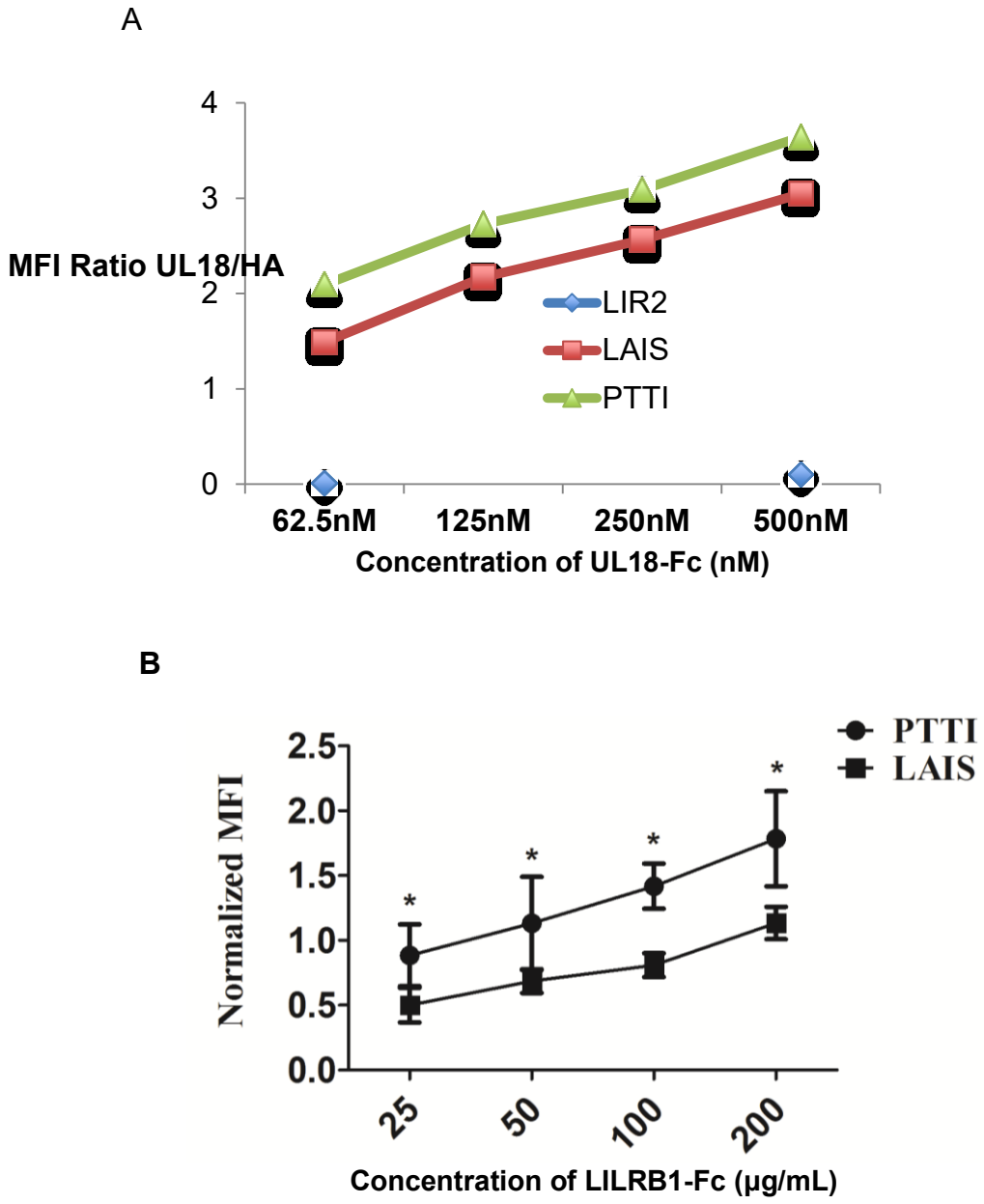


Figure 5.3 Differential binding of the two LILRB1 binding domain variants to UL18.

A. The two LILRB1 variants (PTTI and LAIS) and LILRB2 were stably expressed on RBL cells and binding of a UL18 Fc-fusion protein was measured by flow cytometry. LILRB2 was used as a negative control as LILRB2 binds minimally to UL18. This data is representative of three out of five experiments. **B.** UL18 was expressed on 221 cells and the LILRB1 protein variants as Fc proteins were used for binding assays. Data in B generated by Kang Yu.

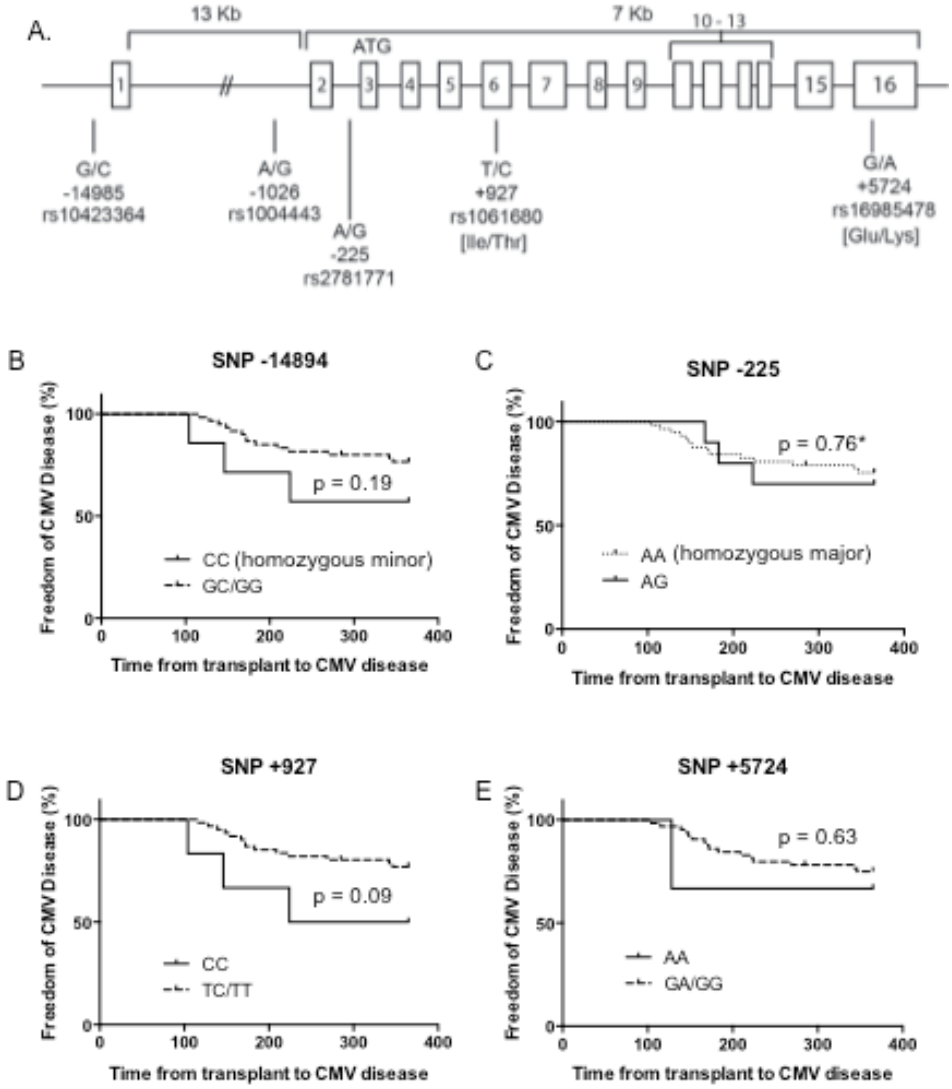


Figure 5.4 Incidence of CMV disease in post-transplant patients grouped by LILRB1 genotype.

A) LILRB1 gene organization and location of five polymorphic residues to be genotyped in post-transplant patients monitored for CMV viremia and disease for one year after transplant surgery. B) Incidence of CMV disease grouped into donors homozygous for the minor allele at -14894 (solid line) versus heterozygous or homozygous for the major allele (dashed line). C) Incidence of CMV disease grouped into donors homozygous for the major allele (dotted line) or heterozygous (solid line) at position -225. *Note: no homozygous minor donors were present. D) Incidence of CMV disease grouped into donors homozygous for the minor allele at +927 (solid line) versus heterozygous or homozygous for the major allele (dashed line). E) Incidence of CMV disease grouped into donors homozygous for the minor allele at +5724 (solid line) versus heterozygous or homozygous for the major allele (dashed line). Statistical analysis and plotting was performed by Luiz Lisboa.

Table 5.1 Patient characteristics in the Canadian Transplant study. From Manuel et. al (92)

	Total N=67	CMV Disease (N=17)	No CMV Disease (N=50)	p-value
Age, y, mean (SD)	48.1 (14.4)	47.7 (14.6)	49.3 (14.2)	0.829
Sex, M/F, No.	50/17	13/4	37/13	1
Type of transplant				
Kidney	31 (46.3)	7 (41.2)	34 (48.0)	0.197
Kidney-pancreas	8 (11.9)	1 (5.9)	7 (14.0)	
Liver	15 (22.4)	5 (29.4)	10 (20.0)	
Lung	7 (10.4)	2 (11.8)	5 (10.0)	
Heart	3 (4.5)	0	3 (6.0)	
Other	3 (4.5)	2 (11.8)	1 (2.0)	
Antiviral Prophylaxis				
Intravenous Ganciclovir	10 (14.9)	3 (17.6)	7 (14.0)	0.709
Valganciclovir	65 (97.0)	17 (100.0)	48 (96.0)	1
Oral ganciclovir	2 (3.0)	0	2 (4.0)	1
Duration of prophylaxis, d, median (IQR)	98 (96-150)	98 (96-125)	98 (92-153)	0.988
Induction therapy				0.973
None	4 (6.0)	1 (5.9)	3 (6.0)	
Basiliximab	37 (55.2)	9 (52.9)	28 (56.0)	
Thymoglobulin	26 (38.8)	7 (41.2)	19 (38.0)	
Alemtuzumab	0			
Maintenance				
Steroids	57 (85.1)	15 (88.2)	42 (84.0)	1
Tacrolimus	58 (86.6)	14 (82.4)	44 (88.0)	0.682
Cyclosporin	6 (9.0)	3 (17.6)	3 (6.0)	0.166
MMF/MPA	58 (86.6)	13 (76.5)	45 (90.0)	0.216
Azathioprine	1 (1.5)	0	1 (2.0)	1
mTOR inhibitors	5 (7.5)	3 (17.6)	2 (4.0)	0.099

Abbreviations: CMV, Cytomegalovirus; MMF, mycophenolate mofetil; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin

disease incidence in patients with different genotypes at positions -225 (rs2781771) and +5724 (rs16985478) (Figure 5.4c and e). Patients homozygous for the minor allele at position -14894 (rs10416697), -1026 (rs1004443) and +927 (rs1061680) had a trend towards a higher incidence of CMV disease, with p values of 0.19, 0.19 and 0.09, respectively (Figure 5.4b and d). Note only -14894 is shown as representative for both it and -1026 since these two SNPs are in perfect linkage disequilibrium. It is likely that the small number of donors with these genotypes, 7 and 6 out of 67, respectively, limits the sensitivity.

We also examined the incidence of CMV viremia for the two polymorphic residues where we observed the trend for CMV disease. We found that when all types of organ transplant are included, no difference is observed between the genotypes (Figure 5.5a and b). However, in specifically the kidney transplant patients when analyzed separately, there is a trend towards increased CMV viremia in donors homozygous for the minor allele (Figure 5.5c and d). These results are suggestive of an influence of LILRB1 polymorphism on CMV activity but the small sample size limits the conclusions that can be drawn.

5.2.3 LILRB1 polymorphism correlates with occurrence of CMV viremia post-kidney transplant: Swiss cohort

Having observed a trend towards increased CMV disease incidence in donors homozygous for the minor allele at -14894 and +927 in a small study of transplant patients, we moved to a much larger cohort of post-transplant patients in a similar study of patients tracked for CMV viremia and disease following surgery.

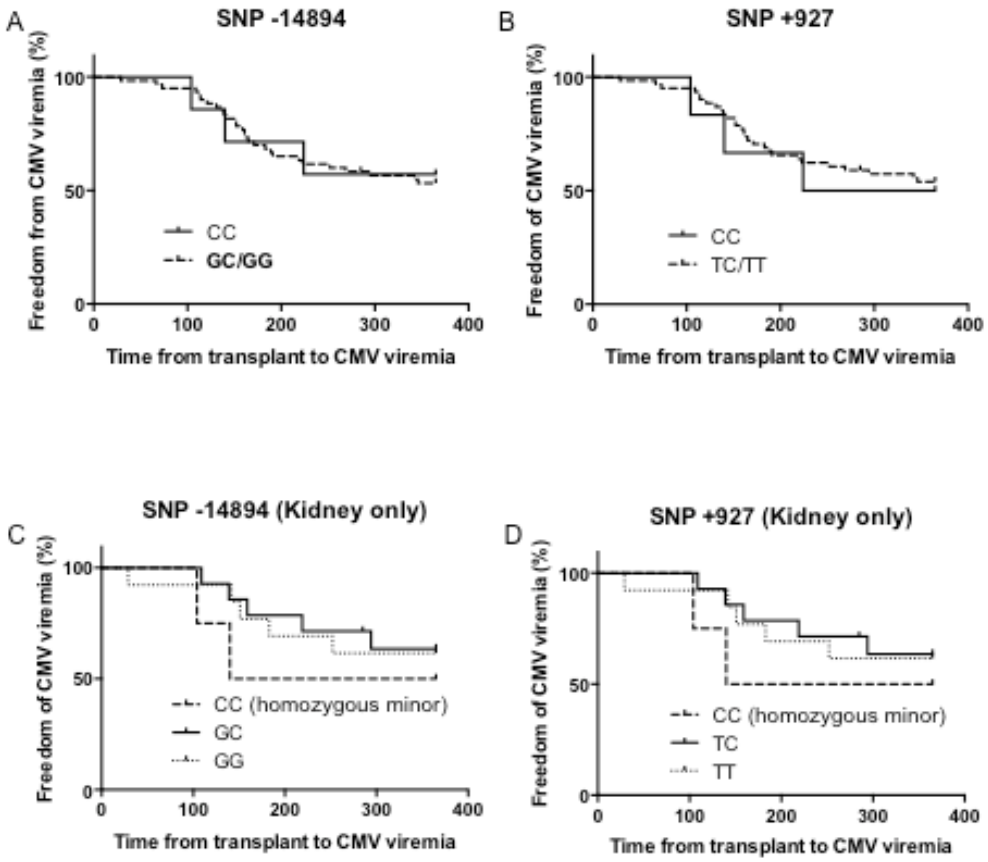


Figure 5.5 Incidence of CMV viremia in post-transplant patients analyzed by LILRB1 genotype.

A and B) Incidence of viremia in all transplant patients relative to LILRB1 genotype at positions -14894 and +927, respectively. C and D) Incidence of viremia in kidney transplant patients relative to LILRB1 genotype at positions -14894 and +927, respectively.

Our collaborators with the Swiss Transplant Cohort Study examined three polymorphic residues within LILRB1 in a large cohort of 1018 post-transplant (Table A1.1). The three residues we examined are rs10411879 (-19219), rs10423364 (-14186) and rs1061680 (+927). The coding SNP, rs1061680, was also examined in the smaller Canadian cohort and results in an amino acid change between Isoleucine and Threonine. The distal promoter SNP, rs10423364, is part of the expression correlated distal promoter haplotype, like rs10416697 in the Canadian study but is approximately 700bp downstream. The SNP located approximately 5Kb upstream of LILRB1, rs10411879, was included to determine the specificity of the LILRB1 SNPs in the role in CMV susceptibility.

Upon analysis of the distal promoter SNP, rs10423364, there was no difference in CMV disease or viremia incidence between transplant patients with different LILRB1 genotypes at this position (Figure A1.1). Interestingly, when exclusively kidney transplant patients were examined, there was a significantly higher incidence of CMV viremia in patients homozygous for the minor allele (Figure A1.2a). In contrast to results with the Canadian cohort, there is no difference in incidence of CMV disease between donors of different genotypes (Figure A1.2b). As a position further upstream of the gene, rs10411879, is examined, the correlation with CMV viremia incidence decreases (Figure A1.2c and d). When kidney transplant patients are examined for CMV viremia in relation to genotype at position rs1061680, a trend ($p=0.06$) is still observed for increased incidence in donors homozygous for the minor allele, however, the association is less significant than for rs10423364 (Figure A1.3). The data from this study are summarized in Table A1.2. Upon finding a stronger

correlation specifically in kidney transplant patients we did the same analysis in the Canadian cohort. This trend was visible when only the kidney transplant patients were examined as well, though this further decreases the number of patients in an already small cohort and therefore the significance also decreases (Figure 5.6).

Our collaborators performed a multivariate analysis to consider other known contributing factors in disease/viremia susceptibility (Table A1.3). In the multivariate analysis, the association of the homozygous minor genotype at rs10423364 with CMV viremia in kidney transplant patients remains significant (Table A1.3). Although the two cohorts provide slight differences in the details, collectively they imply that LILRB1 genotype influences immune control of HCMV.

5.3 Summary

This analysis of the potential correlation between LILRB1 genotype and complications post-transplant due to CMV began due to the obvious association between LILRB1 and this virus, as evidenced by CMV targeting LILRB1 with the immune evasion molecule, UL18. Further evidence for an association came from the recent discovery that LILRB1 genotype and susceptibility to CMV disease is correlated in HIV patients (113), another group with substantial immune deficiency. We first examined the potential influence of changes in the LILRB1 protein on binding to UL18. We found that the PTTI variant binds more strongly to UL18, in contrast to my initial hypothesis that this more "LILRB2-like" variant would have reduced binding to UL18. Next we made use of previously collected DNA samples from a cohort of 67 Canadian transplant patients to examine the possibility that

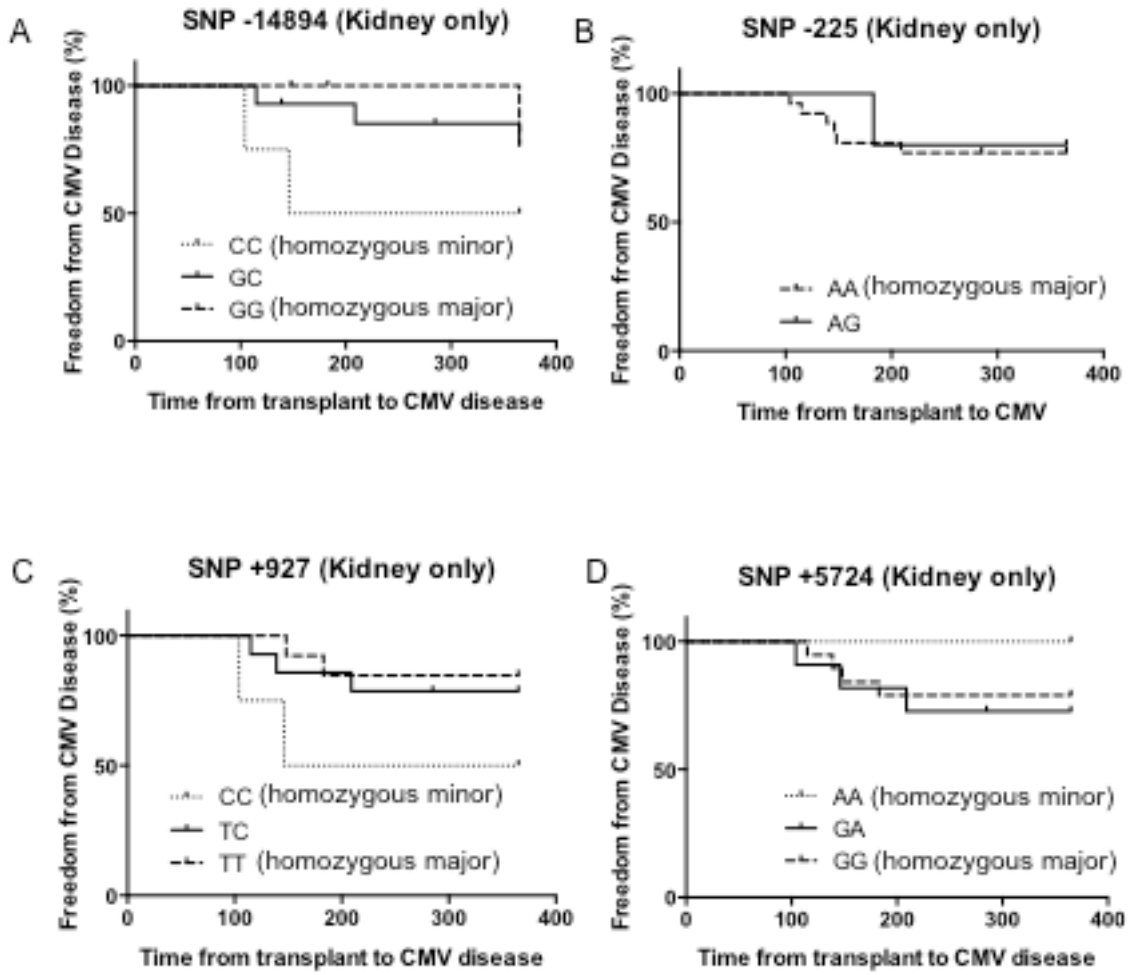


Figure 5.6 Incidence of CMV disease in post-kidney transplant patients grouped by LILRB1 genotype at position -14894 (A) -225 (B) +927 (C) and +5724 (D).

At each position, the patients with a homozygous major (dashed line), heterozygous (solid line) and homozygous minor (dotted line) genotype are shown grouped separately. No donor homozygous for the minor allele at position -225 were present in this cohort.

LILRB1 genotype is associated with CMV disease or viremia post-transplant and found a trend with three of the five polymorphisms examined. One within the distal promoter, one in the proximal promoter and one in the coding region at the 5' end, which produces a non-synonymous change within the D2 domain of the LILRB1 extracellular region. With promising results from our pilot study, we examined a much larger cohort through the Swiss Transplant Cohort Study, which collects data on all organ transplant recipients in Switzerland (114). In this cohort, we found an association between a polymorphism in the LILRB1 distal promoter and susceptibility to CMV viremia, however, only in kidney transplant patients.

CHAPTER 6: Discussion and future directions

6.1 Summary of Research Findings

The research presented in this thesis provides new details of the control of LILRB1 expression in NK cells and the implications for control of infection. I mapped the regions of the distal promoter required for expression of LILRB1 and identified JunD as an essential transcription factor regulating transcription from this element. I determined that there are extended haplotypes that span the distal and proximal promoter. The linkage disequilibrium over the 13 kb intron between the two may explain why polymorphisms in the proximal promoter region associate strongly with LILRB1 expression NK cells even though the distal promoter dominates as the transcriptional start. I began to uncover the role of polymorphism in the variable expression on NK cells observed between people. Finally, I uncovered a potential role for LILRB1 polymorphism in susceptibility to CMV-related complications post-organ transplant by showing a correlation between LILRB1 genotype and CMV complications.

6.2 Implications from characterization of the distal LILRB1 promoter

6.2.1 Distal core promoter and the role of exon 1

I mapped the core promoter activity and identified a core promoter of approximately 120bp (Figure 6.1). My initial studies were done with a promoter fragment ending at -13793 relative to the translational start site based on a bioinformatics prediction of where the exon began. Following 5' RACE results that were published in 2010 (21) indicating the transcriptional start site in lymphocytes, I was able to make constructs excluding exon sequence by ending at -13920, just before the transcriptional start site.

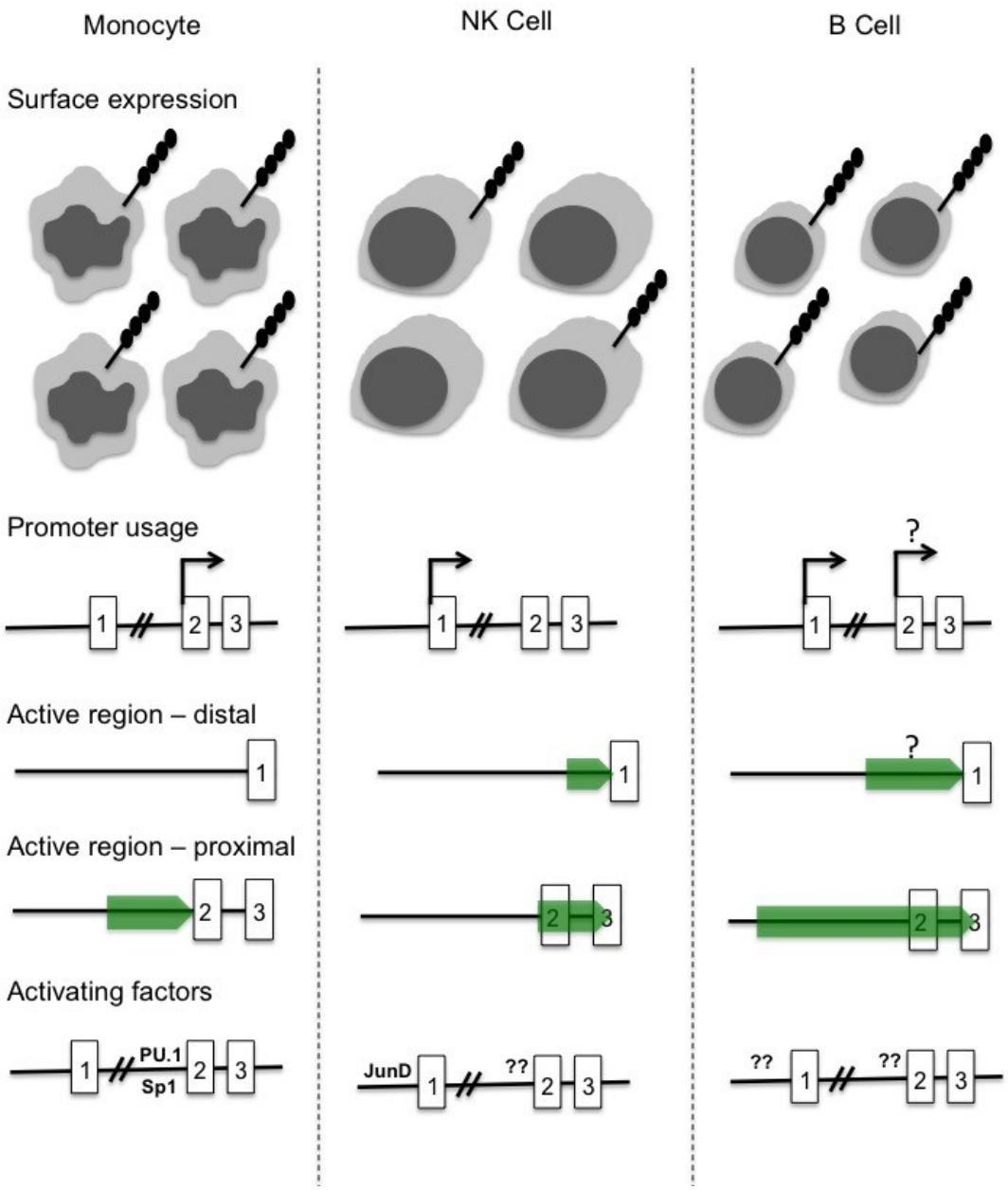


Figure 6.1 Lineage specific expression and gene regulation of LILRB1.

The three leukocyte lineages examined in this thesis are shown. The surface expression is shown in the top panel. What is known about promoter usage is indicated next, with data from this thesis as well as from other groups (20, 21, 29, 32). The core active region of each promoter is indicated by transparent green arrows. The transcription factors known to activate the promoters are shown with data again from this research as well as from other groups (20, 32).

The constructs ending at -13920 exhibit a similar pattern of activity in terms of mapping but much higher reporter activity. This is likely due to the decreased translation efficiency caused by the transcribed LILRB1 sequence prior to the translational start of the luciferase gene. Therefore, our results support findings by Lamar et. al (21) that the presence of exon one sequence lead to less efficient translation. There remain questions regarding the mechanism of how the ATGs and ARE sequence limits translation, but conservation of the exon provides a mode of regulation that limits expression from the transcript in lymphocytes. This is potentially important to prevent overexpression of LILRB1 that could suppress the cells too much during cell:cell interactions. The transcriptional machinery has to read through an additional 13Kb of DNA in order to include this translationally repressive exon. The upstream exon of LILRB1 contains several ATG triplets as well as an AU-rich element (ARE) (21). A large-scale genome analysis indicated that transcripts with long 5'UTRs containing multiple upstream ATGs are often associated with weak initiation of translation (115). Addition of an upstream ATG into the apolipoprotein gene also decreased luciferase expression in a reporter assay (116). AU-rich elements are common in genes responsible for transient responses as they tend to decrease the mRNA stability, making transcripts short-lived, however, this refers to AREs found in the 3' UTR (117-119) and information on the role of AREs in the 5' UTR in mRNA stability is lacking. An ARE element in the 3' UTR of the IL-2 transcript is actively targeted for decay by a protein in T cells (120). If an ARE in the 5'UTR is also indicative of less translation by a mechanism of mRNA degradation, it poses the question of why it would be beneficial for lymphocytes

specifically, and not monocytes, to have a short-lived LILRB1 transcript. This in combination with the variable expression on NK cells may indicate that having too much inhibition through LILRB1 is more detrimental in NK cells than the other LILRB1-expressing leukocytes. This idea will be discussed further in the context of selective forces acting on LILRB1 and its expression level on various cell types in section 6.8.

6.2.2 A role for AML-1?

To dissect the core promoter and identify the relevant transcription factors, I took advantage of bioinformatic analysis of the 126bp core promoter that revealed a region of several highly predicted transcription factor-binding sites, including consensus sequences for AP-1 and AML-1. Mutation of the AP-1 binding site led to a severe decrease in distal promoter activity and as discussed in the following section, became the primary focus of the study. AML-1 has been implicated in regulating the related KIR genes in NK cells (30). There is a predicted AML-1 site that is present within the regulatory bidirectional promoter in the majority of KIR genes (30). This site is interrupted in two KIR genes that are not expressed but for which anti-sense transcript is present, indicating a role for this site in overcoming anti-sense or reverse transcription (100). I identified a weak role for AML-1 in regulating LILRB1 expression with an observable 10% decrease in promoter activity indicating a minor role in directly driving transcription. AML-1 could have a larger role in other cell types or even under different conditions or developmental stages in NK cells but we did not investigate this possibility further beyond identification by EMSA.

AML-1 itself is worth using as a comparison to LILRB1 in terms of gene regulation. AML-1, also known as RUNX-1, is a very well-studied gene. This is due in part to its role in hematopoiesis (reviewed in (121)). AML-1 is also well studied for its role in certain cancers, including acute myeloid leukemia for which it is named. In some leukemias, there is a translocation that allows for a chimerization of the AML-1 gene with other genes to create an oncogene (122-124). Similar to LILRB1, AML-1 has two promoters that regulate its expression (125). The two promoters are active in different lineages (126). We can use what is known about lineage specific regulation of the two promoters in this dual promoter hematopoietic gene to speculate on the potential regulatory mechanisms of LILRB1. Markova et al. found that the two promoters themselves did not seem to account for the tissue specificity but they are differentially used between lineages (126). Two regulatory regions were uncovered in the AML-1 gene. These regions are an enhancer in the large first intron and a region that appears to be responsible for bringing together regulatory regions during active transcription of the gene, which is located in intron 5 (126). This is strikingly similar to some of our current hypotheses of how LILRB1 may be regulated, including a potential enhancer in the first intron (Burshtyn, unpublished observation) as well as a potential regulatory region of unknown function around exon 6. It would be interesting to examine these two regions more thoroughly as well as to look for additional regulatory regions within the gene. We have the advantage of working on a gene that is substantially more tightly packed and smaller than most, making a full analysis manageable. Analysis of the chromatin conformation and which regions are

in contact would be beneficial and in addition to uncovering lineage specific regulatory mechanisms, may provide answers to our questions regarding how SNPs in the proximal promoter influence expression of LILRB1.

6.3 AP-1 regulation of LILRB1 transcription from the distal promoter in NK cells

The AP-1 family of transcription factors has a role in a variety of genes with fairly ubiquitous expression across lineages. To study the role of AP-1 in regulation of LILRB1 expression, I used a Jun-kinase inhibitor, EMSAs to identify family members able to bind this specific DNA sequence and ChIP assays to confirm binding to the endogenous LILRB1 promoter. Through my studies, I identified JunD as part of the complex that binds the distal promoter and confirmed its role in activation of transcription through over-expression and knock down studies. To our knowledge, this is the first report of JunD as a regulator of a gene in NK cells. However, JunD has been studied in other lymphocytes. JunD has a regulatory role in CD4+ T cells (127) as well as playing a role in binding to and activating the IL-2 promoter in these cells (128). It would be interesting to know if JunD acts on the IL-2 promoter in NK cells as well or if the IL-2 gene is regulated in a lineage specific way with different factors driving its expression in NK cells. JunD is also involved in survival signalling through the JNK pathway (129). This potentially explains the documented role of JunD in multiple cancers, including prostate cancer (130, 131) and lymphoma (132, 133). Mice with JunD knocked out were shown to be viable,

with defects in male reproduction, (134) but nothing on immune function in these mice has been reported.

6.3.1 Cytokines and JunD/AP-1

The ability of cytokines such as IL-2 and IL-15 to enhance LILRB1 transcription may be an important means to ensure the levels of LILRB1 are maintained when the cells proliferate as lower levels might fall below a critical level for function or need to be increased during infection to prevent immunopathology. The involvement of JunD might explain our earlier findings that IL-2 and IL-15 can increases LILRB1 expression. Similar to other members of the AP-1 family, JunD is a target of the upstream kinases JNK and Erk that are activated in NK cells by a variety of stimuli including IL-2 and natural cytotoxicity receptors (135-139). In the case of JNK, JunD is required to be in a heterodimer with another AP-1 family member to recruit the kinase (140, 141). I observe a reduction of LILRB1 message when JNK is pharmacologically inhibited in the IL-2 dependent NK cell lymphoma line, NKL. Therefore, cytokine activation of NK cells may alter LILRB1 transcription via the AP-1 system. We previously reported that IL-2 and/or IL-15 treatment of *ex vivo* NK cells increases surface expression of LILRB1 but changes in mRNA were quite variable from one donor to the next (37). I also found an apparent increase in JunD containing-complex(s) binding to the LILRB1 AP-1 site when NK cells were differentiated in culture. This could be due to increases in JunD protein or there may be changes in the levels of the binding partners of JunD and/or the phosphorylation of JunD or its partners, likely in response to signaling through IL-2. In examining NK cells cultured in activating conditions, it does appear that the distal transcript is also

increased, however, this was not studied in enough detail to make a conclusion and could be followed up in future work.

On the other hand, I observed an increase in proximal promoter transcript in primary cells cultured in activating conditions with IL-2 and irradiated target cells (Figure 3.1). This may indicate another potential explanation as many of the NK cell lines are cultured in IL-2 and all may generally be in a more activated state. Transfection of primary NK cells with the proximal promoter reporter would clarify this, however, to acquire large enough numbers and to maintain them after transfection, they would need to be cultured in IL-2, making analysis of resting NK cells difficult. A quantitative measure of the increase in proximal promoter transcript was not possible due to a very short unique sequence for the proximal transcript and thus the need for a touchdown PCR, which cannot be used for quantitative PCR. This issue could be addressed by using copy number PCR to determine total transcript relative to transcript with exon 1 present. Future studies could be directed at characterizing the role of IL-2 in activation of the proximal promoter in NK cells. Additionally, the *in vivo* relevance could be examined by sorting for activated NK cells in blood and comparing the amount of proximal transcript in these cells relative to non-activated NK cells from the same donor.

6.4 Developing new methodology for unbiased transcription factor identification

ChIP-seq is a well-known method to identify binding sites within the genome for a particular transcription factor. In performing the characterization of this promoter

however, I noted a lack of techniques to examine the reverse question of which factors bind to a particular DNA sequence. I developed a separate technique to address this gap and I performed the preliminary experiments to optimize it. As described in chapters two and three, I designed a method to use biotinylated DNA fragments to collect and analyze bound factors from a particular nuclear environment by mass spectrometry. This is a promising tool that could enhance our ability to characterize promoters or other regulatory regions. Further optimization is required to allow for use of larger DNA fragments but the principle is the same and the basic protocol should remain the same. A limitation is that, at this point, bioinformatics must be used in conjunction as a great number of proteins are identified in this unbiased approach and many are likely irrelevant. Further to this, confirmation of each factor bound should be done using traditional techniques as used in chapter three (EMSA, ChIP, knock down, reporter assays). It should also be noted that this technique, like EMSA, only informs us as to what factors can bind but does not guarantee that they do so in the context of chromatin and at more biologically relevant quantities. A ChIP assay should be performed to address this concern and confirm *in vivo* relevance with any factors identified using this technique. This technique could also potentially be used to identify differences between haplotypes in the transcription factors that they bind. A differential band pattern and subsequent identification of any unique bands in a single haplotype could be used. This assay will require further effort to scale up to larger DNA fragments and to perfect the factor identification but the main aspects of the protocol are now in place to expand upon. The development of such a technique could be very useful to efficiently

characterize regulatory regions that have been linked to disease states by genome-wide association studies or expression analysis where transcriptional control is implicated as opposed to alteration of a protein.

6.5 Regulation of lineage specific expression of LILRB1

I explored differences in regulation of LILRB1 between various cell lineages because understanding the differences in LILRB1 regulation between cell lineages could provide useful clues to how SNPs selectively influence expression in NK cells. LILRB1 is the only member of the LILR family that is expressed in lymphocytes and is only expressed by subsets of NK and T, whereas LILRB1 is expressed by all B cells (8, 9, 22, 25, 29). While there is considerable variation in the frequency of LILRB1+ T cells between individuals our previous work did not show a correlation of the expression pattern of LILRB1 on NK and T cells (22). It seems LILRB1 expression on T cells is related to their state of differentiation following activation (98). Therefore, in my studies I focused on examining the differences in expression between NK cells and B cells using the distal promoter and contrasted to monocytes that use the proximal promoter where appropriate.

6.5.1 Differential use of transcription factors

I have mentioned the difference in which promoter is used between myeloid and lymphoid lineages, however, there are also differences in the regions used by each within the same promoter. The difference in regions of the proximal promoter used by monocytes and NK cells according to mapping done in chapter 4 further indicates

a variable role for the proximal promoter in these two lineages (Figure 6.1). The region used by monocytes and activated by Sp1 and PU.1 (32) is not active in the NK cell lines. According to the BioGPS database of expression data (142), PU.1 is expressed in human NK cells but at a much lower level than in monocytes, while Sp1 is expressed in both at a comparable level. It is not clear why these factors would not act on the proximal promoter in NK cells but the high levels of PU.1 as seen in monocytes may be required.

The correlation of genotype with expression on NK cells and not B cells indicates a difference in regions and/or factors used to activate LILRB1 expression in the two lymphocyte lineages. B cells could use additional factors that strongly activate transcription and mask any differences between haplotypes. Alternatively or additionally, NK cells could have a unique factor that is impacted by a polymorphic site. I found different banding patterns by EMSA when nuclear extract was used from primary B cells versus primary NK cells from the same donor suggesting the factors available in the nuclear environment that bind the core promoter are different in the two lineages. This does not rule out other mechanisms that may lead to uniform B and variable NK cell expression of LILRB1 but strongly suggests transcription factors that bind to the promoter in B cells might override the effects of the polymorphisms.

6.5.2 Epigenetic modifications correlate with lineage-specific expression

Another possibility that I examined is a difference in regions used due to lineage specific epigenetic modifications, more specifically, DNA methylation. I found that while methylation within the distal core promoter was comparable between the two lymphocyte lineages, NK cells were more highly methylated at almost all of the CpG sites in the upstream region of the distal promoter (Figure 6.2, top panel). This could indicate that B cells have a more expanded core promoter with additional transcription factor binding sites. Future studies could map the core promoter in B cells using the same techniques used in chapter 3 of this thesis to map the promoter in NK cells. Within exon two, the 3' end of the examined region is slightly less methylated in NK cells. This difference is small and cannot be seen in Figure 6.2 with the way the data is grouped together in the summary, however, it can be observed in figure 4.7 and is significant at position -258. This corresponds to the observation that this exon 2 region is required for any activity from the proximal promoter in NK cells and less so in B cells. Future studies should characterize this region of the proximal promoter active in NK cells to determine key regions and factors, however, this should only be a priority after the function in NK cells is elucidated.

A CpG site within the core distal promoter that is largely unmethylated in NK and B cells is methylated in monocytes. This finding can explain why I found that both

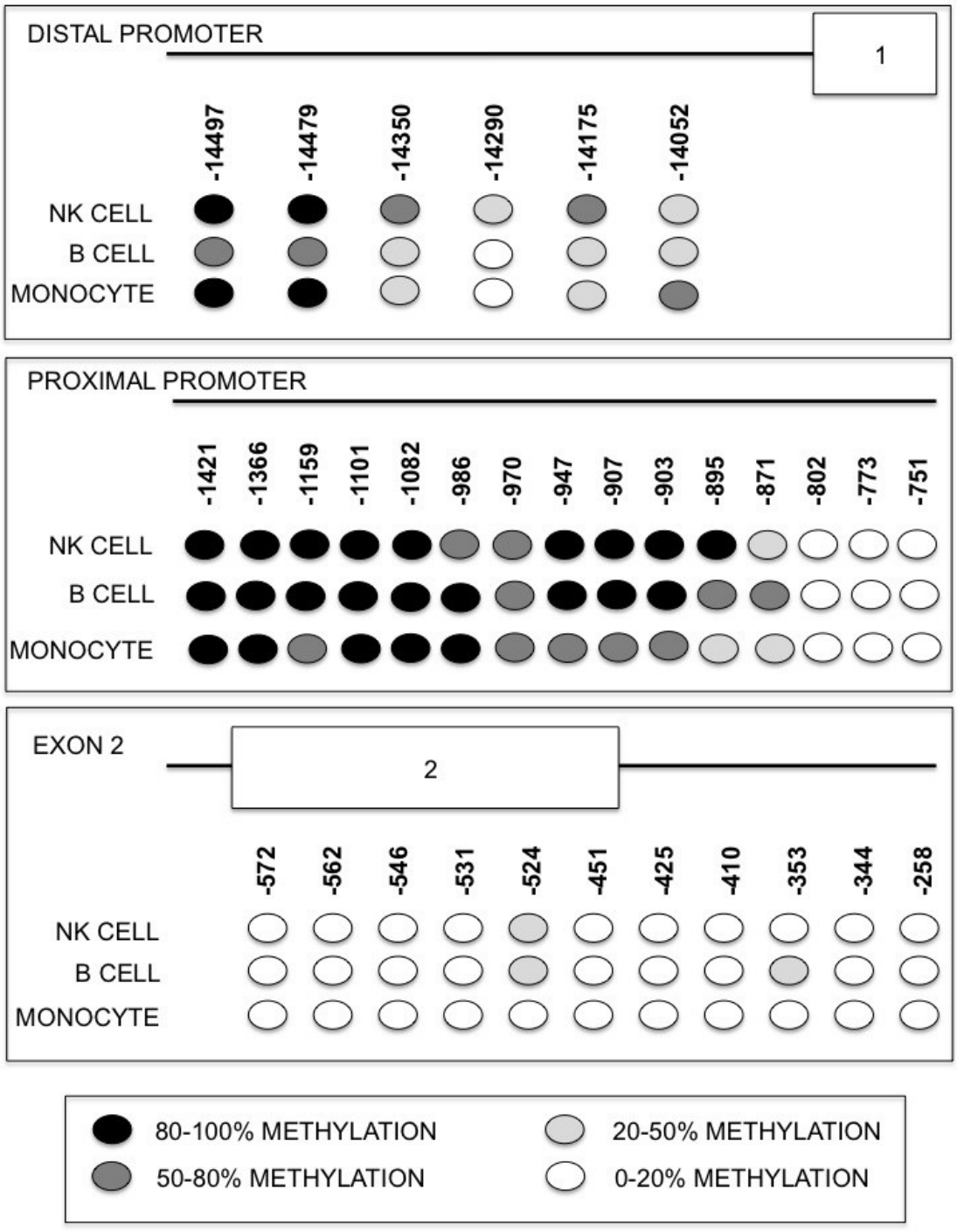


Figure 6.2 Summary of methylation analysis of three regions in LILRB1.

This data is compiled from data in figures 4.5-4.7. The key indicates the meaning of each coloured circle. The region examined is depicted at the top of each panel, followed by the position of each CpG, relative to the translational start site.

promoters are active in the NK and monocytic cell lines when using reporter assays (Figure 3.1). I did confirm reporter assays in at least two cell lines per lineage for both monocytes and NK cells but a different nuclear environment in primary cells is still a possibility. I was surprised by the function of the distal promoter in the monocyte lines due to the lineage specific usage of each promoter in primary cells observed by us and others (20, 21). There are likely differences in regulation of transcription when the LILRB1 core distal promoter is in the context of chromatin as opposed to being encoded within a plasmid. The methylation at the start of the distal promoter I found could be directly involved in repressing the distal promoter in the monocytes by preventing a transcription factor from binding or could be indicative of the mechanisms that lead to its repression. Alternatively, it remains possible the two monocyte cells lines I used aberrantly express the transcription factors that facilitate the distal promoter function because they are transformed cells.

6.6. Role of polymorphism in expression of LILRB1

A primary goal of my thesis work was to understand how polymorphisms selectively control expression of LILRB1 in NK cells relative to other lymphocytes and monocytes. In section 6.3, I discussed the identification of a major transcriptional regulator of LILRB1 expression in NK cells, AP-1, and mapping of the core distal promoter. However, the four SNPs in the distal promoter haplotype are outside of this core promoter region and cannot directly impact AP-1 binding. There could a role for the polymorphism in AP-1 regulation of LILRB1 expression, as AP-1 is known to dimerize with other factors (143-146) and a binding site for one of these partners may

be influenced by the SNPs. However, the correlation between haplotype and expression on NK cells more likely indicates additional regulatory regions and/or mechanisms corresponding to the location of one or more of the extended haplotype SNPs.

6.6.1 Relationship of SNPs to CpG methylation

To further investigate the role of the SNPs in NK-specific expression, I continued to focus on differences in haplotypes and set out to identify the SNPs that directly influence expression. Polymorphisms can influence expression in several ways, most of which are not mutually exclusive so a combination of these mechanisms may play a role in the final result. Along the same lines as just discussed in the context of lineage differences, epigenetic differences could exist between the two haplotypes as well as between lineages. This difference could be due to more general differences between the haplotypes or by the presence or absence of particular CpG sites within a haplotype. Of the 90 SNPs observed in our study of the LILRB1 gene, almost a third of them alter CpG sites. The two altered CpG sites within the regions examined in this study for methylation are within the exon two fragment at positions -572 and -425 relative to the translational start site. In the four donors I examined to compare high and low haplotype methylation, the high haplotype, which has CpGs at both of these positions had little to no methylation. Had the haplotype with the methylation sites been highly methylated, this would increase the potential for a difference in activity between the two haplotypes by direct presence or absence of methylation sites. This may be the case with other methylation sites within the gene as I only

examined four areas for methylation status and examining additional regions could be an area to follow up with future studies. Interestingly, a large-scale analysis of the genome found that the differential methylation of genes correlates with expression is intragenic rather than at the promoter, particularly in the immune system (147).

One of the SNPs that is not in linkage disequilibrium with the promoter haplotype eliminates the CpG site at +1661 and does appear to skew strongly towards lower expression on NK cells in the small subset of 11 donors examined in our initial study (29). The four donors with highest LILRB1 expression on NK cells have no CpG sites at this location on either allele while the other seven all have one or more allele that has this CpG. This observation, in combination with this region being the most CpG rich section of the LILRB1 gene, may indicate a regulatory role. Unfortunately, although I set out to study the methylation of the region around exon six that contains +1661 due to high CpG content, this study was abandoned due to technical problems. This region is worth revisiting for the two reasons just mentioned. One speculation is that this region could represent the start of an intragenic transcript and methylation of this regulatory region, as is possible when a CpG site is present, would lead to decreased activity from this intragenic promoter and apparently less of the coding transcript as well. This is, however, just a hypothesis but future studies should examine this SNP and the surrounding region further. Another possibility is that this region may function, as is seen in the AML-1 gene (126), as an intragenic control region responsible for bringing the regulatory regions together for transcriptional activation. This could be examined using chromosome conformation capture (3C)

(148). Methylation at this site as well as potential binding factors influenced by this change should also be examined to begin to address whether this SNP may have an important role. Preliminary analysis of available data from 20 of our expanded panel of 24 donors is shown in table 6.1 with donors listed from lowest to highest NK expression of LILRB1. It is apparent in this cohort that presence of the C (and thus the CpG) is correlated with lower expression.

6.6.2 Role of SNPs in the proximal promoter

The most straightforward way a haplotype or individual SNP can influence expression is in altering binding of specific transcription factors by altering their binding site. I found that the proximal promoter haplotype associated with lower expression on NK cells has lower activity in reporter assays (Figure 6.3), leading us to further investigate the role of the proximal promoter. The activity difference between proximal promoter haplotypes led me to investigate potential roles for this "myeloid" promoter in NK cells. I found roles for the proximal promoter in activating the LILRB1 gene in NK cells under activating conditions and also identified a potential function as a repressor of the distal promoter. The activating conditions include initial culture with target cells and mitogen and continuous exposure to IL-2. The significance of the proximal promoter being used is further enhanced by the finding that transcript generated from this promoter is more readily translated (21). Even a small increase in transcription from the proximal promoter could have substantial effects on the amount of LILRB1 protein expressed on the surface. CD94, another receptor expressed on NK cells, that also has an upstream or

Table 6.1 Genotype at position +1661 in the LILRB1 gene within a panel of 21 donors. Donors are listed from lowest to highest LILRB1 expression frequency on NK cells**.** The left column indicates the donor number. The right indicates genotype at position +1661. The grey shaded boxes indicate the presence of a C at this position in that donor.

257	C/T
177	C/T
230	C/C
226	C/T
183	C/T
227	C/C
225	C/C
255	T/T
259	T/T
224	C/T
253	C/T
190	T/T
105	T/T
232	T/T
231	T/T
229	T/T
228	T/T
256	C/?
254	T/T
252	C/T
258	T/T

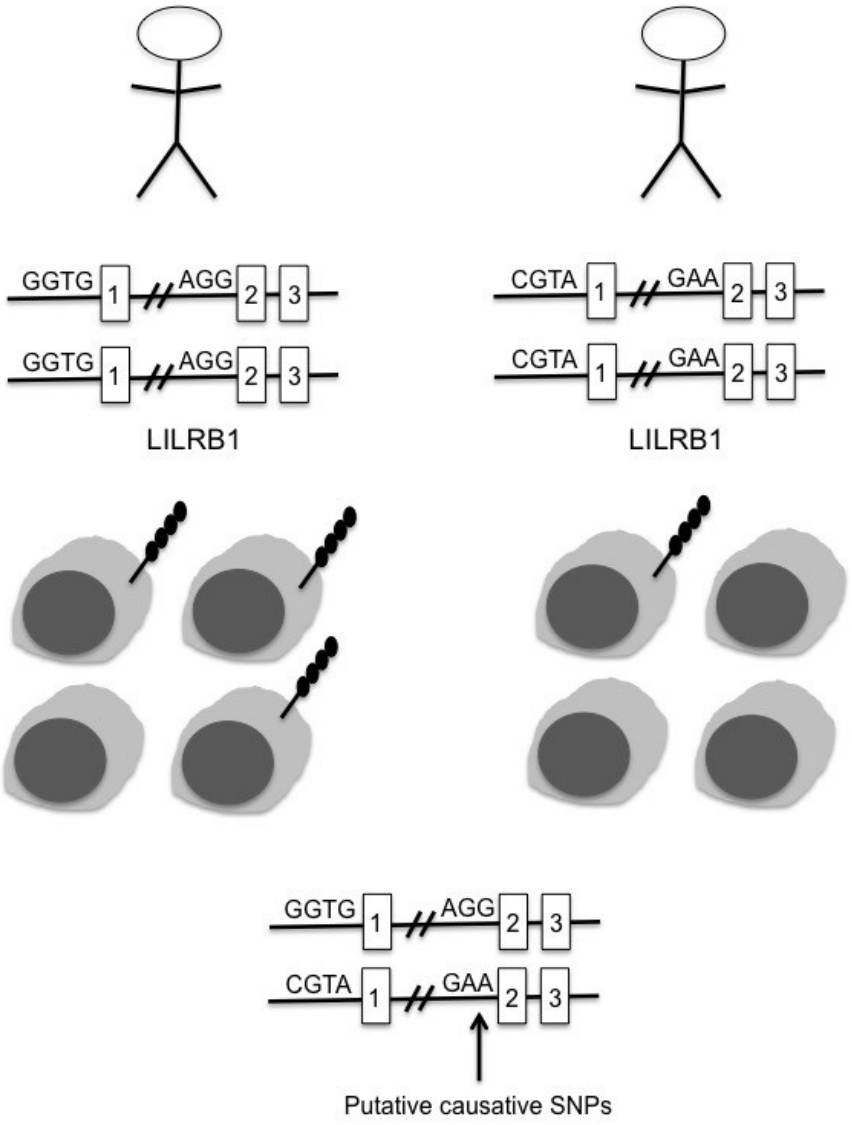


Figure 6.3 Correlation of genotype with expression of LILRB1 on NK cells.

The two homozygous genotypes are indicated at the top, with expression-correlative SNPs in each promoter shown. The correlated NK specific expression level is shown on the cells below each genotype. The bottom panel indicates that the proximal promoter SNPs are causative as discovered by reporter assays with both haplotypes of each promoter region (Figure 4.15).

distal promoter with a unique untranslated exon (149). In the case of CD94, the distal promoter is inactive in NK cells until stimulated with IL-2 or IL-15, at which point it becomes active (149). This represents a similar regulatory mechanism for another gene expressed by NK cells.

It is important to note that there are likely many more SNPs that are part of the extended haplotypes, both within the large first intron and upstream of the distal promoter we examined as well as within other regulatory regions that may or may not be within the LILRB1 gene. Therefore, there may be additional roles for the SNPs within the haplotypes in regulating expression than those I have identified in this research. Unfortunately, I was unable to identify a single SNP responsible for the lower activity of the "low" proximal promoter haplotype. This is something that could be followed up with further experiments as such a small change may need further repetition to determine any differences. Each SNP could also be examined using EMSAs with probes of DNA with each nucleotide variation. This would also clarify whether the regions containing each SNP are able to bind any factors in the NK nuclear environment and thus are worth pursuing. Further, I only examined SNPs in the two promoters here. As mentioned, there may be important haplotype SNPs within other types of regulatory elements, such as enhancers. Investigation of this possibility could begin with identification of regulatory elements beyond the two promoters examined in this thesis.

In addition, the proximal promoter transcript is more abundant in our high LILRB1 donor (D258) with an AGG/AGG genotype than in the low donor with a GAA/GAA genotype (D301) when examining cultured/activated NK primary NK cells. This result is in one donor for each genotype and the PCR is not truly quantitative so should be cautiously interpreted but does warrant further study.

6.6.3 SNPs could influence reverse promoter activity

In addition to a difference in regions responsible for forward gene transcription, I found much stronger reverse promoter activity by the proximal promoter region in the monocyte cell lines examined. This is a potential mechanism for shutting off the distal promoter in monocytes, as is observed in KIR genes as a mechanism of developmental shut off (100). The distal promoter is extremely active in monocyte cell lines when expressed exogenously in a luciferase vector so an epigenetic mechanism, potentially regulated by a developmental switch, is a possible reason for the lack of endogenous distal transcript. Further studies could be done to focus on regulation of the distal promoter in monocytes or other myeloid lineage cells. Reverse activity of the proximal promoter was also identified in an NK cell line, though to a lesser extent than that observed in the monocyte line. This reverse activity tends to be higher from the "high" haplotype, however, this experiment should be further repeated to determine validity of this finding. This observed trend is surprising as I would have hypothesized any haplotype difference to be just the opposite, with more reverse activity from the low expressing haplotype. I did attempt

to detect naturally produced reverse transcript in primary cells using an approach used in identification of the KIR anti-sense transcripts (150) but these preliminary experiments were unsuccessful. Further research on the potential reverse activity could be done to elucidate whether there is a regulatory role for a reverse transcript in monocyte development and during NK cell development. This may include examining reverse activity in an early developmental stage NK cell line. I did use the LNK mouse NK cell line as this has been used to examine KIR reverse activity due to their early developmental stage (100) but no reverse activity was detected. Importantly, if there is role for a reverse transcript in regulating LILRB1 it is quite distinct from that of the KIR promoters.

6.7 Proximal promoter regulation of the distal promoter

Surprisingly, when testing if the proximal promoter was an enhancer of the distal promoter I identified what appears to be a repressive function of the proximal promoter (Figure 6.4). These studies should be followed up with further research to confirm the repressive function as well as to look for potentially different levels of repression based on haplotype. This may be another way that the haplotype of the proximal promoter can impact the surface expression if the two haplotypes repress the distal haplotype to varying levels. This would provide an additional mechanistic role for the proximal promoter SNPs that correlate with expression of LILRB1 on NK cells.

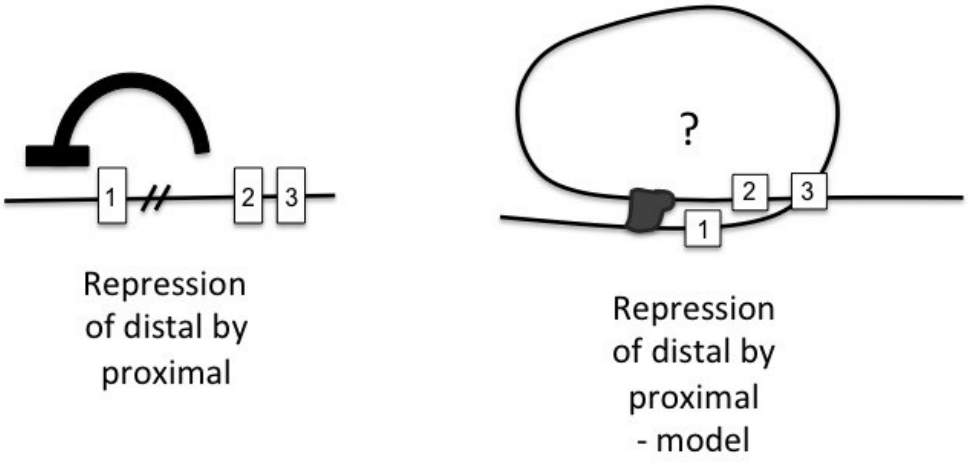


Figure 6.4 Model of proximal promoter repression of the distal promoter.

Reporter assays show that the proximal promoter can repress the distal (Figure 4.20). The right panel proposes a model where the proximal promoter loops around to be in close proximity to the distal promoter. The different proximal promoter haplotypes could differentially bind factors and this could be a mechanism for proximal promoter haplotype driving expression in NK cells.

There is still a great deal of information to be gathered on the regulation of the LILRB1 gene in NK cells as well as other LILRB1-expressing lineages. There are presumably additional, non-promoter regulatory regions that are similarly inclusive of the polymorphisms that are part of the haplotypes that correlate with expression in NK cells and as mentioned, identification of these regions could allow elucidation of SNP(s) that influence expression directly. Future research should involve a more thorough, locus-wide analysis to identify additional regulatory sequences now that the initial mapping and promoter characterization is complete. The large first intron is of particular interest as it is unique to LILRB1 and the presence of the additional upstream exon and large intron appears to allow LILRB1 expression in NK cells. There is in fact a predicted NK-specific enhancer region within this intron that is currently being investigated in the Burshtyn laboratory (Burshtyn unpublished observations).

6.8 Evolutionary pressures driving LILRB1 selection

For the obvious reason of being constantly acted on by pathogens, there is more active selection and rapid evolution in the immune system relative to many other systems (151). This idea has been examined in several studies, a few of which I will mention here (152-154). Ferrer-Admetlla et. al. found evidence for both positive and balancing selection acting on different immune genes upon analysis of nine genes (152). Two Toll-like receptors appeared to be acted on by positive selection while six of the nine genes show evidence of balancing selection. More specifically, within the LRC, KIR gene diversity appears to be maintained by balancing selection (153, 154).

KIR genes are under high selective pressure as evidenced by their rapid evolution. Interestingly, even in three Amerindian tribes with relatively little genetic diversity, the A and B KIR haplotypes are maintained (154, 155). Parham suggests that the more simple KIR A haplotype may be maintained in the population for defense against pathogens, while the expanded and variable KIR B haplotype may be maintained for its role in reproductive success (154). As evidence, he cites the finding that women homozygous for haplotype A have more complications in pregnancy (156, 157) while AA homozygous people are more able to defend themselves against Hepatitis C (158). One can imagine that LILRB1 has similarly conflicting forces acting on it and driving its evolution. By way of comparison, a previous study examined the balancing pressures acting on the IL-10 promoter and the resultant balancing selection (159). As a repressive or inhibitory component of the immune response, this gene has similar issues with extremes in expression: too high and the organism becomes susceptible to infection but too low and uncontrolled immune responses become problematic. Interestingly, as has been largely the focus of this thesis, this variable and intermediate expression of LILRB1 appears to be much more apparent on NK cells than on monocytes or B cells. I will speculate on a few of the potential driving factors in LILRB1 evolution in the following section.

6.8.1 Factors to maintain LILRB1 expression

Two potential balancing factors come to mind and have been eluded to previously. First, like IL-10, complete loss of LILRB1 could lead to an under-inhibited immune response and potentially auto-immunity. There has in fact been a report of a lower

expressing LILRB1 variant associating with higher rheumatoid arthritis (35, 160). Second, as with the KIRs, LILRB1 inhibition plays a role in reproduction. HLA-G is expressed on fetal trophoblast cells and this is thought to play a part in protecting the allogeneic fetus from attack by maternal NK cells (161). While CD94/NKG2A and LILRB1, both of which bind HLA-G, are expressed only on a fraction of peripheral blood NK cells, when decidual NK cells are examined they all express one or both of these receptors (161). LILRB1 on these tissue specific NK cells may be induced once cells are recruited there or alternatively, those with high LILRB1 expression may be recruited to the placenta. The role of LILRB1 on NK cells in the placenta underscores the need for tight regulation of the gene and constraints on diversity.

6.8.2 Potential Selective Pressure from CMV in driving expression of LILRB1 on NK cells

A factor likely to be playing a selective role in the evolution is the CMV virus that specifically targeted LILRB1 by producing an MHC-I mimic, UL18, for which LILRB1 has a high affinity for compared to endogenous MHC-I molecules (57). CMV is relatively prevalent in the population and while generally asymptomatic, it does play a role in situations of immune suppression, such as infection with HIV (113) or pharmaceutical suppression after organ transplant (reviewed in (162)). While CMV plays a role in survival in these groups, this is unlikely to have large historical impacts on the evolution of LILRB1 due to both of these states of immunosuppression being relatively recent in human history as well as often affecting people at an older age. However, CMV is also the most common congenital

infection and causes disease in in 10% of infected infants (reviewed in (163)). Of those, 10%, the fatality rate in the first 6 weeks of life is 12% and several other long term effects are seen in those that do survive, including infants who were initially asymptomatic (reviewed in (163)). This gives CMV the potential to be a strong driver of LILRB1 evolution. It would be logical however that if this was the only driving force in LILRB1 evolution, this inhibitory receptor would be selected against on NK cells and expression driven down, particularly as LILRB1+ NK cells are inhibited by UL18, while LILRB1- NK cells are activated (28).

6.8.3 Other factors influencing LILRB1 evolution

There are other factors that could logically be driving down expression of LILRB1 in addition to CMV. First there are the bacterial and viral ligands mentioned in the introduction. LILRB1 binds to bacterial species such as Staphylococcus aureus and inhibits TLR activity, potentially assisting the bacteria with evasion (62). This initial report has not been followed up and the role of NK cell LILRB1 binding to bacteria is not clear at this point. The bacterial binding study is largely done with a focus on the mouse receptor, PirB. As mentioned, PirB is not expressed on NK cells in mice. LILRB1 also binds to Dengue virus and inhibits the activation signal received through the Fcγ receptor, decreasing the interferon response and allowing for enhanced viral replication in monocytes through antibody-mediated uptake of the virus. Dengue might also interact with LILRB1+ NK cells that have the CD16 and perturb their function (reviewed in (164)). Finally, there is also a potential role for LILRB1 in cancer detection. Some tumor cells upregulate HLA-G on their surface

(reviewed in (165, 166)), in which case high LILRB1 on NK cells would likely result in poor detection and elimination of these transformed cells. However, once again, this may impact evolution minimally as often cancer impacts people after reproductive age.

In this thesis, I began to examine the CMV relationship with LILRB1. I did not address the other factors mentioned above but this is an interesting area and there is a lot to be learned about the many selective pressures acting on LILRB1 evolution.

6.9 Relationship between CMV and LILRB1 polymorphism

6.9.1 Consequences of LILRB1 protein changes in binding to UL18

There are coding changes in the LILRB1 binding domains that are in linkage disequilibrium with the SNPs of the regulatory regions that correlate with expression in NK cells. I wanted to examine these protein variants as well as the previously discussed promoter variants. This study began with an observation that one of the two major protein variants of LILRB1 is more "LILRB2-like". LILRB1 and B2 are very similar in amino acid sequence, with 77% identity overall and 82% identity in D1 and D2, which are the domains involved in binding to both MHC-I and UL18 ligands. Interestingly, while LILRB1 and B2 both bind to their MHC-I ligands similarly, B1 binds UL18 with a 1000 fold higher affinity than it binds MHC-I, while B2 does not have significant binding to UL18 (57, 167). These observations led me to hypothesize that the "PTTI" variant that more closely resembles LILRB2 would bind UL18 with a decreased affinity. Unexpectedly, I identified stronger binding to

UL18 by this PTTI variant. This result was somewhat inconsistent in my hands, however, it has since been reproduced by another student in the lab, Kang Yu, as shown in figure 5.3b. The higher binding PTTI variant is largely associated with the "low" promoter haplotype. This could potentially indicate a balancing mechanism within the gene. The higher binding could have driven this variant to be expressed at a lower level on NK cells to counter this evasion mechanism by HCMV and limit the level of inhibition received.

6.9.2 Association between LILRB1 genotype and CMV susceptibility in solid organ transplant patients

In addition to the relationship with UL18 specifically, we wanted to examine the broader relationship between LILRB1 diversity and CMV. During the course of my research, evidence for an association of LILRB1 polymorphism with controlling CMV was reported in HIV patients, another group with substantial immune deficiency (113). In this study, the association was relatively weak and was only apparent in the Caucasian population.

In collaboration with Dr. Atul Humar, I was provided 67 samples from an existing cohort of Canadian transplant patients to examine LILRB1 genotype and CMV disease or viremia post-transplant. I found a trend with three of the five polymorphisms examined. One SNP within the distal promoter, one in the proximal promoter and one in the coding region at the 5' end, which produces a non-synonymous change within the D2 domain of the LILRB1 extracellular region. The

strongest association was at position +927, which causes a non-synonymous change between an Isoleucine and a Threonine. This is the same residue that the association was observed for in the previously mentioned study in an HIV positive population (113). We observed a trend towards more disease in the absence of the Isoleucine containing allele while in the previous study they found less disease in the heterozygous donors relative to those homozygous for the Isoleucine containing allele. Note that the threonine/isoleucine change studied for association with CMV complications post-transplant is represented by the third of the four amino acids in our two variants, the second "T" in PTTI. It does not reach the standard p value of .05 to consider reliable as the number of patients homozygous for the minor allele is too small. However, the potential association of the Threonine at position 142 with more CMV disease is consistent with the binding data to UL18 wherein the PTTI variant has higher binding. This fits with a model that UL18 provides stronger suppression of the immune response, perhaps through NK cells.

With promising preliminary results from our pilot study, we examined a much larger cohort through the Swiss Transplant Cohort Study, which collects data on all organ transplant recipients in Switzerland (114). In this cohort, we found a stronger association between a polymorphism in the LILRB1 distal promoter and susceptibility to CMV viremia (Appendix and section 5.2.3), however, this was specifically in kidney transplant patients and not significant in the full patient cohort. In view of the multiple reports on association of KIR genotype and CMV

susceptibility (109-112), a multivariate analysis by the STCS group addressed this and confirmed LILRB1 as an independently segregating variable.

It is not clear why there is a specific correlation between LILRB1 genotype and susceptibility to CMV in kidney transplant patients. However, I hypothesize that it is related to the degree of immunosuppression. Heart and lung transplant patients receive the strongest immunosuppression, which could lead to a higher incidence overall of CMV complications in these patients regardless of LILRB1 genotype. In a previous study of the patients within the STCS examining the correlation between KIR haplotype and CMV viremia, it was discovered that the role for KIR genotype is predominantly in patients receiving the highest level of immune suppression (all heart and lung recipients as well as kidney recipients given ATG, a stronger immunosuppressant) (168). In our study, in the sub-population of kidney transplant patients treated with ATG, we would expect to see the effect of LILRB1 genotype absent, as in the heart and lung transplant recipients.

In both CMV cohorts, we examined only LILRB1 genotype. It is possible that the strain of the virus and particularly the UL18 variant may also play a role. In our binding assays, we used the AD169 strain variant of UL18. This variant is reported to have the lowest binding to LILRB1 (169). Ideally in future studies of additional CMV cohorts, genotyping of UL18 would also be done to see if this plays any role in the level of CMV complications observed.

6.10 Model of LILRB1 evolution

Humans have a genetic predisposition to either frequently express LILRB1 or not on NK cells. There are likely several aspects involved in this mechanism, including epigenetic regulation and genotype, as well as environmental aspects both within and around the cell. The chimpanzee may represent the ancestral state of the gene, having the high promoter haplotype (AGG) and a modified high binding variant in domains 1 and 2 of the protein (STTI) (Figure 6.5). It would be beneficial to determine whether the same diversity is present in chimpanzees. Presence of both haplotypes in chimpanzees could indicate whether a similar selection process is occurring in this species parallel to the selection that we presume to be happening in humans. It is possible that this highly expressed and high UL18 binding allele lead to excessive inhibition and thus high replication of the virus when HCMV acquired UL18. Particularly if this were in the early stages of the relationship between humans and HCMV, the virus may not have become attenuated to a point of co-existing in the population and could have been a strong selection pressure in the population. There are two ways this inhibition could have been driven down: one is by decreasing expression frequency in the cell population and the other is by decreased binding to the viral ligand, UL18, as illustrated in Figure 6.5. The two current common alleles represent just that. The AGG-LAIS haplotype has higher expression due to the "high" promoter variant but the LAIS variant of the protein has reduced binding to UL18. The GAA-PTTI haplotype, while maintaining higher binding to the UL18 viral ligand, has the "low" promoter variant, correlated with a decrease in the amount of cells in the population expressing LILRB1. While this model is speculative and makes

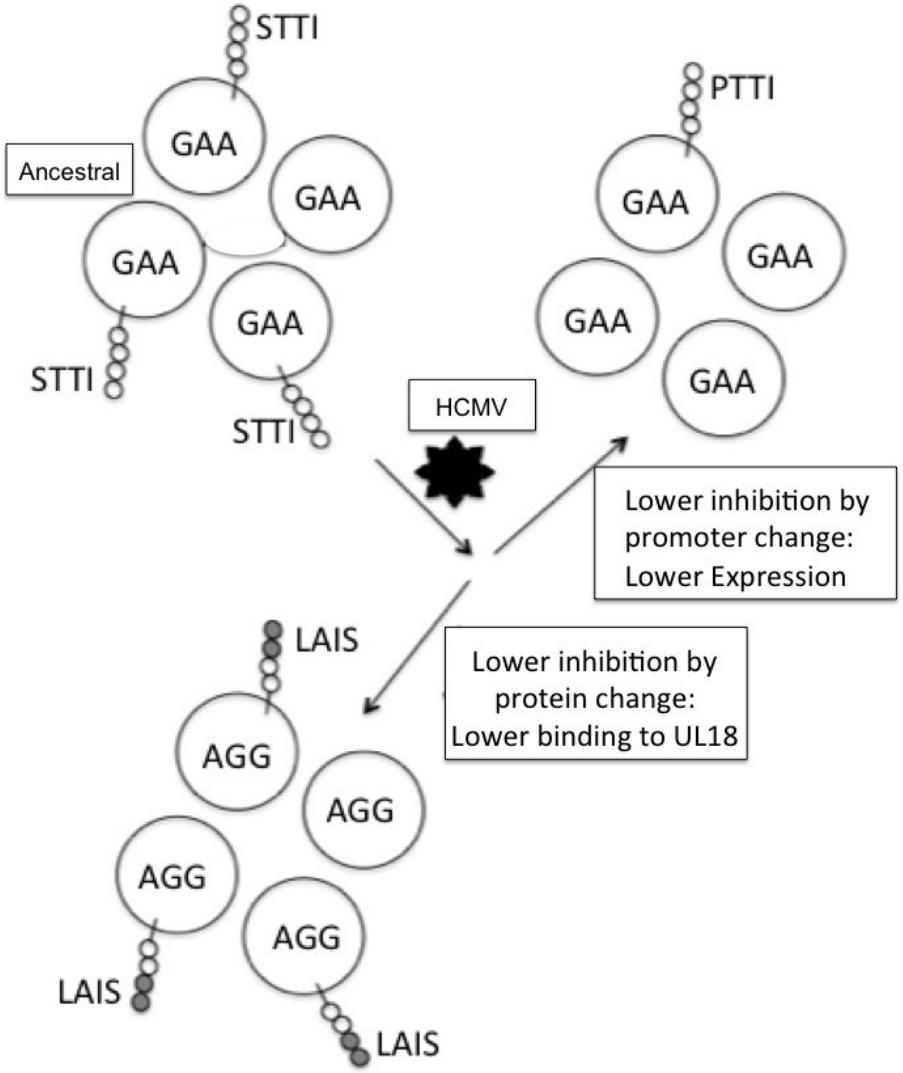


Figure 6.5 Model of HCMV driven evolution of the LILRB1 receptor on NK cells.

The ancestral LILRB1 is predicted based on the available chimpanzee sequence to be AGG-STTI. The AGG indicates the proximal promoter haplotype (nucleotide sequence), while STTI indicates the protein variant of domains D1 and D2 (amino acid sequence). This is a combination of the two major haplotypes we see in humans. We model that in humans, exposure to HCMV has driven LILRB1 to have lower inhibition from UL18 by two mechanisms. One is a haplotype GAA-PTTI, which maintains high binding but through modification of the promoter, exhibits lower expression on NK cells. The other is haplotype AGG-LAIS, which maintains high expression on NK cells but has lowered binding affinity through modifications of the LILRB1 protein.

assumptions about the sequence of events in evolution, it provides a working model for how variation in regulatory elements and the LILRB1 protein may have arisen and potential roles for both.

In a broader context than just CMV, lower LILRB1 expression could potentially lead to higher risk of autoimmunity and complications in pregnancy, while high LILRB1 expression could increase susceptibility to infection as well as certain types of cancers. We have healthy donors in the range of 25-85% LILRB1+ NK cells (22, 29), indicating this is likely all within a functional range. It would be interesting to know if anything different would be observed in a donor with 0 or 100% LILRB1 expression, or if there is a reason we do not see either of these extremes.

6.11 Significance and Conclusions

Our initial identification of two distinct haplotypes associated with low and high expression LILRB1 in NK cells (29) raised the questions of how polymorphisms selectively influence expression in NK cells and if the different alleles influence the immune response. In this thesis, I have provided information on how LILRB1 is regulated in NK cells relative to other cell types and ruled out many possible mechanisms for how the SNPs function to drive the expression levels. The body of work provides new clues to be explored to provide a mechanistic understanding how the SNPs regulate expression, particularly that the proximal promoter region might be a repressor and that it might be relevant in cytokine stimulated NK cells. Knowledge of an inherent genetic predisposition to increased risk of complications due to HCMV

could be used in determining the course of anti-viral treatment and monitoring in transplant patients. Identification of a SNP correlated with susceptibility to CMV in transplant patients provides potential for a simple assay to add to the genetic screening done prior to an organ transplant, particularly in those that are between a CMV+ donor and a CMV- recipient that could be used to guide treatment regimes with available antivirals. Finer mapping of the genetic association could refine and strengthen the prognostic value.

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APPENDIX A1: Results from collaboration with the Swiss Transplant Cohort Study

Table A1.1 Polymorphisms analyzed in Swiss transplant cohort

Gene	rs number	aa/nt change	MAF	HWE_Pex	remarks	N of patients with genotypes
LILRB1	*rs1061680*	C+425T/ I142T	0.29	0.399	Potentially functional SNP	N=1018[a]
LILRB1	*rs10423364*	A-630G	0.33	0.521	Surrogate in GWAS (LD R^2=0.82)	N=1018

a) in order to allow comparison between SNPs, 100 additional patients who had *rs1061680* but not *rs10423364* genotypes were not included in the initial analyses.

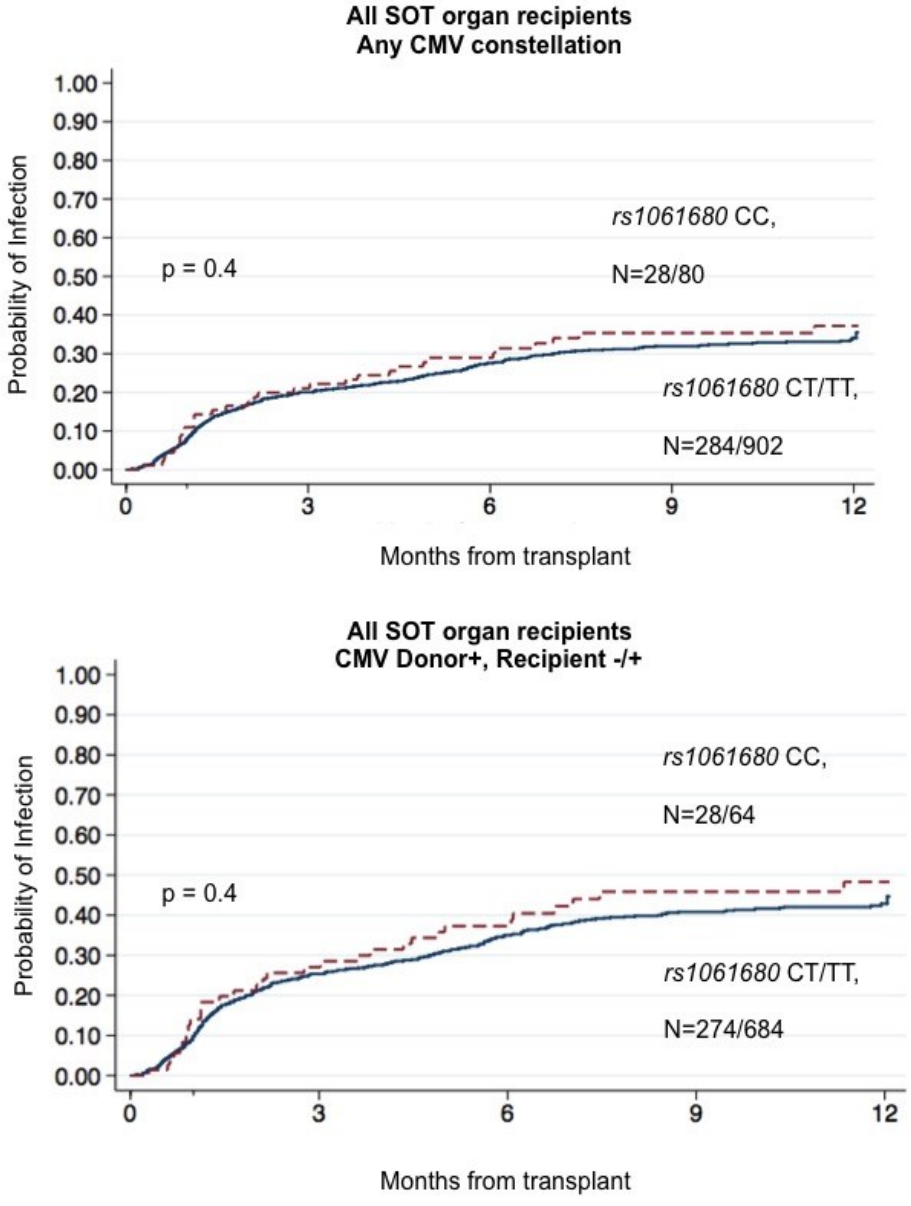

Figure A1.1 Cumulative incidence of CMV infection according to rs1061680 polymorphisms in recipients of all types of organ transplants.

An association between genotype at position rs1061680 and CMV infection was not significant (p=0.4) when all recipients were examined, nor when only those who received organs from HCMV positive donors were examined. The dotted red line shows donors homozygous for the minor allele and the solid blue line indicates the donors homozygous for the major allele grouped with the heterozygous donors. The probability of infection is shown on the y axis and the months post transplant on the x axis.

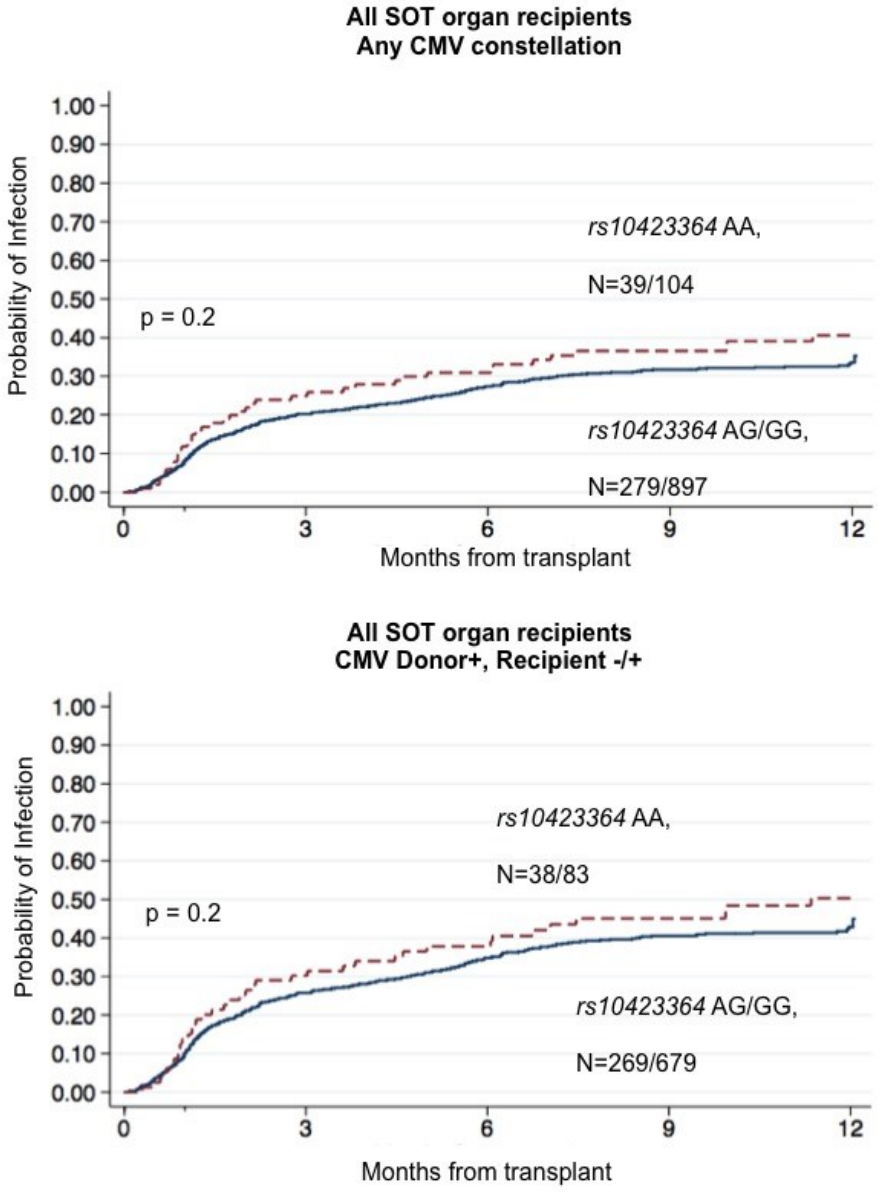

Figure A1.2 Cumulative incidence of CMV infection according to rs10423364 (position -14894) polymorphisms in recipients of all types of organ transplants.

An association between genotype at position rs10423364 and CMV infection was not significant when all recipients were examined (p=0.2), nor when only those who received organs from HCMV positive donors were examined (p=0.2). The dotted red line shows donors homozygous for the minor allele and the solid blue line indicates the donors homozygous for the major allele grouped with the heterozygous donors. The probability of infection is shown on the y axis and the months post transplant on the x axis.

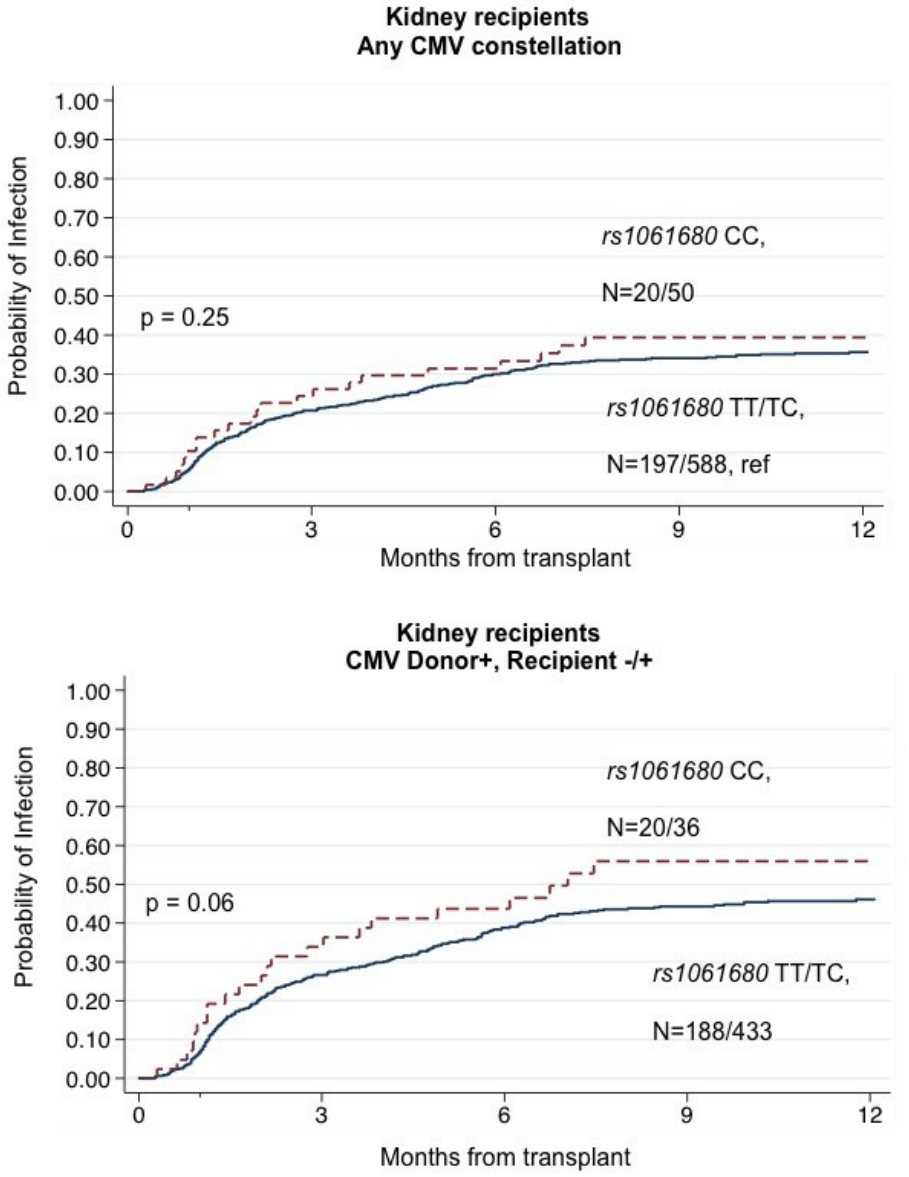

Figure A1.3 Cumulative incidence of CMV infection according to rs1061680 (position +927) polymorphisms in recipients of kidney transplants.

An association between genotype at position rs1061680 and CMV infection was not observed with all patients were examined (p=0.25) but was observed when only those who received organs from HCMV positive donors were examined (p=0.06). The dotted red line shows donors homozygous for the minor allele and the solid blue line indicates the donors homozygous for the major allele grouped with the heterozygous donors. The probability of infection is shown on the y axis and the months post transplant on the x axis.

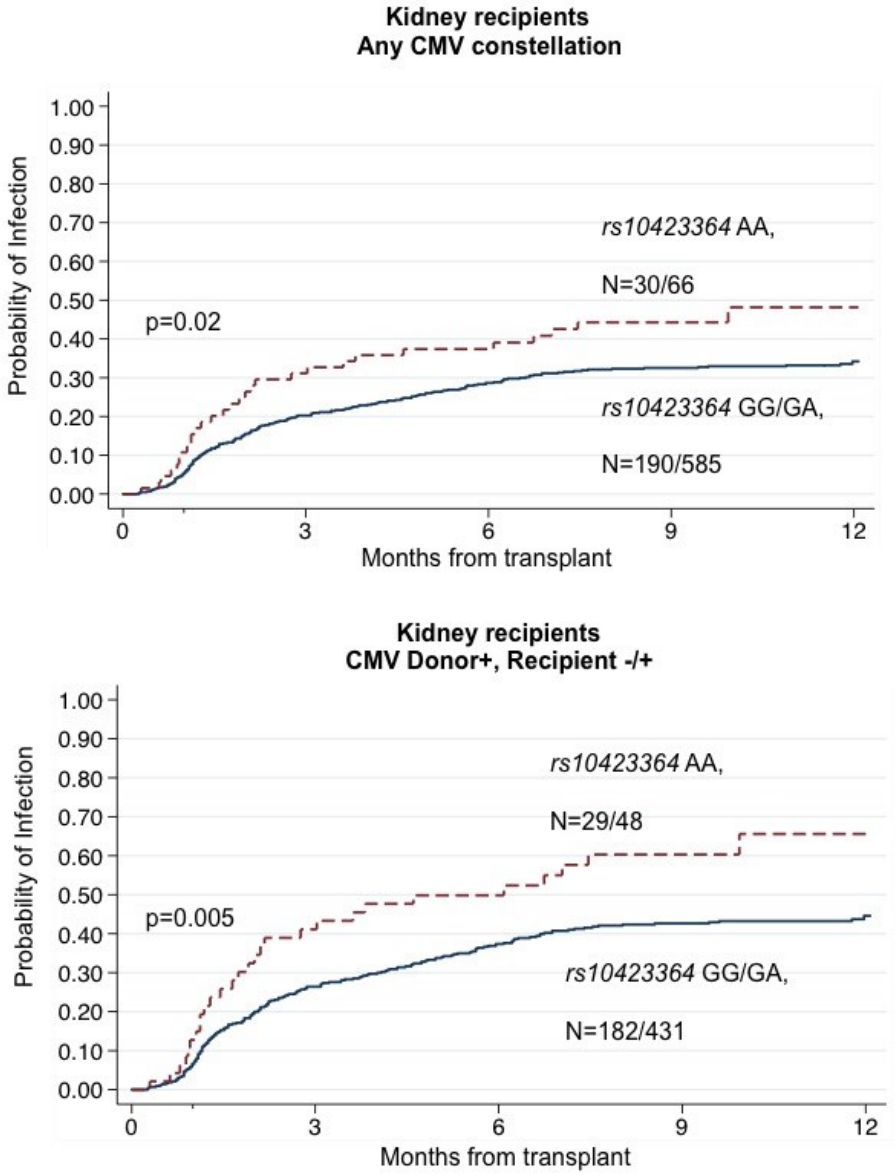

Figure A1.4 Cumulative incidence of CMV infection according to rs10423364 (position -14894) polymorphisms in recipients of kidney transplants.

An association between genotype at position rs10423364 and CMV infection was significant when all recipients were examined (p=0.02), and even more strongly when only those who received organs from HCMV positive donors were examined (p=0.005). The dotted red line shows donors homozygous for the minor allele and the solid blue line indicates the donors homozygous for the major allele grouped with the heterozygous donors. The probability of infection is shown on the y axis and the months post transplant on the x axis.

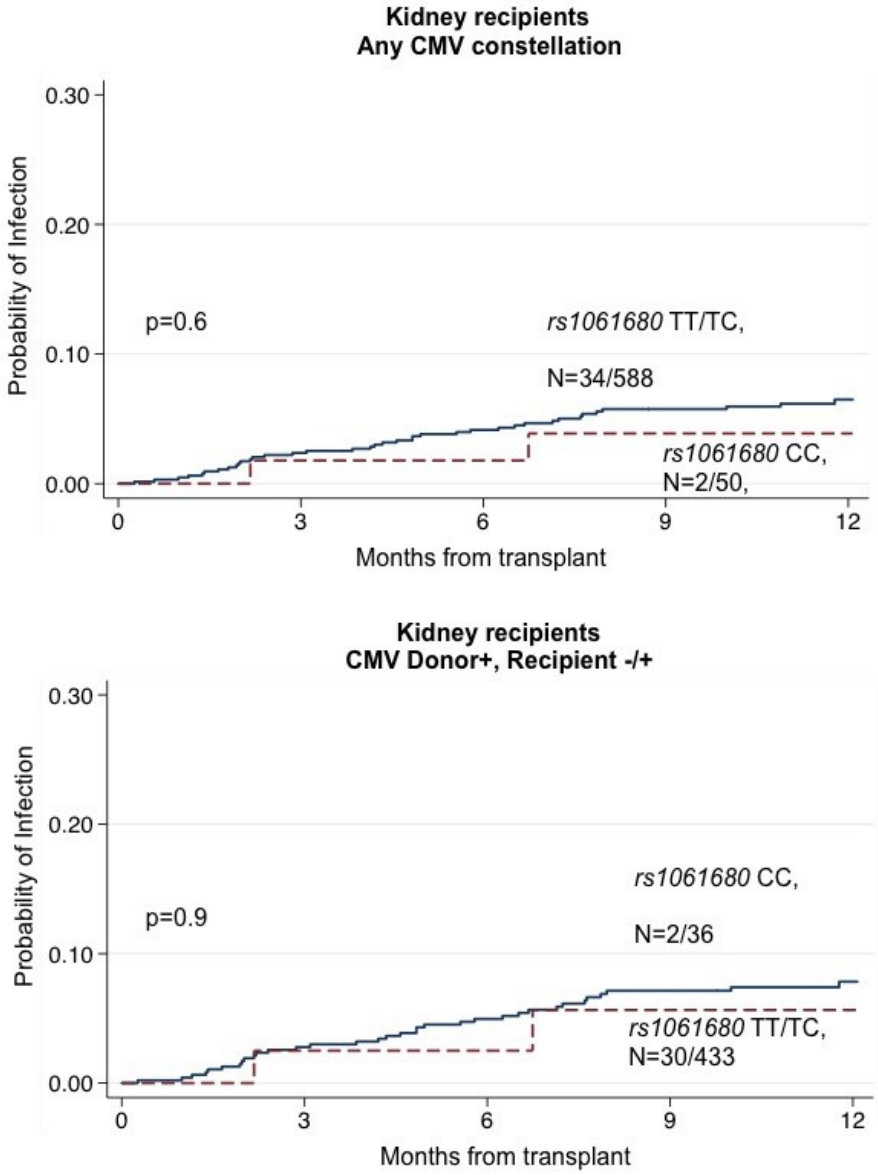

Figure A1.5 Cumulative incidence of CMV disease according to rs1061680 (position +927) polymorphisms in recipients of kidney transplants.

An association between genotype at position rs1061680 and CMV disease was not significant (p=0.6) when all recipients were examined, nor when only those who received organs from HCMV positive donors were examined (p=0.9). The dotted red line shows donors homozygous for the minor allele and the solid blue line indicates the donors homozygous for the major allele grouped with the heterozygous donors. The probability of infection is shown on the y axis and the months post transplant on the x axis.

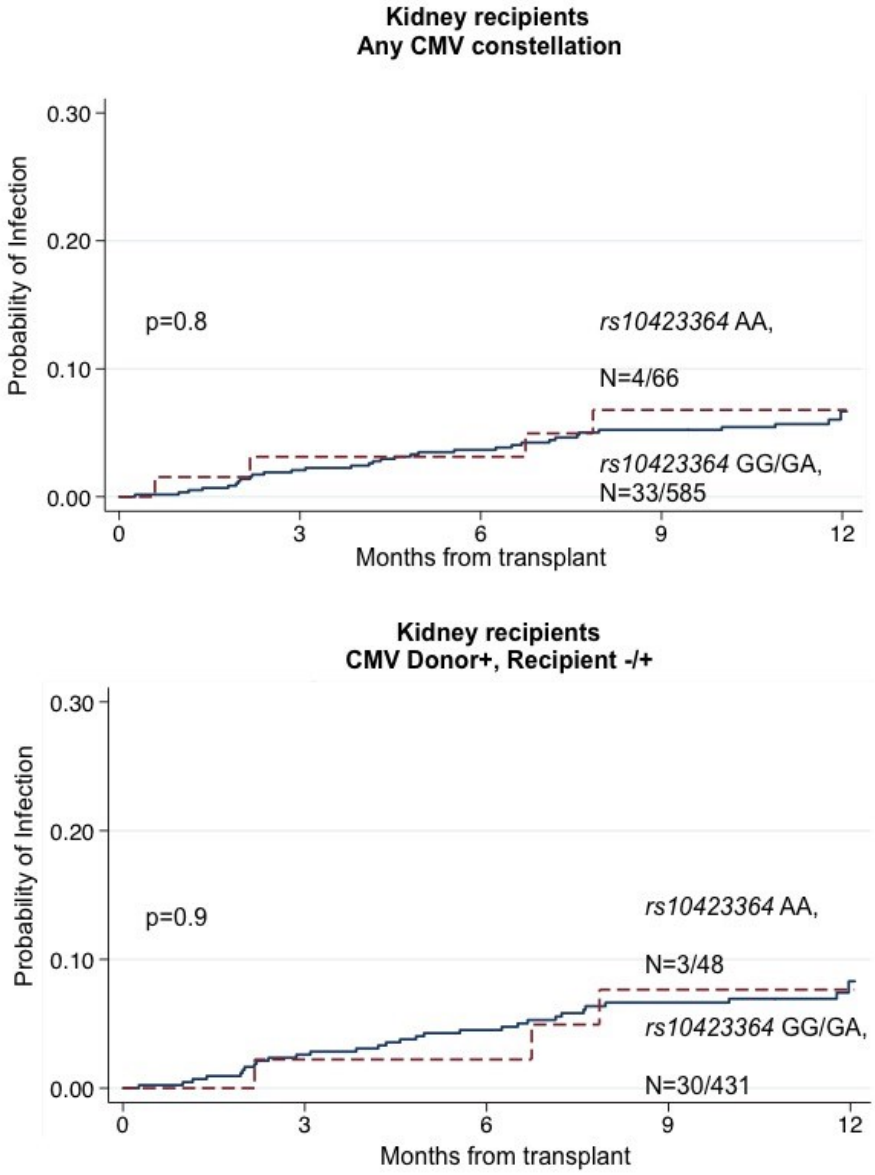

Figure A1.6 Cumulative incidence of CMV disease according to rs10423364 (position -14894) polymorphisms in recipients of kidney transplants.

An association between genotype at position rs10423364 and CMV disease was not significant (p=0.8) when all recipients were examined, nor when only those who received organs from HCMV positive donors were examined (p=0.9). The dotted red line shows donors homozygous for the minor allele and the solid blue line indicates the donors homozygous for the major allele grouped with the heterozygous donors. The probability of infection is shown on the y axis and the months post transplant on the x axis.

Table A1.2 Summary of Swiss cohort data analyzing CMV viremia and disease relative to LILRB1 genotype at positions -14894 (rs10423364) and +927 (rs1061680).

	All organs			Kidney		
SNP	Any CMV constellation N=1018	Only D+ or/and R+ N=776	Only D+/R- N=194	Any CMV constellation N=651	Only D+ or/and R+ N=479	Only D+/R- N=117
CMV infection						
LILRB1 rs1061680[1]	P=0.4	P=0.4	P=0.5	P=0.25	P=0.06[2]	P=0.5
LILRB1 rs10423364[1]	P=0.2	P=0.2	P=0.9	P=0.02	P=0.005	P=0.9
CMV disease						
LILRB1 rs1061680[1]	P=0.8	P=0.9	P=0.6[3]	0.6	0.9	P=0.3[3]
LILRB1 rs10423364[1]	P=1.0	P=0.7	P=0.2[3]	0.8	0.9	P=0.16[3]

[1] P values assessed by log-rank test, recessive mode
[2] P=0.2 when all the patients with *rs1061680* (N=1118)
[3] These results should be treated with caution cause numbers of events were very small

Table A1.3 Multivariate analysis in kidney transplant recipients (CMV Donor+, Recipient+/-)

	CMV infection in kidney CMV D+/R- or R+					
	Univariate analysis			Multivariate analysis		
Variable	HR	(95%CI)	P value [1]	HR	(95%CI)	P value [1]
LILRB genotypes available (N=476)						
Recipient male sex	0.93	(0.70-1.22)	0.6	-	-	-
Recipient age (per year)	1.02	(1.01-1.03)	**0.0003**	1.02	(1.01-1.03)	**<0.00001**
LILRB1 genotypes						
rs10423364 AA vs. GG/GA	1.74	(1.18-2.58)	**0.005**	1.69	(1.13-2.54)	**0.01**
rs1061680 CC vs. TT/TC	1.56	(0.98-2.47)	0.06	-	-	-
CMV serostatus						
D+/R-	reference					
D-/R+	0.87	(0.59-1.29)	0.5	-	-	-
D+/R+	1.50	(1.07-2.10)	**0.02**	0.59	(0.43-0.81)	**0.001**
Corticosteroids	2.25	(1.11-4.58)	**0.03**	2.17	(1.06-4.46)	**0.03**
Ciclosporine	2.33	(1.73-3.12)	**<0.00001**	2.00	(1.46-2.76)	**0.00002**
Tacrolimus	0.47	(0.35-0.63)	**<0.00001**	-	-	-
Rejection episodes	1.46	(1.05-2.05)	**0.03**	1.94	(1.37-2.75)	**0.0002**
Exposure to antiviral drugs	0.27	(0.19-0.39)	**<0.00001**	0.25	(0.17-0.36)	**<0.00001**
LILRB and KIR genotypes available (N=263)						
Recipient male sex	0.71	(0.45-1.12)	0.14	-	-	-
Recipient age (per year)	1.02	(1.00-1.03)	**0.05**	1.02	(1.00-1.04)	**0.02**
LILRB1 genotypes						
rs10423364 AA vs. GG/GA	2.13	(1.15-3.95)	**0.02**	2.10	(1.11-3.98)	**0.02**
rs1061680 CC vs. TT/TC	1.64	(0.79-3.43)	0.18	-	-	-
KIR haplotype B[2]	1.40	(0.82-2.40)	0.2	-	-	-
KIR gene *KIR2DS4*	0.69	(0.42-1.13)	0.14	0.68	(0.40-1.13)	0.14
CMV serostatus						
D+/R-	reference					
D-/R+	0.60	(0.29-1.20)	0.15	0.44	(0.25-0.76)	**0.004**
D+/R+	1.33	(0.74-2.38)				
Corticosteroids	2.76	(1.00-7.58)	**0.05**	3.16	(1.12-8.87)	**0.03**
Ciclosporine	3.18	(1.94-5.21)	**<0.00001**	2.48	(1.46-4.19)	**0.001**
Tacrolimus	0.37	(0.22-0.60)	**0.00006**	-	-	-
Rejection episodes	1.25	(0.71-2.22)	0.4	-	-	-
Exposure to antiviral drugs	0.33	(0.18-0.61)	**0.0004**	0.36	(0.19-0.68)	**0.002**

[1] P value assessed by multivariate Cox regression model

[2] KIR haplotype B was defined by the presence of one or more of the following genes: *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5* and *KIR3DS1*

APPENDIX A2: Results from mass spectrometry analysis

Table A2.1 Raw data from mass spectrometry analysis on band 2 (see figure 3.8).

Family	Member	Database	Accession	Score	Mass	#Distinct matches	#Significant matches	#Sequences	#Significant Sequences	emPAI	Description
1	1	SwissProt	TRYP_PIG	439	24394	34	21	4	4	1.18	Trypsin OS=Sus scrofa PE=1 SV=1
2	1	SwissProt	K2C1_HUMAN	372	65999	10	10	10	10	1.07	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
3	1	SwissProt	EF1A1_BOVIN	326	50109	23	14	11	8	1.16	Elongation factor 1-alpha 1 OS=Bos taurus GN=EEF1A1 PE=2 SV=1
4	1	SwissProt	K1C9_HUMAN	237	62027	9	5	8	5	0.47	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3
5	1	SwissProt	ACTB_BOVIN	209	41710	8	6	7	6	0.99	Actin, cytoplasmic 1 OS=Bos taurus GN=ACTB PE=1 SV=1
6	1	SwissProt	ARP3_BOVIN	196	47341	9	8	9	8	1.25	Actin-related protein 3 OS=Bos taurus GN=ACTR3 PE=1 SV=3
7	1	SwissProt	HNRPF_HUMAN	166	45643	4	3	4	3	0.37	Heterogeneous nuclear ribonucleoprotein F OS=Homo sapiens GN=HNRNPF PE=1 SV=3
8	1	SwissProt	EF1G_HUMAN	131	50087	7	5	7	5	0.62	Elongation factor 1-gamma OS=Homo sapiens GN=EEF1G PE=1 SV=3
9	1	SwissProt	SAV_STRAV	129	18822	3	3	3	3	1.13	Streptavidin OS=Streptomyces avidinii PE=1 SV=1
10	1	SwissProt	TBA_TORMA	121	50136	4	3	4	3	0.33	Tubulin alpha chain OS=Torpedo marmorata PE=2 SV=1
11	1	SwissProt	FEN1_HUMAN	108	42566	3	3	3	3	0.4	Flap endonuclease 1 OS=Homo sapiens GN=FEN1 PE=1 SV=1
12	1	SwissProt	CALX_HUMAN	99	67526	2	2	2	2	0.15	Calnexin OS=Homo sapiens GN=CANX PE=1 SV=2
13	1	SwissProt	1A74_HUMAN	95	40865	3	1	3	1	0.12	HLA class I histocompatibility antigen, A-74 alpha chain OS=Homo sapiens GN=HLA-A PE=1 SV=1
14	1	SwissProt	GPX1_MOUSE	85	22316	3	3	3	3	0.9	Glutathione peroxidase 1 OS=Mus musculus GN=Gpx1 PE=1 SV=2
15	1	SwissProt	TERA_BOVIN	81	89274	2	2	1	1	0.06	Transitional endoplasmic reticulum ATPase OS=Bos taurus GN=VCP PE=2 SV=1
16	1	SwissProt	RCC1_HUMAN	69	44941	3	3	3	3	0.38	Regulator of chromosome condensation OS=Homo sapiens GN=RCC1 PE=1 SV=1
17	1	SwissProt	FA98B_HUMAN	63	37167	1	1	1	1	0.14	Protein FAM98B OS=Homo sapiens GN=FAM98B PE=1 SV=1
18	1	SwissProt	K1C10_CANFA	63	57676	6	4	4	3	0.28	Keratin, type I cytoskeletal 10 OS=Canis familiaris GN=KRT10 PE=2 SV=1
19	1	SwissProt	DX39B_CAEEL	62	48462	2	1	2	1	0.1	Spliceosome RNA helicase DDX39B Homolog OS=Caenorhabditis elegans GN=hel-1 PE=2 SV=1
20	1	SwissProt	RS7_BOVIN	59	22113	3	2	3	2	0.54	40S ribosomal protein S7 OS=Bos taurus GN=RPS7 PE=2 SV=1
21	1	SwissProt	PRDX2_MOUSE	53	21765	3	2	2	1	0.24	Peroxiredoxin-2 OS=Mus musculus GN=Prdx2 PE=1 SV=3
22	1	SwissProt	NHRF1_BOVIN	51	39579	1	1	1	1	0.13	Na(+)/H(+) exchange regulatory cofactor NHE-RF1 OS=Bos taurus GN=SLC9A3R1 PE=2 SV=1
23	1	SwissProt	IRF4_HUMAN	50	51739	3	2	3	2	0.2	Interferon regulatory factor 4 OS=Homo sapiens GN=IRF4 PE=1 SV=1
24	1	SwissProt	DNAJ1_CHLAE	47	44853	2	1	2	1	0.11	DnaJ homolog subfamily A member 1 OS=Chlorocebus aethiops GN=DNAJA1 PE=2 SV=1
25	1	SwissProt	A2MG_BOVIN	45	167470	1	1	1	1	0.03	Alpha-2-macroglobulin OS=Bos taurus GN=A2M PE=1 SV=2
26	1	SwissProt	IFAA1_BOVIN	43	46125	4	1	4	1	0.11	Eukaryotic initiation factor 4A-I OS=Bos taurus GN=EIF4A1 PE=2 SV=1
27	1	SwissProt	PA2G4_HUMAN	42	43759	1	1	1	1	0.12	Proliferation-associated protein 2G4 OS=Homo sapiens GN=PA2G4 PE=1 SV=3
28	1	SwissProt	GDIB_HUMAN	39	50631	3	1	3	1	0.1	Rab GDP dissociation inhibitor beta OS=Homo sapiens GN=GDI2 PE=1 SV=2
29	1	SwissProt	PS4_PINST	38	870	19	2	1	1	56.21	Putative LRR disease resistance protein/transmembrane receptor kinase PS4 (Fragment) OS=Pinus strobus PE=1 SV=1
30	1	SwissProt	PGDH_BOVIN	36	29007	1	1	1	1	0.18	15-hydroxyprostaglandin dehydrogenase [NAD+] OS=Bos taurus GN=HPGD PE=2 SV=1
31	1	SwissProt	SKI_MOUSE	21	80069	1	1	1	1	0.06	Ski oncogene OS=Mus musculus GN=Ski PE=2 SV=2
32	1	SwissProt	ITB1_BOVIN	21	88036	1	1	1	1	0.06	Integrin beta-1 OS=Bos taurus GN=ITGB1 PE=1 SV=3
33	1	SwissProt	RS7_LACSS	19	17965	1	1	1	1	0.3	30S ribosomal protein S7 OS=Lactobacillus sakei subsp. sakei (strain 23K) GN=rpsG PE=3 SV=1
34	1	SwissProt	DNAK_ALKMQ	17	66452	2	1	1	1	0.08	Chaperone protein DnaK OS=Alkaliphilus metalliredigens (strain QYMF) GN=dnaK PE=2 SV=1

Table A2.2 Raw data from mass spectrometry analysis on band 3 (see figure 3.8).

Family	Member	Database	Accession	Score	Mass	#of matches	#Significant	#Sequences	#Significant	emPAI	Description
1	1	SwissProt	TRYP_PIG	645	25078	48	33	4	4	1.59	Trypsin OS=Sus scrofa PE=1 SV=1
2	1	SwissProt	ACT2_MOLO	253	42521	14	11	9	8	1.76	Actin, muscle-type OS=Molgula occulta PE=3 SV=1
2	2	SwissProt	ACT1_AEDAE	232	42045	12	9	8	7	1.22	Actin-1 OS=Aedes aegypti GN=ACT-1 PE=2 SV=2
2	3	SwissProt	ACT2_XENLA	194	42304	14	9	9	7	1.48	Actin, alpha 3, skeletal, muscle-2 OS=Xenopus laevis GN=act2 PE=2 SV=1
2	4	SwissProt	ACT17_DICD	132	41773	7	7	5	5	0.99	Actin-17 OS=Dictyostelium discoideum GN=act17 PE=3 SV=1
3	1	SwissProt	MYSS_CHICK	198	223976	4	4	3	3	0.07	Myosin heavy chain, skeletal muscle, adult OS=Gallus gallus PE=1 SV=4
3	2	SwissProt	MYH1_BOVIN	114	223764	2	2	2	2	0.04	Myosin-1 OS=Bos taurus GN=MYH1 PE=2 SV=2
4	1	SwissProt	SEPT2_BOVIN	184	41830	4	3	4	3	0.41	Septin-2 OS=Bos taurus GN=SEPT2 PE=2 SV=1
5	1	SwissProt	1A01_HUMAN	153	41105	3	3	3	3	0.42	HLA class I histocompatibility antigen, A-11 alpha chain OS=Homo sapiens GN=HLA-A PE=1 SV=1
6	1	SwissProt	A2MG_BOVIN	144	168953	6	6	5	5	0.15	Alpha-2-macroglobulin OS=Bos taurus GN=A2M PE=1 SV=2
7	1	SwissProt	ALDOA_HUMAN	139	39851	6	4	6	4	0.62	Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2
8	1	SwissProt	K2C1_HUMAN	131	66170	4	3	4	3	0.24	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
9	1	SwissProt	PGK1_HUMAN	120	44985	9	7	9	7	1.11	Phosphoglycerate kinase 1 OS=Homo sapiens GN=PGK1 PE=1 SV=3
10	1	SwissProt	EF1A1_BOVIN	118	50451	8	4	5	4	0.46	Elongation factor 1-alpha 1 OS=Bos taurus GN=EEF1A1 PE=2 SV=1
11	1	SwissProt	CALX_HUMAN	108	67982	2	1	2	1	0.07	Calnexin OS=Homo sapiens GN=CANX PE=1 SV=2
12	1	SwissProt	HNRPD_HUMAN	78	38581	3	2	3	2	0.28	Heterogeneous nuclear ribonucleoprotein D0 OS=Homo sapiens GN=HNRNPD PE=1 SV=1
13	1	SwissProt	SAV_STRAV	77	18822	3	3	3	3	1.13	Streptavidin OS=Streptomyces avidinii PE=1 SV=1
14	1	SwissProt	THIL_BOVIN	70	45146	1	1	1	1	0.11	Acetyl-CoA acetyltransferase, mitochondrial OS=Bos taurus GN=ACAT1 PE=2 SV=1
15	1	SwissProt	JUNB_HUMAN	68	36028	6	2	6	2	0.3	Transcription factor Jun-B OS=Homo sapiens GN=JUNB PE=1 SV=1
16	1	SwissProt	ACTN1_BOVIN	64	103486	1	1	1	1	0.05	Alpha-actinin-1 OS=Bos taurus GN=ACTN1 PE=2 SV=1
17	1	SwissProt	PGDH_BOVIN	59	29235	1	1	1	1	0.18	15-hydroxyprostaglandin dehydrogenase [NAD+] OS=Bos taurus GN=HPGD PE=2 SV=1
18	1	SwissProt	CH60_BOVIN	51	61240	1	1	1	1	0.08	60 kDa heat shock protein, mitochondrial OS=Bos taurus GN=HSPD1 PE=1 SV=2
19	1	SwissProt	PCBP2_HUMAN	51	38955	4	3	4	3	0.45	Poly(rC)-binding protein 2 OS=Homo sapiens GN=PCBP2 PE=1 SV=1
20	1	SwissProt	PS4_PINST	51	870	21	3	1	1	56.21	Putative RRD disease resistance protein/transmembrane receptor kinase PS4 (Fragment) OS=Pinus strobus PE=1 SV=1
21	1	SwissProt	AATM_HUMAN	49	47886	1	1	1	1	0.11	Aspartate aminotransferase, mitochondrial OS=Homo sapiens GN=GOT2 PE=1 SV=3
22	1	SwissProt	K1C10_BOVIN	47	54986	3	1	3	1	0.09	Keratin, type I cytoskeletal 10 OS=Bos taurus GN=KRT10 PE=3 SV=1
23	1	SwissProt	ARP2A_DANRE	45	44878	2	1	2	1	0.11	Actin-related protein 2-A OS=Danio rerio GN=actr2a PE=2 SV=1
24	1	SwissProt	THIM_HUMAN	42	42354	1	1	1	1	0.12	3-ketoacyl-CoA thiolase, mitochondrial OS=Homo sapiens GN=ACAA2 PE=1 SV=2
25	1	SwissProt	ILF2_DANRE	39	43110	1	1	1	1	0.12	Interleukin enhancer-binding factor 2 homolog OS=Danio rerio GN=ilf2 PE=2 SV=1
26	1	SwissProt	ITB1_BOVIN	32	91343	1	1	1	1	0.05	Integrin beta-1 OS=Bos taurus GN=ITGB1 PE=1 SV=3
27	1	SwissProt	RSSA_BOVIN	31	32977	2	1	2	1	0.16	40S ribosomal protein SA OS=Bos taurus GN=RPSA PE=2 SV=4
28	1	SwissProt	MBNL1_CHICK	30	40385	1	1	1	1	0.13	Muscleblind-like protein 1 OS=Gallus gallus GN=MBNL1 PE=2 SV=1
29	1	SwissProt	TADBP_HUMAN	27	45053	1	1	1	1	0.11	TAR DNA-binding protein 43 OS=Homo sapiens GN=TARDBP PE=1 SV=1
30	1	SwissProt	SYL_METTP	22	109589	1	1	1	1	0.05	Leucyl-tRNA synthetase OS=Methanosaeta thermophila (strain DSM 1939 / FT) GN=leuS PE=3 SV=1
31	1	SwissProt	G3P1_JACOR	20	39616	1	1	1	1	0.13	Glyceraldehyde-3-phosphate dehydrogenase, muscle OS=Jaculus orientalis PE=1 SV=2

Table A2.3 Raw data from mass spectrometry analysis on band 4 (see figure 3.8).

Family	Member	Database	Accession	Score	Mass	#DifferentMatches	#Significant	#Sequences	#Significant	emPAI	Description
1	1	SwissProt	G3P_HUMAN	735	36201	24	17	17	11	9	2.76 Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3
2	1	SwissProt	TRYP_PIG	630	25078	52	37	37	5	5	2.13 Trypsin OS=Sus scrofa PE=1 SV=1
3	1	SwissProt	K2C1_HUMAN	406	66170	12	10	12	10	10	1.07 Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
4	1	SwissProt	K1C9_HUMAN	261	62255	9	8	9	8	8	0.86 Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3
5	1	SwissProt	SAV_STRAV	160	18822	4	4	4	3	3	1.13 Streptavidin OS=Streptomyces avidinii PE=1 SV=1
6	1	SwissProt	CALX_HUMAN	148	67982	3	3	3	3	3	0.24 Calnexin OS=Homo sapiens GN=CANX PE=1 SV=2
7	1	SwissProt	ALDOA_HUMAN	131	39851	5	4	5	4	4	0.62 Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2
8	1	SwissProt	ROA2_BOVIN	116	36041	4	3	4	3	3	0.49 Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Bos taurus GN=HNRNPA2B1 PE=2 SV=1
9	1	SwissProt	ROAA_MOUSE	76	30926	2	2	2	2	2	0.36 Heterogeneous nuclear ribonucleoproteins A/B OS=Mus musculus GN=Hnrnpab PE=1 SV=1
9	2	SwissProt	HNRDL_CHICK	44	33594	2	2	2	2	2	0.33 Heterogeneous nuclear ribonucleoprotein D-like OS=Gallus gallus GN=HNRPDL PE=2 SV=1
10	1	SwissProt	NCPR_HUMAN	74	77097	2	2	2	2	2	0.13 NADPH--cytochrome P450 reductase OS=Homo sapiens GN=POR PE=1 SV=2
11	1	SwissProt	CH60_BOVIN	69	61240	1	1	1	1	1	0.08 60 kDa heat shock protein, mitochondrial OS=Bos taurus GN=HSPD1 PE=1 SV=2
12	1	SwissProt	PGDH_BOVIN	64	29235	2	1	2	1	1	0.18 15-hydroxyprostaglandin dehydrogenase [NAD+] OS=Bos taurus GN=HPGD PE=2 SV=1
13	1	SwissProt	TERA_BOVIN	64	89958	2	1	1	1	1	0.06 Transitional endoplasmic reticulum ATPase OS=Bos taurus GN=VCP PE=2 SV=1
14	1	SwissProt	RTN3A_XENLA	59	23551	1	1	1	1	1	0.22 Reticulon-3-A OS=Xenopus laevis GN=rtn3-a PE=2 SV=2
15	1	SwissProt	ROA3_HUMAN	58	39799	1	1	1	1	1	0.13 Heterogeneous nuclear ribonucleoprotein A3 OS=Homo sapiens GN=HNRNPA3 PE=1 SV=2
16	1	SwissProt	ENPL_BOVIN	57	92654	1	1	1	1	1	0.05 Endoplasmin OS=Bos taurus GN=HSP90B1 PE=2 SV=1
17	1	SwissProt	ANXA2_BOVIN	56	38873	2	2	2	2	2	0.28 Annexin A2 OS=Bos taurus GN=ANXA2 PE=1 SV=2
18	1	SwissProt	NPM_HUMAN	48	32726	3	2	3	2	2	0.34 Nucleophosmin OS=Homo sapiens GN=NPM1 PE=1 SV=2
19	1	SwissProt	ACT2_SUIBO	49	41979	2	1	2	1	1	0.12 Actin-2 OS=Suillus bovinus GN=ACT2 PE=2 SV=1
20	1	SwissProt	KCAB2_BOVIN	47	41244	2	1	2	1	1	0.12 Voltage-gated potassium channel subunit beta-2 OS=Bos taurus GN=KCNAB2 PE=1 SV=1
21	1	SwissProt	PDIA3_CHLAE	46	57143	1	1	1	1	1	0.09 Protein disulfide-isomerase A3 OS=Chlorocebus aethiops GN=PDIA3 PE=2 SV=1
22	1	SwissProt	PS4_PINST	45	870	22	1	1	1	1	56.21 Putative ERBB4 tyrosine kinase receptor protein/transmembrane receptor protein kinase PS4 (Fragment) OS=Pinus strobus PE=1 SV=1
23	1	SwissProt	ARF1_BOVIN	42	20741	1	1	1	1	1	0.26 ADP-ribosylation factor 1 OS=Bos taurus GN=ARF1 PE=1 SV=2
24	1	SwissProt	HACD3_BOVIN	42	43276	1	1	1	1	1	0.12 3-hydroxyacyl-CoA dehydratase 3 OS=Bos taurus GN=PTPLAD1 PE=2 SV=1
25	1	SwissProt	APEX1_BOVIN	41	35946	1	1	1	1	1	0.14 DNA-(apurinic or apyrimidinic site) lyase OS=Bos taurus GN=APEX1 PE=1 SV=2
26	1	SwissProt	AT2A1_BOVIN	36	110532	1	1	1	1	1	0.04 Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 OS=Bos taurus GN=ATP2A1 PE=2 SV=1
27	1	SwissProt	EF1AO_XENLA	36	50524	1	1	1	1	1	0.1 Elongation factor 1-alpha 3, somatic form OS=Xenopus laevis GN=eef1as PE=2 SV=1
28	1	SwissProt	HNRH3_HUMAN	33	36960	2	1	2	1	1	0.14 Heterogeneous nuclear ribonucleoprotein H3 OS=Homo sapiens GN=HNRNPH3 PE=1 SV=2
29	1	SwissProt	ITB1A_XENLA	26	91416	1	1	1	1	1	0.05 Integrin beta-1-A OS=Xenopus laevis GN=itgb1-a PE=2 SV=1
30	1	SwissProt	ARC1B_HUMAN	24	41722	1	1	1	1	1	0.12 Actin-related protein 2/3 complex subunit 1B OS=Homo sapiens GN=ARPC1B PE=1 SV=3
31	1	SwissProt	TOM40_BOVIN	23	38043	1	1	1	1	1	0.13 Mitochondrial import receptor subunit TOM40 homolog OS=Bos taurus GN=TOMM40 PE=2 SV=1
32	1	SwissProt	ESYT1_HUMAN	21	123293	1	1	1	1	1	0.04 Extended synaptotagmin-1 OS=Homo sapiens GN=ESYT1 PE=1 SV=1