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Cell-type specific promoters drive expression of the inhibitory receptor CD85j by hematopoietic lineages

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Cell-type specific promoters drive expression of the inhibitory receptor CD85j by hematopoietic lineages

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Abstract:

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CD85j (ILT2/LILRB1/LIR-1) is an inhibitory receptor that recognizes MHC class Ia and Ib alleles that are widely expressed on all cell types. Upon ligand recognition, CD85j diminishes kinase activity by recruiting phosphatases to motifs within its cytoplasmic domain. Within the hematopoietic system, CD85j is expressed with cell-specific patterns and cell surface densities which reflect the different roles of cell contact-mediated inhibition in these lineages. While monocytes ubiquitously have high cell surface expression, B lymphocytes start to express CD85j at intermediate levels during maturation on both early B cells in the bone marrow and, as we show here, on transitional B cells in the periphery. T cells and NK cells gain low expression only on a subset of cells. On NK cells, CD85j is one component of a repertoire of MHC class I-binding regulatory receptors, each of which may or may not be expressed on a given cell. CD85j expression by T cells is exclusive to memory cells and is more likely on CD8 T cells. We show that CD85j expression is predominant on CD45RA effector T cells and that expression increases with age in all memory subsets. The cell-specific expression pattern is accomplished by two complementing but not independent mechanisms. We show that lymphocytes and monocytes utilize distinct promoters to drive CD85j expression. The previously undescribed lymphocyte promoter maps 13 kb upstream of the monocyte promoter; its use results in the inclusion of a distant exon into the 5'UTR. A short sequence stretch within this exon has the unique function of repressing CD85j protein translation and is responsible for the subdued expression in lymphocytes. These cell-specific mechanisms allow tailoring of CD85j levels to the distinct roles it plays in different hematopoietic lineages.

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Chapter 1: Introduction and Literature Review

Introduction

Cellular activation within the immune system requires detection of external signals that trigger intracellular changes culminating in a response. There is an enormous array of ways to send and receive signals, and a coordinate diversity of possible cellular responses. In each case, the underlying requirement to respond to a stimulus is an alteration of the steady state molecular and chemical milieu of the cell. These can include perturbations of the basic physiology of the cell: e.g., changes in membrane permeability and/or intracellular ion concentrations;[1] changes targeted to particular proteins, such as a phosphorylation event disrupting a protein-protein interaction; or, most likely, a combination of different types of changes in response to the same stimulus. These initial alterations rarely constitute the ultimate reaction of a responding cell. Rather, they are part of a cascade of events, each in response to an earlier change, and resulting in subsequent changes to other features within the cell, that all together sum up to a meaningful functional outcome.

Each immune cell-type features a key signal:receptor interaction that initiates intracellular signals leading to the cell's flagship function. For example, the T cells receptor (TCR) recognizes a protein complex on opposing antigen-presenting cells (APC) or target cells comprised of a peptide displayed within the binding-groove of a particular major histocompatibility complex (MHC) molecule. Signals generated by the TCR-peptide:MHC interaction lead to T cell activation

and characteristic responses including proliferation, differentiation, cytokine production, and target-cell lysis. The analogous interaction on B cells involves the B cell receptor (BCR) bound to its cognate antigen which is usually a foreign protein or carbohydrate. Antigen recognition by the BCR initiates intracellular signals that culminate in B cell activation and its accompanying functions including proliferation, differentiation, and immunoglobulin (Ig) secretion.

In the above examples, some signals generated by the TCR and BCR alone are not enough to activate the cell. The strength of the signal must be enough to push the cell away from the equilibrium of its resting state. An isolated signaling event in a resting cell, while no different in quality from a signal capable of cellular activation, will be too weak and will dissipate before full signal propagation has occurred. Mechanisms that maintain cellular equilibrium will simply restore the minor perturbations caused by the signal to their prior resting state. For cellular activation to occur, a signal must overwhelm the cell's equilibrium by delivering a continuous dose of stimuli that each act on downstream factors triggering a chain reaction such that cellular changes are amplified beyond the point of dissipation. At this point the cell has become activated and a program of characteristic cellular changes is initiated that allow the cell to perform its key functions.

Whether a signal will lead to activation is determined by both the strength of the activating signal and the threshold of signaling required to break equilibrium within the cell. The former is influenced by the number of receptor-ligand interactions, as well as the stability of individual interactions. The later is

largely influenced by conditions within the cell. For many activation stimuli, phosphorylation events are required for signal propagation. Given time, a signal-induced phosphorylation event, which usually requires kinase activity, will be reversed by the action of cellular phosphatases. Therefore, one factor that influences activation threshold is the balance between kinase- and phosphatase-activity within the cell. Other factors, such as the activity of ion pumps that restore perturbed membrane gradients or enzymes that destroy chemical signals, also play a role in determining thresholds.

A third key determinant of cellular activation in the immune system is the strength of signals delivered by receptors that are not directly involved in activation. Known as co-regulatory receptors or co-receptors, these molecules may enhance or counteract the effects of activating signals. Some co-receptors such as CD19 and CD20 in B cells and CD28 in T cells, aid in cellular activation and, in fact, are required for successful signaling through the BCR or TCR. Alternatively, many inhibitory receptors exist whose signaling acts to increase the strength of activating signals required to cross a cell's activation threshold. Depending on the context, inhibitory receptors can restrict cellular activation to only those cells receiving the strongest activating signals or prevent activation altogether. Also, as is the case with CD152 (CTLA-4) which is expressed late during the course of T cell activation, inhibitory receptors can act to wind down a successful cellular response. Each hematopoietic cell expresses a repertoire of co-activating and co-inhibitory receptors available to influence a diverse array of cellular activities. The exact role of these receptors depends on the expression

levels of each and the affinities for and expression patterns of the ligands that they recognize.

Many different co-regulatory receptor genes are clustered within a region on human chromosome 19 known as the leukocyte receptor complex (LRC). These genes include those coding for the killer Ig-like receptors (KIRs), the leukocyte-associated Ig-like receptors (LAIRs), and the CD85 family of receptors. These receptor families, whose members are Ig-superfamily receptors, exhibit considerable homology to one another and are thought to have arisen from a series of gene duplications.[2] Within the KIR and CD85 families, both inhibitory and activating receptors exist. As is the case for many inhibitory receptors, inhibitory KIRs, CD85 family members, and the two LAIR receptors exert their effects by recruiting phosphatases to amino-acid motifs within their cytoplasmic tails. These motifs, known as immunoreceptor tyrosine-based inhibitory motifs (ITIMs) usually conform to the consensus sequence S/I/V/LxYxxI/V/L where "x" represents any amino acid. The tyrosine within these motifs becomes phoshorylated upon receptor ligation and acts as a docking site for the SH2 domains of phosphatases. The activity of enzymes, commonly SHP-1, SHP-2 or SHIP, suppresses cellular activation by reversing the action of activation-dependent kinases. By contrast, activating receptors within the LRC lack cytoplasmic domains, and hence lack ITIMs. In order to exert their effects, they associate with adaptor proteins via charged residues within their transmembrane domains. These adaptor proteins, such as DAP12 and FcγR, contain immunoreceptor tyrosine-base activation motifs of (ITAMs) with the consensus

sequence YxxI/L$x_{(6–12)}$YxxI/L. Whereas phosphorylated ITIMs recruit phosphatases, ITAMs, upon ligand-induced tyrosine-phosphorylation, recruit kinases such as Syk and Zap70 that enhance the propagation of activating signals. ITIMs and ITAMs are not exclusive to LRC receptors and molecules containing them are crucial players in many cellular processes. For example, ITAMs on CD3 and Igα/Igβ mediate the chief activation signals of T cells and B cells, respectively.

All members of the KIR family and several CD85 family members recognize MHC class I molecules, which are expressed by all nucleated cells. As such, many of these receptors are expressed by natural killer (NK) cells which survey the body for virally-infected and neoplastic cells, which are eliminated if detected. When NK cells encounter a normal cell, MHC class I molecules expressed on that cell engage inhibitory KIRs and CD85 members and the target cell is spared. Although NK cells also express co-activating receptors that recognize MHC class I, when faced with a healthy cell, it seems the inhibitory signals predominate. Many viruses try to evade detection by T cells by down-regulating MHC class I molecules in the cells they infect. Similarly, many tumor cells express low levels of MHC class I. An NK cell encountering cells that have lost MHC class I expression fails to engage inhibitory receptors and the default cytotoxic program, which is facilitated by activating receptors that do not depend on MHC class I recognition, is initiated and the unhealthy cell is destroyed.

Each NK cell expresses a repertoire of inhibitory and activated receptors, and the exact composition differs from cell to cell. Each MHC class I-binding

receptor recognizes a defined subset of the possible MHC class I alleles. Presumably, the diversity of co-regulatory receptor expression ensures that NK cells can adequately survey the tissues of all individuals, regardless of which MHC haplotype they express. The KIRs tend to recognize a limited spectrum of MHC class I alleles. In contrast, one inhibitory member of the CD85 family, CD85j (also called ILT2, LIR-1, LILRB1, and MIR-7), recognizes nearly all MHC class I alleles, including alleles of classical (HLA-A, B, and C) and non-classical (HLA-G) genes. In addition, CD85j recognizes UL18, a human cytomegalovirus (CMV) MHC class I homolog, with very high affinity, which suggests that targeting CD85j may be a mechanism of immune evasion by CMV.

While the study of LRC co-regulatory receptors initially focused on their roles in NK cells, many are also expressed on a subset of T cells. As we will discuss, these T cells tend to be CD8 T cells and the population expressing them expands with age. We have discovered that this phenomenon is most potent for CD85j which is encoded by the gene *LILRB1*. CD85j is also remarkable among the LRC members for its broad expression on cells of hematopoietic lineage. While, as is the case for KIRs, only a subset of NK cells and T cells express CD85j, its expression is ubiquitous on B cells, monocytes, and dendritic cells (DC). The level of CD85j protein expressed in these cell types varies from moderate (T cells and NK cells) to intermediate (B cells) to quite high (monocytes). Prior to our work, very little was known about how the characteristic patterns of CD85j expression are achieved in these cell types. Here we report a novel promoter that drives CD85j expression in lymphocytes.

This promoter lies 13 kilobases (kb) upstream of known *LILRB1* promoter, which drives expression in monocytes, and the main *LILRB1* exon cluster. We also show that the lymphocyte promoter introduces an addition exon in *LILRB1* transcripts and that sequences in this exon mediate translational repression of CD85j protein, helping to explain the disparity in protein levels between lymphocytes and monocytes.

CD85j Literature Review

Original descriptions of CD85j

CD85j was first described in 1997 in two independent reports in *Immunity* and *Journal of Experimental Medicine*. The *Immunity* paper by Cosman et al.[3] identifies CD85j (called leukocyte immunoglobulin-like receptor 1, or LIR-1, in the paper) as a UL18-binding protein. The goal of the paper was to test the hypothesis that UL18, a MHC class I homolog encoded by human CMV, binds to KIRs expressed on NK cells. CMV, in an effort to avoid detection by T cells, down-regulates class I expression on infected cells, leaving them vulnerable to NK cell lysis. Thinking that UL18 may protect infected cells from lysis by serving as a ligand for inhibitory NK cell receptors, the authors tested whether UL18 binds to KIRs. A UL18-Fc (IgG1) fusion protein (co-expressed with β2m) bound a subset of NK cells and to all CB23, U937, and Daudi cell lines. UL18-Fc immunoprecipitated (IP) a 110-120 kDa protein (a size that does not correspond to KIRs) from surface-biotinylated CB23 cells that was also seen in lysates from

U937, THP-1, Daudi, Raji, MP-1 cells and primary monocytes. A CD85j-encoding cDNA was isolated by UL18-binding to CV-1/EBNA cells transfected with a CB23 cell cDNA library. The sequence revealed a type 1 transmembrane receptor with four extraceullar C2-type Ig-like domains, a spacer region, a transmembrane domain and a cytoplasmic tail containing four putative intracellular ITIMs.

Anti-CD85j monoclonal antibodies (mAb) were generated and used to detect CD85j expression on PBMCs. All CD14+ and CD19+ cells and almost no CD3+ or CD56+ cells were CD85j+. Particularly high staining was seen on CD14loCD16hi cells (monocytes), which are the chief producers of interferon (IFN)-α. To identify an endogenous CD85j counter structure, a CD85j-Fc fusion protein was used to stain various cell types and to screen a HSB-2 cell cDNA library. The authors reported nearly ubiquitous staining in all cell types tested. The cDNA library screen revealed CD85j-binding to the MHC class I alleles HLA-A2 and -B44, and binding was blocked by anti-HLA-A, -B, -C antibody (W6/32), thus establishing CD85j as a MHC class I-binding receptor.

To determine if CD85j, like KIRs, binds to SHP-1, IP was performed on lysates from pervanadate-treated CB23 cells. Anti-SHP-1 antibody pulled down CD85j only with pervanadate treatment. Also, immunoprecipitated CD85j was only detected with anti-phosphotyrosine antibody after pervanadate treatment. These data suggested that CD85j could function as an inhibitory receptor analogous to KIRs.

The second independent description of CD85j came several months later in *Journal of Experimental Medicine*. In this report, Colonna et al.[4] explored expression and function of CD85j (called immunoglobulin-like transcript 2, or ILT2, in the paper) after having introduced its sequence in a prior paper.[5] The authors suggested that the behavior of the NKL cell line could not be explained by contemporary knowledge. Lysis of 721.221 cells by NKL cells is inhibited by transfection with HLA-B*2702, -B*2705, -B*5101, -A*0301, -B*0702, -Cw*0301 and -G1. At the time, no known molecules expressed by NKL could account for this; NKL do not express p70 KIR which recognizes HLA-B*2702, -B*2705, and -B*5101, and anti-CD94 antibodies only partially restored lysis of HLA-A*0301, -B*0702, -Cw*0301 and -G1 transfectants. To identify the inhibitory molecules responsible, NKL cells were used as immunogens against which to raise mAbs capable of fully restoring NKL lysis of transfectants. mAb HP-F1 achieved these results. Additionally, in a redirected killing assay, HP-F1 inhibited basal and anti-CD16-enhanced lysis of Fc receptor-positive P815 cells by NKL cells (presumably by binding to Fc receptors on P815 cells and cross-linking the HP-F1 target on NKL cells).

The HP-F1 antibody detected a ~110 kDa product (under reducing and non-reducing conditions; 90 kDa after N-glycosidase treatment) from NKL lysates. HP-F1 bound a subset of human T cells (CD3+, α/β, γ/δ), and NK cells (CD56+) and bound all B cells (CD19+) and monocytes (CD14+). Indicating that no known KIRs share the molecular weight or cellular distribution of the HP-F1 counterantigen, the authors speculated that HP-F1 may recognize the recently

cloned CD85j (ILT2)[5] whose sequence and distribution are consistent with HP-F1's characteristics. Indeed, HP-F1-probing of lysates from COS cells transfected with CD85j was identical to result found with NKL lysates, confirming CD85j as the HP-F1 counterantigen. To confirm that CD85j binds to MHC class I molecules, a CD85j-Fc fusion protein was used to stain 721.221 transfectants by flow cytometry. HLA-A (*0301), B (*2702 and *2705), and G transfectants were stained but HLA-C (w*0301) transfectants were not. The authors next examined whether the putative ITIMs within the CD85j cytoplamic domain recruit phosphatases as is predicted for a KIR-like inhibitory receptor. CD85j was immunoprecipitated from NKL and C1R (EBV-transformed B cell line) cells exposed, or not, to pervanadate and blots were probed with anti-SHP1, -SHP2, and -SHIP antibodies (some data was not shown). Only SHP1 was pulled down with CD85j and only after pervanadate treatment. The specificity of CD85j for SHP-1 has been confirmed by other groups.[6]

In addition to the initial NK cytotoxicity assays, several functional assays were performed to examine the inhibitory potential of CD85j in a variety of cell-types and settings. Serotonin release by CD85j-transfected FcεRI-expressing rat basophil leukemia (RBL) cells was abolished by adding HP-F1 to plates coated with IgE. CD85j+ Vβ2+ CD8 T cell clones killed TSST-1 pulsed 721.221 cells (superantigen binds to MHC class II on target cells and activates TCR on T cells) but not HLA-B*2705 transfectants. The authors next examined the effect of CD85j cross-linking on IgG-triggered Ca2+ mobilization in B cells. CD85j co-ligation in C1R cells completely prevented Ca2+ mobilization, but, interestingly,

this effect was only marginal in primary B cells (this data was not shown in the report).

Several additional minor reports introduced CD85j before it was clear what molecule they were examining. In an attempt to identify human homologs of the mouse inhibitory receptor gp49B, Wagtmann, et al.[7] isolated an expressed sequence tag exhibiting 46% identity to gp49B within the 52 amino-acids coded by the sequence fragment. Further analysis including 5'RACE and sequencing of full-length cDNA clones identified cl-7 which corresponds to what is now called CD85j (accession number AF004230). Using cl-7 as a probe, the authors found strong hybridization to RNA from monocytes, hince they named it monocyte/macrophage immunoglobulin-like receptor (MIR)-7. By Southern blotting and hybridization to cosmids, the authors were able to map the genomic location of MIR-7 to human chromosome 19q13.4.

In what could also be considered an "original description" of CD85j, two mAbs recognizing an antigen on hairy cell leukemia cells were introduced in a 1991 report.[8] The mAbs VMP55 and GHI/75, raised in BALB/C mice against homogenized splenic tissue from a hairy cell leukemia patient, were selected for their staining of frozen sections of hairy cell leukemic spleens. Further analysis suggested that both mAbs recognize the same antigen, a 72 kDa unreduced product found by western blotting and immunoprecipitated from leukemic spleen lysate. By immunohistochemistry and flow cytometry, these antibodies stained all B cells and monocytes and no T cells or NK cells. A variety of cell lines and other leukemia and lymphoma cells were also tested. U937, Daudi, and Raji cell

lines, as well as some K562 cell line cells, were positive. Other hematopoietic and all non-hematopoietic cell lines tested were GHI/75- and VMP55-negative. Hairy cell leukemia cells from 12 donors were all positive, as were some chronic lymphocytic leukemia, plasmacytoma, multiple myeloma, and some non-Hodgkin B cell lymphoma cells. Amongst the hematological cancers that were not stained were all Hodgkin disease, all non-Hodgkin T cell lymphomas, lymphoblastic and immunoblastic non-Hodgkin B cell lymphomas, acute and chronic myeologenous leukemias, and prolymphocytic leukemia.

The defining of the LILR gene family

The antigen for mAbs VMP55 and GHI/75 was defined as CD85 (no "j" at this point) at the 5th Human Leukocyte Differentiation Antigens (HLDA) workshop in 1993. In 1999, the same group discovered, by peptide sequencing of CD85 protein affinity-purified with GHI/75, that CD85 was the same antigen as ILT2 and LIR-1.[9] Finally, CD85 became CD85j at the 7th HLDA workshop in 2000. It was decided that CD85 would refer to the entire family of receptors that include all ILTs and LIRs. Each individual family member would be defined by an additional letter, assigned alphabetically, based on the centromeric-to-telomeric location of its gene on chromosome 19.[10]

The symbol for each gene encoding proteins in the CD85 family begins with the stem symbol *LILR*, which stands for "leukocyte immunoglobulin-like receptor." The stem is followed by an A or a B and a number. The A and B designation separates the subfamilies into activating and inhibitory receptors,

respectively. Genes from subfamily A encode transmembrane proteins lacking intracellular ITIMs while subfamily B members contain ITIMs. Each member within a subfamily is also given a number. For example CD85j is encoded by the gene *LILRB1*. This convention was adopted in 2000 following a discussion amongst experts in the field (see http://www.genenames.org/genefamily/lilr.php).

Table 1: LILR gene family members and aliases

Gene Name	CD85 name	ILT name	LIR name	MIR name
LILRA1	CD85i		LIR-6	
LILRA2	CD85h	ILT1	LIR-7	
LILRA3	CD85e	ILT6	LIR-4	
LILRA4	CD85g	ILT7		
LILRA5	CD85f	ILT11	LIR-9	
LILRA6	CD85b	ILT8		
LILRB1	**CD85j**	**ILT2**	**LIR-1**	**MIR-7**
LILRB2	CD85d	ILT4	LIR-2	MIR-10
LILRB3	CD85a	ILT5	LIR-3	
LILRB4	CD85k	ILT3	LIR-5	
LILRB5	CD85c		LIR-8	

LILR gene family members are located within the so-called leukocyte regulatory complex (LRC) of human chromosome 19. As discussed in the Introduction, the LRC also includes members of another gene family, the killer cell Ig-like receptors (KIRs), as well as the molecules LAIR, FCαR, and NCR1, all of which share significant structural and functional homology and expression

within the hematopoietic system. Unlike the KIRs, which, like MHC molecules, are inherited as distinct haplotyopes,[11-12] all individuals receive the complete LILR gene family.[13]

CD85j expression profile

CD85j is broadly expressed by cells of hematopoietic origin and many studies have examined its distribution on primary cell populations and common cell lines.[3-4,9,14-19] CD85j expression is often described as ubiquitous, or nearly so, on peripheral B cells, monocytes, and DCs, while only a subset of T cells and NK cells are found to be CD85j-positive. In a representative study, Young et al.[18] examined CD85j expression on PBMC subsets. B cells and monocytes were essentially ubiquitous for CD85j, while a subset of T cells (23% of αβ, 48% of γδ, <1% of CD4, and ~20% of CD8) and NK cells (~40%) expressed CD85j.

CD85j expression on NK cells

Several studies report that only a subset of NK cells expresses CD85j and the proportion of CD85j+ cells differs from donor to donor. Vitale et al.[20] examined CD85j expression (using mAb M401) on a population of lymphocytes enriched for NK cells (depleted of CD3+ and HLA-DR+ cells) from several donors. The authors report a range of 5% to 85% CD85j+ NK cells in these donors. They also report that no apparent correlation exists between CD85j expression and KIR or CD94/NKG2A expression on the NK cell-enriched population. A UL18-Fc

fusion protein stained these cells with a similar pattern that also did not correlate with KIR or CD94/NKG2A expression.

CD85j expression on T cells

CD85j is expressed on subsets of CD4 and CD8 primary and in vitro-maintained T cells. Its expression is much more likely on CD8 T cells and is enriched in highly differentiated populations. A 1999 report by Speiser et al.[16] represents the first indication that CD85j expression on T cells is confined largely to CD28- cells. In addition to CD85j (detected by mAb HP-F1), the authors examine several other NK cell receptors such as KIRs and the C-type lectin-like receptors NKRP1A, ZIN176, CD94 and CD94/NKG2A. For each receptor, expression is more likely on CD8+CD28- T cells but the trend is most pronounced for CD85j. As an indication that aging may be important for NK cell receptor expression by T cells, a phenomenon studied by our lab, the authors also show that T cells from cord blood are nearly all CD28+ and that almost no NK cell receptor expression is found, including CD85j. Another group found similar results in cord blood.[18] These findings foreshadow results by our lab (see Chapters 2 and 3) and others that CD85j expression on T cells correlates with aging[21] and transition to effector phenotype[18,21].

Young et al.[18] examined inhibitory receptor expression in T cells and found that CD85j expression is largely confined to CD8 cells and, using three-color staining including CD3, found CD85j preferentially in CD27-, CD28-, CD56+ and CD57+ populations. This profile, along with a relative absence of CD85j in cord

blood T cells, suggests that CD85j expression correlates with differention to memory/effector phenotype in T cells. This report also assessed the extent to which CD85j and the KIRs (using pooled panel of mAbs to several KIRs) are expressed together by the same cells. In T cells, cells expressing KIRs are likely to also be CD85j-positive (~60%) while a minority of CD85j+ cells co-express KIRs (~30%). This is in contrast to NK cells, in which each inhibitory receptor is as likely to be expressed on cells expressing the other (~60%). In nearly every donor tested, a greater percentage of T cells were CD85j-positive than were KIR-positive. The inverse was true for NK cells from a majority of donors.

Young et al.[18] also generated CD85j+ T cell clones by first using FACS to isolate single CD85j+ cells and then stimulating with IL-2 and feeder cells. Clones shared features of primary CD85j+ T cells including CD8, CD56 and CD57 expression and lack of CD28.

To examine CD85j expression and function, Saverino et al.[17] generated CD4 and CD8 T cell clones from PBMCs by PHA and IL-2 stimulation. In most CD8 clones, CD85j surface expression was bimodal, however, a percentage of clones were ubiquitously positive (3/30) or negative (5/30) for HP-F1 staining. A large majority of CD4 clones were wholly negative for CD85j; only 4 clones out of the 34 clones generated exhibited bimodal CD85j expression. Surprisingly, the authors found that all cells from all clones, whether CD4 or CD8 and regardless of CD85j surface staining, expressed CD85j intracellularly, as determined by confocal and flow cytometry following fixation and permeabilization. This phenomenon was also found in resting T cells isolated from PBMCs and was

seen with several different anti-CD85j mAbs. In general, protein levels found by surface flow cytometry correlated with IP and Western blotting, and with mRNA levels detected by RT-PCR.

Ubiquitous intracellular staining for CD85j among T cells has been found by other groups, including ours (see Chapter 3). Nikolova et al.[22] compared CD85j staining (GHI/75 mAb) on CD4-gated PBMCs from Sézary syndrome patients and healthy controls. Surface staining was largely absent in controls and, as they show is common in Sézary syndrome, present on a significant fraction of CD4 cells. In both groups, all CD4 cells were positive for intracellular CD85j. In logical support of intracellular CD85j expression by all cells, the authors claim that CD85j mRNA levels did not differ between patients and controls (using the same primers used by Saverino et al.[17]) despite the difference in CD85j-surface expression. However, because RNA was isolated from total PBMCs, we question their interpretation.

The concept that all T cells express CD85j is controversial and most groups maintain its expression is restricted to only a subset of T cells. After some analysis of our own, we agree with the consensus that CD85j is not expressed by all T cells. Our experiments addressing this issue and the rationale leading to our conclusion are presented as part of Chapter 3.

CD85j expression on B cells

CD85j is found on the surface of all peripheral B cells at an intermediate level.[3-4,8-9,23] An analysis of CD85j expression on tonsillar B cell subpopulations

suggests that germinal center B cells and memory cells all express CD85j at levels similar to circulating B cells.[9] Expression remains stable for at least 6 days after ex vivo B cell stimulation by CD40L or lipopolysaccharide (LPS) plus IL-4.[23] B cell precursor populations within the bone marrow begin to express CD85j as they mature from pro/pre-B cell (CD10+CD20-; 28% CD85j+) to immature B cells (CD10+CD20+; 74% CD85j+) to mature B cells (CD10-CD20+; 91% CD85j+).[9] The maturation-dependent CD85j expression on B cells is a feature that is shared with T cells. We will examine this phenomenon in Chapter 2.

Ligand-binding properties

Several studies have established that CD85j recognizes a broad range of classical and non-classical MHC class I molecules, including the human CMV-encoded MHC class I homolog UL18. Beginning with a 1999 report by Chapman et al.,[24] a series of analyses have defined molecular and structural details that help explain CD85j's recognition of a broad array of MHC class I alleles. The Chapman et al. report used synthesized proteins to perform a variety of biochemical analyses and binding assays to identify domains responsible for CD85j:UL18 and CD85j:MHC class I binding. The authors began by combining soluble CD85j (extracelluar Ig-like domains (D) 1-4) and UL18 complexed with β2-microglobulin (β2m) and peptide to show that the CD85j:UL18 bound complex contains only one of each species. Next, apparent affinities were determined by surface plasmon resonance (SPR) using CD85j D1-D4 and an array of β2m-complexed and peptide loaded classical and non-classical MHC class I

molecules, included UL18. Strikingly, CD85j bound UL18 with an affinity (K_D) in the nM range. This was >1000 times the affinity found for the endogenous class I molecules tested, which included HLA-B*2702 and several HLA-C alleles as well as HLA-G1 and -E. HLA-G1 exhibited the lowest affinity at K_D=100μM which, as is discussed below, is contradicted by a later report by another group.[25] In these experiments, UL18 was included with and without peptide and in several distinct glycosylation states leading the authors to suggest that CD85j binds to a protein moiety and the domain is distinct from the UL18 peptide binding domain.

To isolate domains within CD85j required for binding, the authors performed SPR using CD85j protein containing only some Ig-like domains. CD85j D1-D2 bound UL18 with a similar affinity to the full-length soluble CD85j while D3-D4 did not bind at all. CD85j D1 alone, but not D2 alone, also bound UL18 nearly as strongly as full-length CD85j, implicating D1 as the main binding-domain. CD85j D1 alone also bound an HLA-C allele. Next, the authors performed domain swapping experiments to isolate the required domains on MHC class I molecules. For both UL18 and MHC class I, the α3 domain was required for binding. The authors speculate that by binding to the largely non-polymorphic, non-peptide-binding α3 domain, CD85j can recognize a wide array of MHC class I alleles. This is in contrast to the KIRs which each bind a limited subset of MHC class I alleles and whose binding epitope on MHC class I involves the α1 and α2 domains.[26]

These findings were further clarified one year later when the same group reported the crystal structure of CD85j D1-D2.[27] The analysis revealed Ig-like

domain structures similar to the KIRs, as well as a similarly acute interdomain hinge angle. Using the CD85j D1-D2 crystal structure and taking advantage of that fact that CD85d, while highly similar to CD85j at the amino acid level, does not bind to surface UL18,[28] the authors next attempted to more precisely define the CD85j ligand-binding domain. Reasoning that solvent-exposed residues that differ between CD85j and CD85d may account for differences in UL18-binding, the authors tested UL18-binding following mutatgenesis of these residues. Indeed, CD85j residues 38, 79, 80, and 84 (but not 29 and 30) within D1, when mutated in CD85j or used to replace the corresponding residues in CD85d, profoundly affected UL18-binding causing a ~20 fold decrease (mutated in CD85j) or ~10 fold increase (mutated in CD85d) in UL18-binding, respectively. These residues are distinct from the key ligand-binding residues of the KIRs despite structural similarity between CD85j and the KIRs at the domain level. Of note, the authors also found that unaltered CD85d does in fact bind to soluble UL18 but at a much lower affinity than does CD85j (K_D=12μM for CD85d; K_D=2nM for CD85j).

A similar analysis of CD85j and MHC class I binding affinities was done by a separate group.[25] SPR was again used to calculate binding affinities of CD85j to several MHC class I alleles. The authors found that CD85j binds HLA-B*3501, -Cw*0401, and -G1 with an affinity in the low μM range (K_D=2 to 9 μM). In contrast to Chapman et al.,[24] the highest affinity interaction is with HLA-G1, a discrepancy that the authors speculate is due to differences in MHC class I protein immobilization during the SPR assay. CD85j exhibited no binding with

HLA-Cw*0702 complexed with two different peptides. The study examined CD85d in parallel and found that, although affinities were roughly 10 fold lower than for CD85j, it displays a similar binding profile, including showing the highest affinity for HLA-G1 (K_D=5μM). These data are important because CD85j and CD85d are the major inhibitory receptors for HLA-G which, due to its expression on placental trophoblast cells, is thought to be important for maintaining fetal tolerance by NK cells and T cells.[29]

A later report, again by the Bjorkman group, provided an even more definitive characterization of ligand binding by CD85j.[30] The authors solved the structure of CD85j D1-D2 bound to the HLA-A*0201:β2m complex which confirmed some earlier findings and provided some surprises. As suggested from the prior report, CD85j binds to the α3 domain of HLA-A2 and most of the CD85j residues responsible are found within domain 1. Unexpectedly, much of the interface includes residues from β2m and the interdomain hinge region between CD85j D1 and D2. This finding validated a suggestion by Shiroishi et al. that CD85j might interact with β2m.[25] The involvement of β2m, which is included in nearly all MHC class I and class I-like molecules expressed at the cell surface, and the α3 domain, which is far less polymorphic than the α1 and α2 domains of MHC class I alleles, explains why CD85j recognizes most, if not all, MHC class I alleles. In support of this implication, the authors showed that residues analogous to the CD85j-binding residues of HLA-A2 in other class I alleles are largely conserved while they diverge in somewhat homologous but non-CD85j-binding proteins, including FcRn, HFE, and ZAG.

The crystal structure[30] revealed binding details that have implications about the types of interactions in which CD85j is likely to participate. First, CD85j and HLA-A2 form an anti-parallel binding complex, an orientation suggestive of *trans*, rather than *cis*, interactions. Secondly, full-length CD85j bound in *trans* to a MHC class I molecule would prevent, due to steric hindrance rather than direct competition for binding sites, the T cell co-receptor CD8 from simultaneously binding class I. Indeed, an earlier report by Shiroishi et al.[25] showed that CD85j binds to MHC class I molecules with at least a 10 fold higher affinity than CD8αα and can completely inhibit CD8 binding to MHC class I proteins in SPR assays. Thus, CD85j expressed on CD8 T cells has inhibitory potential derived both from preventing a normal TCR:CD8:MHC class I complex and by recruiting phosphatases to the immunological synapse.

As noted above with regards to HLA-G recognition, the binding partners of the structurally similar CD85 family member CD85d (LILRB2, ILT4, LIR-2, MIR-10) widely overlap those of CD85j.[14] CD85d also contains cytoplasmic ITIMs and recruits SHP-1.[14]

Function of CD85j

Recruitment of SHP-1 by ITIMs

CD85j is an inhibitory receptor which functions by recruitment of phosphatases to motifs within its cytoplasmic domain. Tyrosines within the motifs, called immunoreceptor tyrosine based inhibitory motifs (ITIMs), are phosphorylated upon receptor engagement and serve as docking sites for SH2

domains within phosphatases. Several studies have confirmed that CD85j can recruit SHP-1 but not SHP-2 or SHIP. This recruitment can be achieved by pervanadate treatment[3-4,14] or receptor ligation.

Although the CD85j cytoplasmic tail contains four potential ITIMs, several reports suggest their roles in inhibition and effectiveness at SHP-1 recruitment differ. Dietrich et al.[31] tested the SHP-1-binding capacity of each of the four CD85j ITIMs by incubating Jurkat lysates with 12-14mer peptides containing the sequences surrounding the ITIMs. Strongest SHP-1 interactions were observed for ITIM #2 (VTYAEV, Y562) while ITIMs #1 (NLYAAV, Y533) and #3 (VTYAQL, Y614) exhibited weaker and similar binding; in each, case binding required phosphorylation of the tyrosine within the peptide. ITIM #4 (SIYATL, Y644) seemed not to recruit SHP-1 at all. Another report,[32] using slightly different phosho-peptides, found no SHP-1 interactions with ITIM#1 and similarly strong interactions with both ITIM#2 and #3; ITIM#4 was not tested. Additionally, this report showed that anti-SHP-1 antibodies failed to immunoprecipitate CD85j from Jurkat cells transfected with a CD85j construct featuring the Y562F mutation.

A more detailed analysis from another group of CD85j ITIM function within the context of the entire molecule is contradictory to the above reports in some ways. Bellón et al.[33] transfected wild-type and mutant CD85j constructs into RBL cells and tested tyrosine phosphorylation, SHP-1 recruitment and inhibition of serotonin release in response to IgE-ligation. Truncated CD85j constructs lacking the entire cytoplasmic domain or the last two ITIMs (CD85jΔ2Y) failed to recruit SHP-1 following pervanadate treatment (although CD85jΔ2Y was

tyrosine-phosphorylated) or inhibit serotonin release when exposed to plate-bound HP-F1. This result, as well as the failure of a Y562F mutation in the full-length CD85j construct to prevent inhibition of serotonin release, led the authors to focus the remainder of the report on tyrosines 614 and 644. Constructs were made with Y>F mutations of Y614 and Y644, both individually and together in the same construct. The Y614F construct was only slightly less effective at inhibiting serotonin release, while Y644F construct inhibited very poorly. The double mutant construct did not inhibit serotonin release at all. Of note, in each case, pervanadate treatment resulted in tyrosine-phosphorylation of CD85j and SHP-1 recruitment.

Similar to results obtained by another group[31] using Jurkat cells, Bellón et al.[33] also showed that Lck and Fyn can tyrosine-phosphorylate CD85j leading to recruitment of SHP-1 in transfected COS-7 cells. SHP-1 recruitment following Fyn-mediated tyrosine-phoshorylation of CD85j in constructs mutated for a single ITIM was maintained. Likewise, SHP-1 was recruited in a construct with both Y533F and Y562F mutations. In support of data from RBL cells, SHP-1 could not be recruited using Y614F, Y644F double mutants, despite tyrosine-phoshorylation of CD85j. Curiously, in the RBL system the authors found that the Y533F mutant impairs CD85j inhibition of serotonin release, as does the Y533F, Y562F double mutant. Additionally, no CD85j-tyrosine-phophorylation was seen following pervanadate treatment of CD85j-Y533F transfectants. In COS-7 cells, Fyn-mediated phosphorylation was 6-fold less efficient in Y533F transfectants compared to wt CD85j transfectants prompting the authors to

speculate that Y533 plays a role in CD85j tyrosine-phosphorylation which subsequently affects SHP-1 recruitment to other ITIMs. It is this "processive amplication" of tyrosine phosphorylation that these authors, in a later report,[32] cite as a likely cause for the contradictory data regarding the most important SHP-1-binding ITIMs of CD85j.

In addition to SHP-1, Csk interacts with ITIMs within CD85j. Sayós et al.,[32] using the CD85j cytoplasmic domain as bait in a yeast three-hybrid screen of an EBV-transformed B cell cDNA library, isolated a clone containing the SH2 and kinase domains of Csk. This interaction required the presence of Fyn, which was included in the screen. IP experiments in Jurkat cells confirmed the CD85j-Csk interaction, which required pretreatment with pervanadate. Of note, CD85j co-precipitated with both Csk and SHP-1 but neither Csk nor SHP-1 was pulled-down with IP of the other, suggesting they might compete for a binding site on CD85j. Returning to the hybrid system, the authors used CD85j cytoplasmic tail mutants, each with a Y>F change in a single ITIM, in an attempt to identifiy which ITIMs in CD85j were important for Csk binding. Each mutation weakened the interaction but Y562F completely prevented it. Y644F affected the CD85j-Csk interaction the least (~50% loss of β-galactosidase activity) and Y533F and Y614F had a similar, intermediate effect. The cytoplasmic tails of CD85j and CD85d are very similar except that, due to a 52-amino-acid gap between ITIMs #1 and #3, CD85d lacks ITIM#2. In support of Y562 (ITIM#2) as the best candidate for the site of the CD85j-Csk interaction, the authors found 3-fold weaker interaction between Csk and CD85d in the three-hybrid system.

Using phosho-peptides (similar to those used by Dietrich et al.[31]) spanning Y533, Y562, and Y614 the authors found a strong Csk interaction with Y562; Y533 and Y614 interacted only very weakly with Csk. IP of Csk in Jurkat cells transfected with a full-length CD85j-Y562F mutant construct failed to pull-down CD85j, further implicating Y562 as a key factor in the CD85j-Csk interaction. It is unclear what function a CD85j-Csk interaction serves. Csk, itself a Src kinase, generally acts to inactivate other Src kinases.[34] The authors suggest that Csk may directly regulate CD85j function by altering the kinase activity required to phosphorylate tyrosines within its ITIMs. Alternatively, the CD85j-Csk interaction may act to position Csk in the vicinity of other molecules it regulates.

In summary, although the CD85j cytoplasmic tail contains four ITIMs, they are not interchangeable and they play an unequal role in orchestrating CD85j's inhibitory effects. It appears that SHP-1 can be recruited to several ITIMs but it is most efficient at ITIM#4. Additionally, activity of ITIMs that are not dominant SHP-1 recruiters may be required for proper phosphorylation of other ITIMs. Csk recruitment is most dependent on ITIM#2.

CD85j function in NK cells

Many reports have examined the role of CD85j in target cell lysis by NK cells. In most cases, target cells lacking HLA class I molecules are transfected with various HLA class I alleles which serve as potential ligands for inhibitory receptors on NK cells or cell lines. The most commonly used targets are

721.221 cells which lack all class I molecules. The most common NK cell line, NKL, expresses CD85j and readily lyses untransfected 721.221 cells.

Navarro et al.[35] found that mAb HP-F1 blocks NKL cell lysis of .221-B27 transfectants but not .221-AEH (HLA-E expresser) or .221-G1 (HLA-G1 and -E expresser). The authors show that HLA-G1 is actually a functional ligand for CD85j but that transfected HLA-G1 provides the signal peptide needed for surface expression of HLA-E, a functional ligand for the inhibitory complex CD94/NKG2A. A CD85j+ CD94/NKG2A- NK cell clone did not lyse .221-G1 cells, a finding reversed with blocking by HP-F1.

Vitale et al.[20] identified KIR-CD94/NKG2A+CD85j+ NK cell clones and tested their ability to lysis cell lines transfected with HLA class I alleles. C1R cells transfected with HLA-A1, -B27, and -B24 were protected from lysis. This protection was more robust for the HLA-B alleles than HLA-A1. Lysis was strongly, but only partially, restored with anti-CD85j mAB M401. Similarly, transfection of 721.221 cells with HLA-A2, -A3, -B7, and -G1 inhibits lysis by the NK cell clones and lysis is partially restored with anti-CD85j mAb M401. Of note, HLA-Cw3 and -Cw4 transfectants were only weakly protected, a finding that was true for clones that, in addition to being CD85j+ and KIR-, were either CD94/NKG2A-positive or negative.

CD85j molecules expressed on NK cells are only one component of a MHC class I-detecting inhibitory receptor repertoire that includes other Ig-like superfamily members, such as KIRs, and lectin-like receptors, such as CD94/NKG2A. The particular repertoire of receptors differs among individual NK

cells. Among these receptors, CD85j recognizes the broadest spectrum of MHC class I alleles. As such, the MHC class I alleles targeted by the other receptors expressed by a given NK cell will overlap with those of CD85j. Therefore, the relative importance of CD85j is likely to depend on which other receptors are expressed by a CD85j+ NK cell. Indeed, Kirwan et al.[36] have shown that HLA-Cw15-mediated inhibition of the NK92 cell line expressing both CD85j (endogenously) and KIR2DL1 (by transfection) is dominated by KIR2DL1; anti-CD85j or anti-MHC class I α3 domain antibodies do not reverse inhibition. Even in the absence of KIR2DL1 transfection, CD85j did not inhibit HLA-Cw15+ target cell lysis. Furthermore, lysis of 721.221 cells by the NK cell line was not inhibited by transfection with HLA-Cw3, an allele recognized by CD85j but not KIR2DL1 cells. However, overexpression of CD85j in NK92 cells results in inhibition of lysis of both HLA-Cw3- and HLA-Cw15-tranfected target cells. These findings highlight how CD85j expression levels can affect its ability to function, especially in the context of other inhibitory receptors. It is likely that the importance of CD85j on a given cell depends both on the number of CD85j molecules present and the threshold of cellular activation established by the relative quantities and MHC class I allele binding affinities of all receptors present.

In summary, CD85j expression on NK cells functions to inhibit lysis of MHC class I-expressing target cells. Due to its broad specificity for different MHC class I alleles, CD85j has the potential to influence NK cell interactions with most healthy target cells, but its ultimate impact on cytotoxicity will depend on other receptors expressed on the same NK cell.

CD85j function in T cells

CD85j function has been studied in primary cells, T cell clones, and Jurkat cells by several groups. In general, all functions exerted by CD85j act to down-regulate normal T cell functions, whether cells are CD4- or CD8-positive. Because CD85j expression within T cells is skewed towards CD8 T cells, and because of the crucial role MHC class I molecules play in CD8 T cell stimulation, functional insights from CD8 T cells may have more physiological relevance.

Dietrich et al.[31] examined CD85j function in Jurkat T cells transfected with CD85j. After TCR and CD85j cross-linking, tyrosine-phosphorylation was observed on immunoprecipitated CD85j. Interestingly, very little phosphorylation was seen when only CD85j was cross-linked, suggesting a requirement for TCR co-ligation. Subsequent experiments, in which Lck was inhibited or lacking (using J. CaM 1.6 cells), revealed that Lck activity is also required for CD85j tyrosine-phosphorylation. CD85j also failed to recruit SHP-1 in these situations. An implication of this result is that CD85j may not be able to interfere with the very initial signals of T cell activation because some activating signal is required for CD85j ITIMs to be phosphorylated (the authors note that data from KIRs in NK cells[37] and paired Ig-like receptor B in B cells[38] are consistent with this as well). A report by another group[33] contradicted this assertion in demonstrating that CD85j-ligation, in the absence of other stimuli, on peripheral blood T cells and transfected RBL cells results in phosphorylated tyrosines within CD85j. The authors[33] cite increased Fyn activity in their systems as an explanation. Also, it

seems that tyrosine-phosphorylation following only CD85j-engagement exists in a very narrow window of time. For example, CD85j-phosphorylation is clearly evident in peripheral T cells 1 minute after CD85j cross-linking but is much fainter by two minutes and completely absent by 5 minutes. In RBL transfectants, CD85j-phosphorylation is only present at 2 minutes after CD85j-ligation and not before or after. Therefore, it could easily be the case that in Jurkat cells, CD85j-phoshorylation following CD85j-ligation was simply missed.

Dietrich et al.[31] next examined the effect of CD85j ligation on phosphorylation-dependent intracellular events. They found that TCR-CD85j co-ligation diminished ζ-chain, LAT, and ERK1/2 phosphorylation as well as recruitment of Zap70 to the ζ-chain. Similar results were found using a CD8+CD85j+ T cell clone. Jurkat and CTL contact with anti-CD3/anti-CD85j mAb, but not anti-CD3/control mAb, coated beads resulted in diminished actin cytoskeleton rearrangement as detected by phalloidin staining. The authors also examined CD85j staining by confocal microscopy during TSST-1-dependent contact between OKT8-24 cells and 721.221-HLA-B27 cells. They found that, like CD3, CD85j was localized to the interface between contacting cells. This staining pattern was only observed in HLA-transfected 721.221 cells but was not dependent on the addition of TSST-1. This data suggest that CD85j can be positioned at the immunological synapse during T cell contact with target cells. In CD8 T cells, this theoretically provides a context for CD85j to compete for the CD8:MHC class I α3 domain interaction and an opportunity for CD85j to influence the initiation of T cell responses.

Indeed, similar to its role on NK cells, CD85j inhibits target cell lysis by CD8 T cells. Saverino et al.[17] assessed the ability of cross-linked HP-F1 to inhibit CD3-activated murine P815 cell lysis. Among the several dozen CTL clones tested, the degree to which HP-F1 treatment prevented CD3-mediate P815 lysis ranged from complete inhibition to no inhibition. Over half of the clones inhibited specific lysis by at least 40% and an additional third of clones exhibited a moderate degree (20-40%) of inhibition. Inhibition was also found with GHI/75 and M402, but HP-F1 was most efficient. Each anti-CD85j mAb prevented specific lysis to a degree that was similar to the inhibition with an anti-CD152 mAb. Similar results were found when testing CTLs lysis of autologous EBV-transformed LCLs.

T cell proliferation in a variety of setting is also inhibited by CD85j ligation. Saverino et al.[17] tested the effect of CD85j on CD3-induced proliferation of PHA-generated CD4 clones. Upon addition of HP-F1 and cross-linking goat anti-mouse (GAM), proliferation in nearly half of the clones was decreased by >40% while in an additional third, proliferation decreased by 20-40%. In a few clones, addition of HP-F1 in the absence of GAM enhanced proliferation. Recall antigen-induced proliferation of total PBMCs stimulated with tetanus toxoid, *Candida albicans*, or purified protein derivative (PPD) was slightly to moderately inhibited by HP-F1 cross-linking. More strikingly, addition of HP-F1 without GAM resulted in enhanced proliferation in response to each recall antigen tested. In some cases, especially where the baseline recall response was weak, this enhancement was quite dramatic. A similar pattern of inhibition and

enhancement was found using an anti-CD152 mAb but not an anti-CD8 mAb. The same group repeated these result, without PPD and with the addition of *Cryptococcus* as a recall antigen, in a later report.[39] Also in the second, the authors conclude, based on PI staining of CD4 T cells, that CD85j engagement results in accumulation cells at the G_0/G_1 phase rather than an increase in apoptosis.

The cutaneous T-cell lymphoma known as Sézary syndrome is characterized by malignant clonal expansion of CD4+ T cells, many of which express CD85j. Nikolova et al.[22] examined the role of CD85j on a cell-line derived from Sézary cells that proliferates in response to immobilized anti-CD3 mAb or treatment with exongenous IL-7. CD85j cross-linking in the presence of anti-CD3 stimulation, but not IL-7, impaired proliferation.

In the 2002 report, Saverino et al.[39] also measured the effects of CD85j (and CD152) engagement and blockade on cytokine production by PBMCs two days after recall antigen challenge. In general, levels of immune stimulatory cytokines, including IL-2 and IFN-γ, were enhanced by blocking CD85j (no GAM added) and inhibited by GAM-mediated crosslinking of CD85j. By contrast, IL-10 and TGF-β, which down-regulate immune responses, exhibited a reciprocal pattern. The increase in TGF-β, which was largely absent in the presence of recall antigen alone, following CD85j cross-linking was particularly striking. The effects of CD85j on proliferation and cytokine production (this time including the immune stimulatory IL-13) persisted when cells were re-challenged ten days after the initial challenge and HP-F1 treatment. Similar findings were seen when long-

term (four re-stimulations) antigen-specific CD4 T cell lines were restimulated in the presence, for the first time, of HP-F1 blockade or cross-linking. Also, anti-CD3 restimulation, in the absence of APCs, yielded consistent results, suggesting that CD85j effects were T cell-intrinsic. Again, each of these results was also seen for CD152. Of note, each of these effects was reversed by addition of exogenous IL-2.

In summary, functions of both CD4 and CD8 T cells are inhibited by CD85j ligation. Among the intracellular effects of CD85j engagement are dimished LAT and CD3ζ-chain phosphorylation, decreased Zap70 recruitment, decreased ERK1/2 phophorylation, and decreased actin cytoskeletal rearrangement. Proliferation in response to recall antigens and CD3-stimulation, but not IL-7 treatment, is diminished by CD85j cross-linking. Pro-inflammatory cytokine production decreases and anti-inflammatory cytokine production increases. Like in NK cells, cytotoxicity by CD8 T cells is inhibited by CD85j recognition of MHC class I molecules on target cells. Of note, CD85j co-localizes with CD3 at the point of cell-cell contact which potentially allows CD85j to compete with CD8 for MHC class I binding during initiation of TCR stimulation.

CD85j function in B cells

In one of the original reports,[4] CD85j ligation was found to inhibit BCR-triggered Ca2+ mobilization in primary B cells and C1R cells. Another report[23] showed that CD85j-crosslinking dampens IgG and IgE production by primary B cells following activation. The percentage of B cells expressing IgG and IgE was

also decreased 6 and 12 days after stimulation, respectively. This was true for stimulation with recall antigens, CD40-engagement plus IL-4, and LPS-treatment plus IL-4 engagement. IL-8, IL-10, and TNF-α cytokine production was also reduced by CD85j-crosslinking. Interestingly, when anti-CD85j mAb was added to the recall antigen assays without cross-linking GAM antibody, IgG production was enhanced. These assays included antigen-specific autologous CD4 T cell clones and this enhancement may result from blocking the interaction of CD85j on the B cell and MHC class I molecules on the T cell.

CD85j function in monocytes

Monocytes ubiquitously express very high levels of CD85j and its ligation has been shown to affect short term cellular responses as well as, differentiation events over the course of a week.[40] Fanger et al.[14] demonstrated decreased global tyrosine phosphorylation by co-ligation of CD85j and CD64 (FcγRI) on peripheral blood monocytes (anti-CD85j mAbs are either M402 or M405, unspecified). These experiments also included CD85d co-ligation and CD85d-CD85j co-ligation which, in each case, resulted in diminished tyrosine phosphorylation. CD85d-CD85j co-ligation also inhibited CD64-dependent tyrosine phosphorylation of the common γ chain and Syk, which is recruited and activated by common γ chain activation[41-42]. In addition, CD64-dependent calcium mobilization was inhibited by CD85j (70% inhibition), CD85d (53%), and CD85j-CD85d co-ligation (85%).

Many mature cell-types are derived from monocyte precursors. As all monocytes express a large amount of CD85j protein and MHC class I expression is widespread, CD85j is poised to influence maturation events. Indeed, CD85j ligation on monocytes has been shown to decrease in vitro generation of osteoclasts[6] and to alter the character of monocyte-derived dendritic cells.[40] In these studies, resting monocytes were found to have constitutively tyrosine-phosphorylated CD85j which was able to recruit SHP-1.[6,40] As these results imply, CD85j is capable of *cis* interactions with its ligand. This assumption was confirmed by fluorescence-resonance energy transfer (FRET) experiments using labeled anti-CD85j and anti-MHC class I mAb.[6,40] CD85j is expressed throughout the in vitro differentiation of these and other (macrophages[6]) cell-types, and because it can, in theory, be ligated both in *cis* or by MHC I class I on neighboring or stromal cells, CD85j may play an important role in many pathways of hematopoiesis. In support of this, our group has found CD85j expression on up to 50% of peripheral CD34+ hematopoietic stem cells (Ines Colmegna, unpublished results).

Cell-type specific implications of CD85j expression and preview of our findings

In vitro cross-linking of CD85j with the activating stimuli is not a physiological representation of how CD85j functions in different cellular contexts. In CD8 T cells, MHC class I molecules represent the ligand for both the TCR and CD8 co-receptor and the inhibitory receptor CD85j. TCR and CD8 engage MHC

class I molecules within a tightly organized and spatially focused synapse that serves to strengthen and stabilize T cell-activating signals and more precisely direct effector molecules toward the target cell.[43] If CD85j is recruited to the synapse,[31] it can be expected to deliver a strong inhibitory signal even at low cell surface concentrations. In NK cells, the engagement of inhibitory receptors and their aggregation at the point of contact with target cells is considered a primary event forming an inhibitory synapse and thereby preventing lysis of healthy cells.[44]

In other cell-types, such as B cells and monocytes, whose classic functions do not require MHC class I interactions, in vivo engagement of CD85j may be more dispersed and not directly linked to the activating signal, in particular, if the activating stimulus is a soluble molecule. For these cells, either CD85j will engage in *cis*-binding to MHC class I molecules expressed on the same cell, as has been shown to occur in monocytes,[40] or in *trans*-binding to MHC class I molecules on neighboring cells during cell-to-cell contacts that do not directly involve or require MHC class I. Because MHC class I is expressed by all nucleated cells, *trans* interactions could involve a wide range of cell-types including stromal cells, endothelial cells, T cells, and other B cells or monocytes. Obviously, CD85j on NK and CD8 T cells can participate in these *cis* and *trans* interactions in addition to the more precise scenarios mentioned above.

Given that CD85j function in different cellular contexts likely requires different levels of cell surface expression, it is intriguing to hypothesize that the regulation of CD85j is cell-specific. Despite extensive analysis of CD85j function

and distribution within hemotpoietic cell-types, the mechanisms controlling *LILRB1* expression have received very little attention. In fact, only a single report has addressed the *LILRB1* promoter. By use of luciferase reporter assays, Nakajima et al.[45] found that a ~160 nt core promoter drives *LILRB1* expression and, by electromobility shift assays (EMSA) and co-transfection studies, that the transcription factors PU.1 and Sp1 are involved. Only weak promoter activity was found in Jurkat T cells compared to myeloid cell lines such as THP-1 and U937. In fact, activity in Jurkat T cells was no more than that found in HEK 293 cells, which are of epithelial origin and do not express CD85j.

Importantly, the promoter analyzed by Nakajima et al. lies upstream of the *LILRB1* exon that precedes the exon containing the protein translation start codon. While the majority of *LILRB1* transcripts submitted to NCBI begin with this exon, several others (such as BC015731) begin, instead, with an additional exon that maps to a sequence ~13 kb upstream of the main *LILRB1* exon cluster. This upstream exon is spliced to an acceptor site within what Nakajima et al., and many other groups, consider the first *LILRB1* exon. The presence of transcripts such as BC015731 in the database implies an alternative *LILRB1* promoter that remains uncharacterized.

As described in Chapter 2 and a recently published report,[46] we establish that an upstream *LILRB1* promoter does indeed exist and its activity drives CD85j expression in lymphocytes. We also show that *LILRB1* expression in monocytes originates from the downstream promoter. Importantly, we find that sequences within the first *LILRB1* exon, that is exclusive to transcripts generated

from the lymphocyte promoter, confer poor CD85j translational efficiency compared to transcripts generated in monocytes. Our data provide a mechanism for tissue specific regulation of both *LILRB1* gene expression and CD85j protein levels.

In Chapter 3, we examine in detail CD85j expression by T cells. We show evidence that CD85j increases with age on CD8 T cells and that its expression marks aging at least as well as the loss of CD28. Furthermore, we suggest that increased transcriptional activity from the upstream lymphocyte promoter accounts for CD85j expression in CD8 T cells. The expanding presence with age of an inhibitory receptor on CD8 T cells has implications for vaccine responses, infection control, and cancer surveillance in the vulnerable eldery population. Our findings clarify how and when CD85j is acquired on T cells and will be important to consider when developing future immune manipulation strategies.

Chapter 2: Distinct promoters drive expression of CD85j in hematopoietic lineages*

CD85j expression differs among peripheral blood subsets

CD85j is widely expressed within the hematopoietic system, but its expression pattern differs considerably among cell-types (Figure 2.1A-B).[3-4,14,18] Expression on monocytes is essentially ubiquitous. By contrast, CD85j is expressed on a subset of NK cells and T cells.[3-4] As we[21] and others[18] have shown, CD85j expression within the T cell compartment is far more likely for CD8 T cells than CD4 T cells and is restricted to memory cells and, in particular, CD45RA+ effector cells (Figure 2.1C). CD85j expression on peripheral B cells is widespread; however, a detailed analysis suggests it, like in T cells, is dependent on maturation. We examined circulating human transitional B cell populations which are in the process of completing their maturation after exiting the bone marrow.[47-48] Early transitional B cells (T1) express CD85j in lesser frequency than late transitional B cells (T2) (Figure 2.1D). These results are consistent with an earlier report that developing B cells within the bone marrow acquire CD85j expression during maturation.[9] Among the other peripheral B cell populations we examined (naïve, non-switched memory, and switch memory B cells as reviewed by Sanz et al.[49]), CD85j was ubiquitously expressed at similar levels (Figure 2.1E).

*Portions of this chapter have been adapted from an article published in *Blood* in 2010.[46]

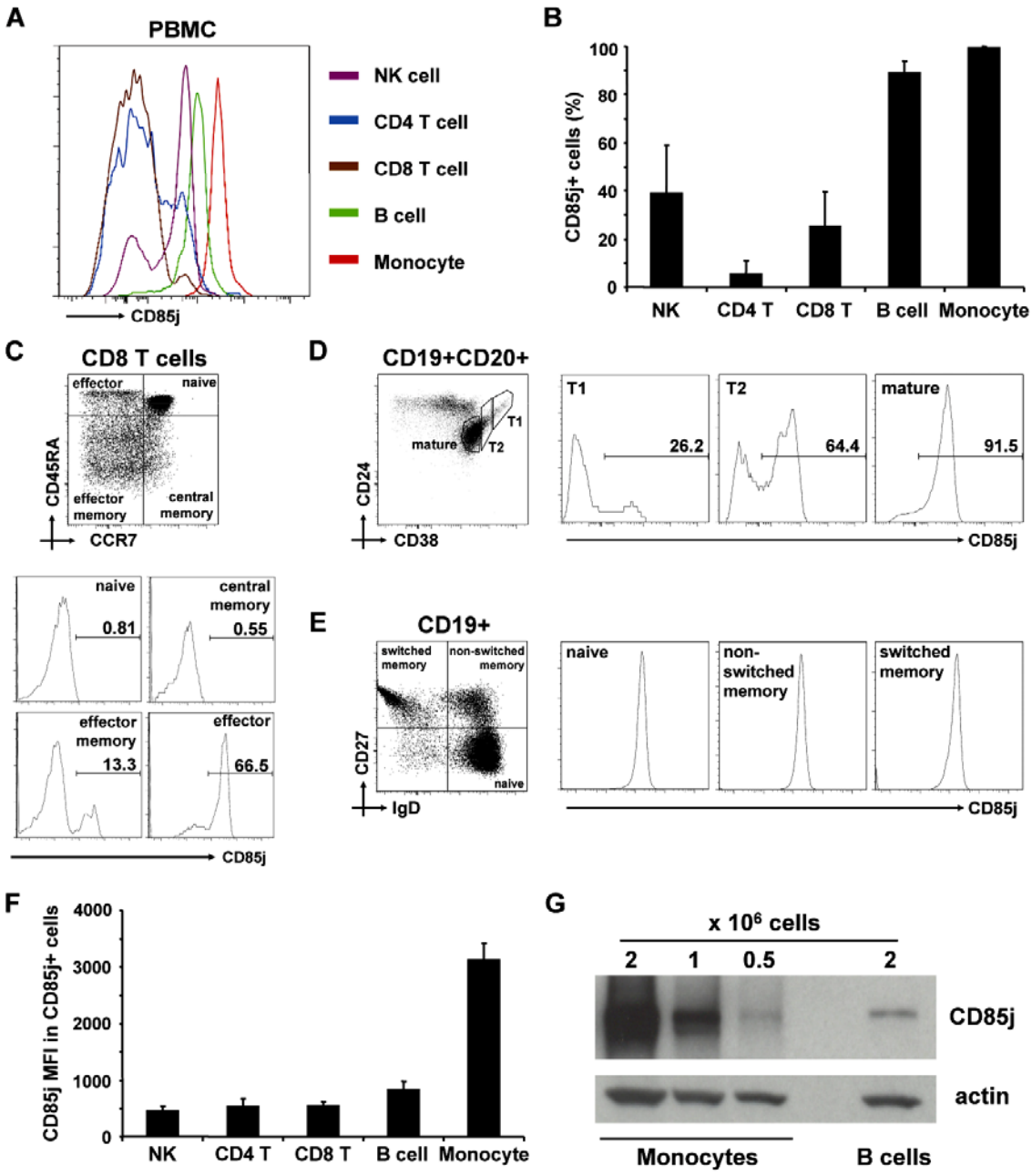

Figure 2.1 Differential CD85j expression on human PBMC subsets. (A) Representative histograms showing CD85j expression on NK cells, CD4 T cells, CD8 T cells, B cells and monocytes. (B) Frequencies of CD85j positive cells within subpopulations are shown as mean ± S.D. of 12 donors. (C) CD85j expression on

CD8 T cell subsets. Histograms (bottom) show CD85j expression on the CD8 T cell subpopulations based on the expression of CCR7 and CD45RA as shown in the dot plot at top. Numbers in the histograms (bottom) indicate the percentage of cells expressing CD85j. (D) CD85j expression on peripheral transitional B cells. Histograms (right) show CD85j expression on the populations defined in density plot to the left (gated on CD19+CD20+ cells). T1, early transitional B cells; T2, late transitional B cells. (E) CD85j expression on peripheral naïve and memory B cells. Histograms (right) show CD85j expression on the populations defined by the quadrants in dot plot to the left (gated on CD19+ cells). (F) CD85j cell surface densities (MFI, mean fluorescence intensity) by flow cytometry on the CD85j positive cells are shown as mean MFI $\pm$ S.D. of 12 donors. (G) CD85j expression on monocytes and B cells are compared by western blot. Blot is representative of comparisons of 4 donors.

In addition to distinct CD85j expression patterns, PBMC subpopulations express characteristic levels of CD85j protein. Monocytes express nearly 4 times as much CD85j protein as B cells (Figure 2.1F-G). NK cells and T cells express CD85j to a lesser degree than B cells, but levels are similar among NK cells and CD4 and CD8 T cells that are CD85j-positive (Figure 2.1F).

We hypothesized that the distinct CD85j expression profiles within PBMC subsets result from differences in transcription of the *LILRB1* gene. As expected, quantitative real-time (qRT)-PCR of exon 8 within the coding region showed *LILRB1* transcripts to be lower in CD8 T cells compared to B cells and monocytes (Figure 2.2A). Surprisingly, B cells and monocytes express similar

levels of *LILRB1* transcripts (Figure 2.2A) despite a considerable difference at the protein level (Figure 2.1F-G).

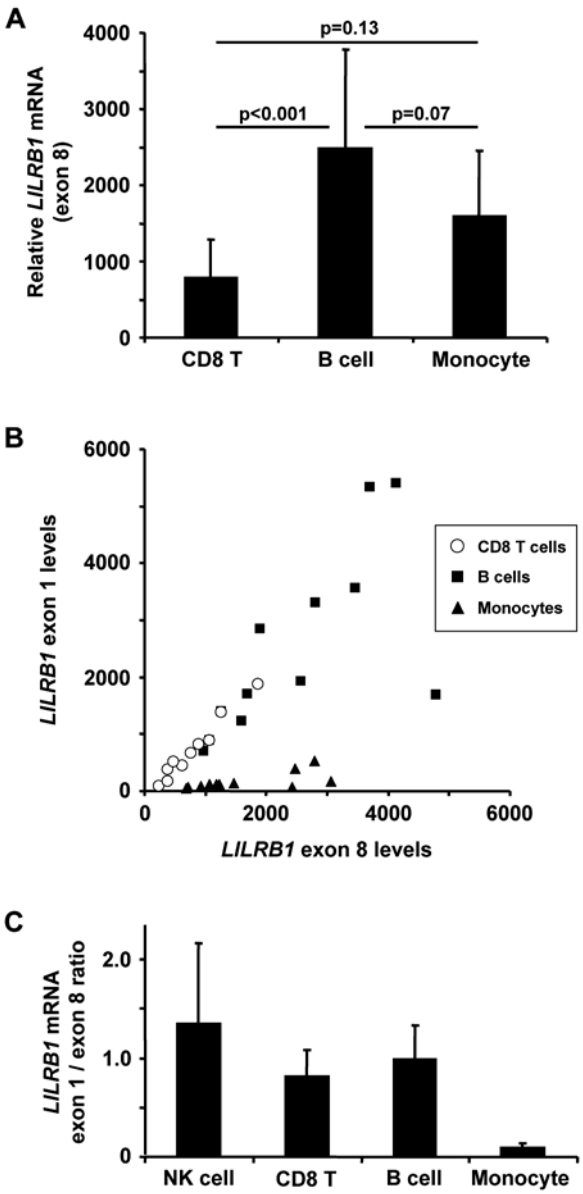

Figure 2.2 *LILRB1* mRNA levels does not account for different subset-specific CD85j protein expression. *LILRB1* transcripts were quantified by RT-qPCR in RNA from magnetic-bead separated CD8 T cells, CD19 B cells and CD14 monocytes, and NK cells purified by negative selection. (A) Results for a primer set within exon 8 are shown as mean transcript numbers ± S.D. of 10-12 donors per group relative to $2x10^5$ beta-actin copies. Compared to protein expression, transcript numbers in monocytes were disproportionately low. (B) *LILRB1* transcripts were compared for exon 1-3 and exon 8 sequences. Results are shown as a scatter plot for CD8 T cells (opened circles), B cells (squares) and monocytes (triangles). (C) Transcript comparisons are quantified as the ratio of *LILRB1* exon 1-3 to exon 8 copies. Results are shown as mean ± S.D. of 10-12 donors per group. NK cells data are from 3 donors.

LILRB1 transcripts in lymphocytes contain 5'UTR sequences that are absent in monocytes

An examination of *LILRB1* cDNA sequences submitted to NCBI revealed several transcript variants, some of which differ within the 5'-untranslated regions (UTR) (see Figure 2.3A for a schematic). We hypothesized that B cells and monocytes express *LILRB1* transcripts with distinct 5'UTRs which affect CD85j translation. Indeed, submitted sequences differ in the number of potential start codons positioned upstream of the accepted start codon within exon 3 (eg, seven ATG sequences in BC015731[50] compared to four ATG sequences in AF283984[13]). As the primer pair we used to quantify *LILRB1* transcripts is specific for a sequence within the coding region, we designed an additional primer pair (subsequently referred to as "exon 1 primers") targeting the 5'-most exon that is present in some submitted sequences (such as BC015731) but not in others (such as AF283984). The antisense primer of this pair binds to a sequence within exon 3 that is present in all *LILRB1* transcripts. To assess whether B cells and monocytes express similar 5'UTRs, we performed qRT-PCR using both exon 1 primers and exon 8 primers simultaneously on the same cDNA sample. B cells and monocytes yielded strikingly different results. Comparison of *LILRB1* exon 1 and exon 8 transcript numbers suggested that most *LILRB1* transcripts in B cells included both exons (Figure 2.2B-C). Monocyte values, however, fall on a much shallower slope implying that the vast majority of *LILRB1* transcripts from monocytes do not include exon 1 (Figure 2.2B-C). The same qRT-PCR test performed on cDNA from CD8 T cells and NK cells revealed a ratio of *LILRB1*

exon 1 to exon 8 levels similar to that seen in B cells (Figure 2.2B-C) and significantly larger than the ratio found in monocytes ($p<0.001$).

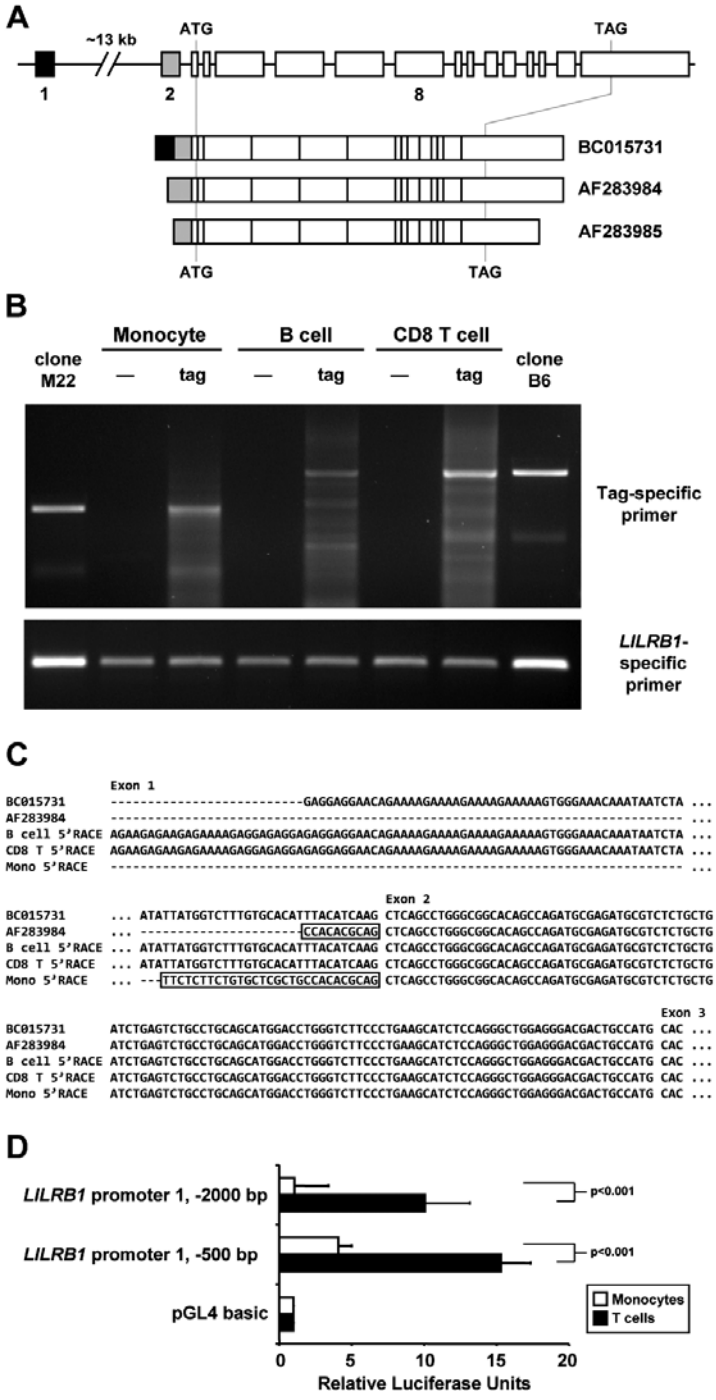

Figure 2.1 *LILRB1* transcription initiation sites in CD8 T cells, B cells, and monocytes. (A) Schematics of the *LILRB1* locus on human chromosome 19 and selected mRNA sequences currently posted to NCBI. Lines are introns and boxes are exons (roughly to scale). Exons 1 and 2 are separated by ~13 kb as indicated by a gap. The *LILRB1* coding region is flanked by a start ("ATG") and stop ("TAG") codon. (B) PCR of 5'RACE products generated from monocyte, B cell, and CD8 T cell RNA isolated from magnetic-bead separated cells. The enzyme TdT was excluded ("—") or included ("tag") when tagging the 5' end of *LILRB1* cDNA. PCR products were amplified using a tag-specific sense primer and an *LILRB1*-specific antisense primer (top) or *LILRB1*-

specific sense and antisense primers (bottom). Templates for lanes indicated by "clone M22" and "clone B6" were the 5'RACE clone from monocytes and B cells, respectively, used to obtain the sequences shown in panel C. (C) Sequencing of 5'RACE products. Total PCR products were TOPO-cloned and sequenced. The sequence corresponding to the major tag-specific band for each cell-type in panel B is compared to *LILRB1* sequences BC015731 and AF283984. Boxed sequences indicate contiguous sequences upstream of exon 2 in the genome. (D) Luciferase reporter assay of sequences upstream of *LILRB1* exon 1. The 500 bp and 2000 bp sequences found immediately upstream of *LILRB1* exon 1 on chromosome 19 were amplified and placed upstream of the firefly luciferase ORF of pGL4.10. Freshly isolated primary T cells and monocytes were co-transfected with the reporter constructs and a control *Renilla* luciferase expression vector. Data represent firefly luciferase activity, normalized to *Renilla* luciferase activity, relative to that seen with the promoterless basic pGL4.10 vector. Data from 3 donors are represented as mean $\pm$ S.D.

Lymphocytes initiate LILRB1 transcription from a site 13 kb upstream of the major site used by monocytes

The above results, when considered together with the genomic structure of the *LILRB1* gene, imply that lymphocytes and monocytes do not use the same promoter to transcribe *LILRB1*. Figure 2.3A depicts the genomic organization of the *LILRB1* gene and indicates the corresponding exons included in *LILRB1* transcript sequences submitted to NCBI. As is shown, exon 1 is separated from the other *LILRB1* exons by a 13 kb intron. The schematic also shows that only

some of the published *LILRB1* transcripts include exon 1 while others begin with exon 2. Little is known about the mechanisms regulating *LILRB1* transcription. The only report of an *LILRB1* promoter analysis examined a roughly 1 kb region just upstream of what is called exon 2 in Figure 2.3A.[45] Therefore, we reasoned that monocytes, whose cDNA yielded very low exon 1 signals by qRT-PCR, may initiate *LILRB1* transcription from the described promoter upstream of exon 2, while lymphocytes initiate *LILRB1* transcription from an undescribed promoter upstream of exon 1. To further address this question and identify *LILRB1* transcription initiation sites, we performed 5'RACE analysis of cDNA from monocytes, B cells, and CD8 T cells. As shown in Figure 2.3B, B cells and CD8 T cells share a major 5'RACE product that is larger than the major product from monocytes. Sequencing of products from all three cell-types confirmed that B cells and CD8 T cells initiate *LILRB1* transcription with exon 1 while monocytes transcripts begin with exon 2 (Figure 2.3C). The major B cell and CD8 T cell product corresponds to a transcription initiation site 26 nt upstream of the first nucleotide of submitted *LILRB1* cDNAs that begin with exon 1 (BC015731). Conversely, the major product in monocytes identifies a transcription initiation site 19 nt upstream of submitted *LILRB1* sequences beginning with exon 2 (AF283984), and 18 nt upstream of the transcription initiation site identified by Nakajima et al.[45] The additional upstream nucleotides we identified all correspond to contiguous genomic sequences.

To further support our assertion that lymphocytes and monocytes,use distinct promoters to drive CD85j expression, we generated luciferase reporter

constructs using genomic sequences upstream of *LILRB1* exon 1 and compared activity of these constructs in T cells and monocytes. Indeed, these sequences exhibited strong activity in transfected T cells but only weak activity in monocytes (Figure 2.3D). In T cells, constructs including the 2000 bp or 500 bp sequences directly upstream of *LILRB1* exon 1 showed 10 and 15-times stronger activity, respectively, than a luciferase construct lacking a promoter. In monocytes, the 500 bp construct showed only modest activity compared to the basic vector while the activity seen with the 2000 bp construct was negligible. These results strongly suggest that a second, yet undescribed, promoter rests 13 kb upstream of the main *LILRB1* exon cluster (exons 2 to 16) and directs *LILRB1* transcription in lymphocytes.

Exon 1 sequences inhibit translation of CD85j

Our initial observation that, despite similar transcript levels, peripheral blood monocytes express far more CD85j protein than B cells suggests that CD85j is not translated as efficiently in B cells. We hypothesized that the unique *LILRB1* 5'UTR that results from usage of the upstream promoter by B cells contributes to this diminished protein production. To test this hypothesis, we generated expression vectors containing either *LILRB1* coding region cDNA alone or including an exon 1-containing 5'UTR (from BC015731). CD85j protein production by cells transfected with these vectors was assessed by flow cytometry. Because CD85j is expressed by a subset of CD8 T cells and by nearly all B cells and monocytes, these experiments were carried out in CD4 T

cells. Indeed, we found that transfection with the 5'UTR-containing vector resulted in diminished CD85j expression compared to cells transfected with the *LILRB1* coding region vector (Figure 2.4A). Interestingly, transfection with the full-length *LILRB1* cDNA (BC015731) resulted in the same diminished expression suggesting that the 5'UTR dominates any effect the 3'UTR may have on CD85j protein levels.

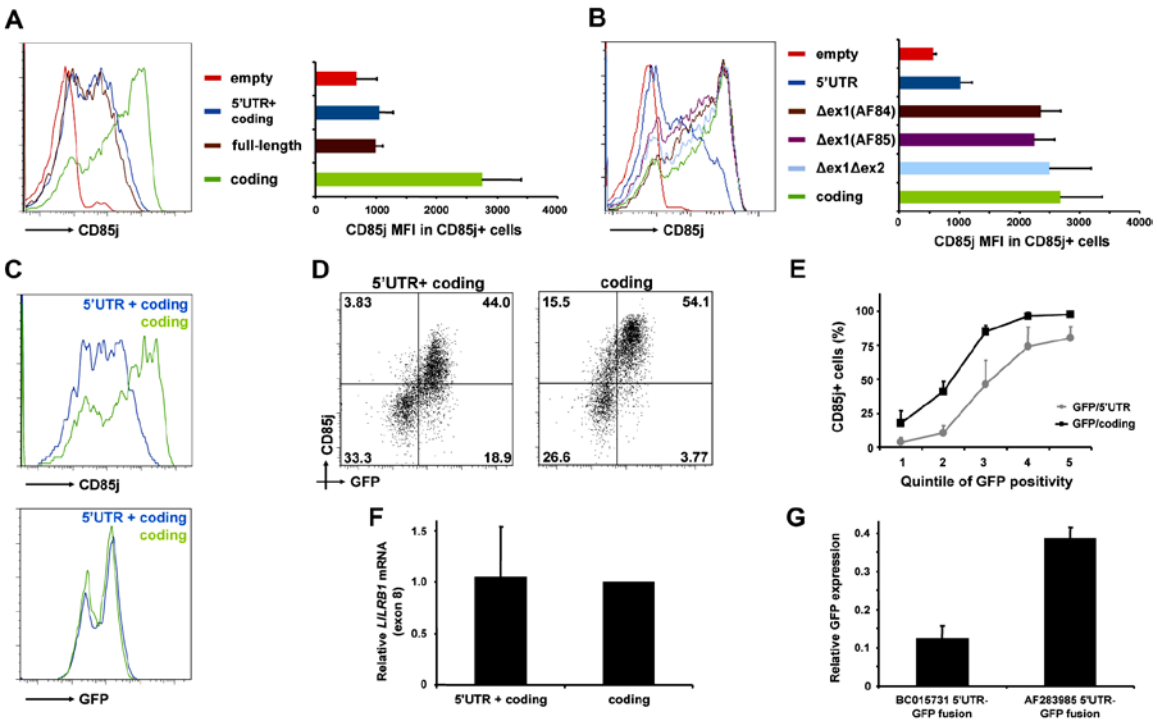

Figure 2.2 Exon 1 sequences repress CD85j expression. Human PBMCs were transfected with expression constructs containing varying *LILRB1* cDNAs and/or GFP. CD85j and GFP expression in CD4 T cells was analyzed 24 hours post-transfection by flow cytometry. (A) Histograms and bar graph comparing CD85j expression by full-length (BC015731) cDNA, 5'UTR plus coding region and coding region alone. Results are representative of six experiments. (B) Histograms and bar graph comparing various portions of the *LILRB1* 5'UTR plus coding region and coding region alone. Δex1(AF84) and Δex1(AF85) contain the *LILRB1* 5'UTR from AF283984 and

AF283985 sequences, respectively. Δex1Δex2 begins with exon 3 and continues through the *LILRB1* coding region. For panels C-F, a plasmid expressing GFP was co-transfected along with a *LILRB1* 5'UTR plus coding or coding region alone plasmid. Results are representative of six experiments. (C) Representative histograms showing CD85j and GFP expression in co-transfected CD4 T cells. (D) Flow cytometry plot of samples shown in panel C. (E) Graph of percent CD85j positive CD4 T cells in cells co-transfected with GFP. Samples were divided into quintiles based on GFP expression and means ± S.D. from 3 transfections were calculated for each quintile. (F) Relative *LILRB1* mRNA (exon 8) in co-transfected cells. cDNA was treated with DpnI prior to real-time PCR to digest plasmid DNA. n=3 transfections. (G) *LILRB1* 5'UTR sequences that include (BC015731) or exclude (AF283985) exon 1 were cloned upstream of the GFP ORF of pmaxGFP. Data showing GFP MFI relative to an unaltered GFP control vector are presented as mean ± S.D. from 3 transfections.

To address the hypothesis that distinct 5'UTRs in B cells and monocytes account for their differences in protein expression, CD85j protein expression from transcripts containing these distinct 5'UTRs were compared. We generated a series of vectors with *LILRB1* coding region cDNA linked to various portions of the 5'UTR. These vectors include the full 5'UTR (containing exon 1) and 5'UTRs from AF283984 and AF283985[13], both of which begin within exon 2, and a truncated 5'UTR that begins with exon 3. Analysis of cells transfected with these vectors demonstrates that exon 1 is responsible for the diminished protein expression conferred by the full-length *LILRB1* 5'UTR (Figure 2.4B). This exon 1 effect was reliably observed by transfection of cells from many different donors; even transfecting double the amount of exon 1-containing DNA failed to reach

CD85j levels seen with vectors lacking exon 1 (data not shown). Co-transfection with a separate GFP vector suggests that cells receiving the full *LILRB1* 5'UTR plus coding region construct are as efficiently transfected as cells receiving the *LILRB1* coding region construct (Figure 2.4C). Additionally, in cells co-transfected with the 5'UTR plus coding construct, only those cells with high GFP expression, reflecting high delivery of plasmid DNA, exhibited high CD85j-positivity (Figure 2.4D-E). In contrast, cells receiving *LILRB1* coding region constructs begin to express CD85j even before GFP is detectible. qRT-PCR analysis from these cells suggest that co-transfected cells transcribe similar levels of *LILRB1* mRNA despite the differences in protein expression (Figure 2.4F). Furthermore, the poor protein expression conferred by the *LILRB1* 5'UTR can be transferred to another protein (GFP) and, in this context, is also exon 1-dependent (Figure 2.4G).

The sequence conferring translational repression of CD85j is mapped to 30 nt of exon 1

To further define the sequences within *LILRB1* exon 1 that may be preventing full protein expression, we made a series of 5'UTR plus coding region constructs containing alterations in the exon 1 sequence (Figure 2.5A). First, we examined the possibility that ATG sequences within exon 1 might act as false start codons, thereby interfering with ribosomal binding to the true start codon within exon 3. By site-directed mutagenesis, we destroyed each of the three ATG sequences within exon 1 by changing the T to an A. When constructs containing just one of

these changes were transfected, CD85j expression did not improve compared to constructs containing the unaltered exon 1 (Figure 2.5B). To prevent all false translation initiations within exon 1, a construct was made where all ATGs were destroyed. Transfection with this construct yielded only slightly higher CD85j expression (Figure 2.5B).

Next, we made a series of constructs with progressive truncation of exon 1. These constructs revealed that as little as 30 nt of exon 1 sequence can prevent the full CD85j expression seen when cells are transfected with coding region constructs (Figure 2.5C). This 30 nt sequence contains one of the three ATGs found in exon 1 as well as the sequence ATTTA, a motif found in so-called AU-rich elements (ARE) that is known to mediate translational repression in other genes. We generated constructs lacking one or both of these sequences and tested CD85j expression following transfection. Similar to our findings using ATG mutants within the entire exon 1, disruption of the ATG within ex1Δ145 resulted in a slight enhancement of CD85j expression (Figure 2.5D). Disruption of the ARE sequence (ATTTA > ATCTA) yielded an even higher CD85j expression. When both the ATG and the ARE sequences were destroyed, CD85j expression nearly matched the strong expression seen when transfecting the *LILRB1* coding region alone. However, when we disrupted the ARE sequence alone within the complete exon 1, we found no enhancement of CD85j expression (Figure 2.5B). The observed effect in the truncated constructs is in contrast to the neglible enhancement of the same mutations in the context of the entire exon 1,

suggesting that false start codons and/or the ATTTA sequence only play an indirect role in the poor CD85j protein expression.

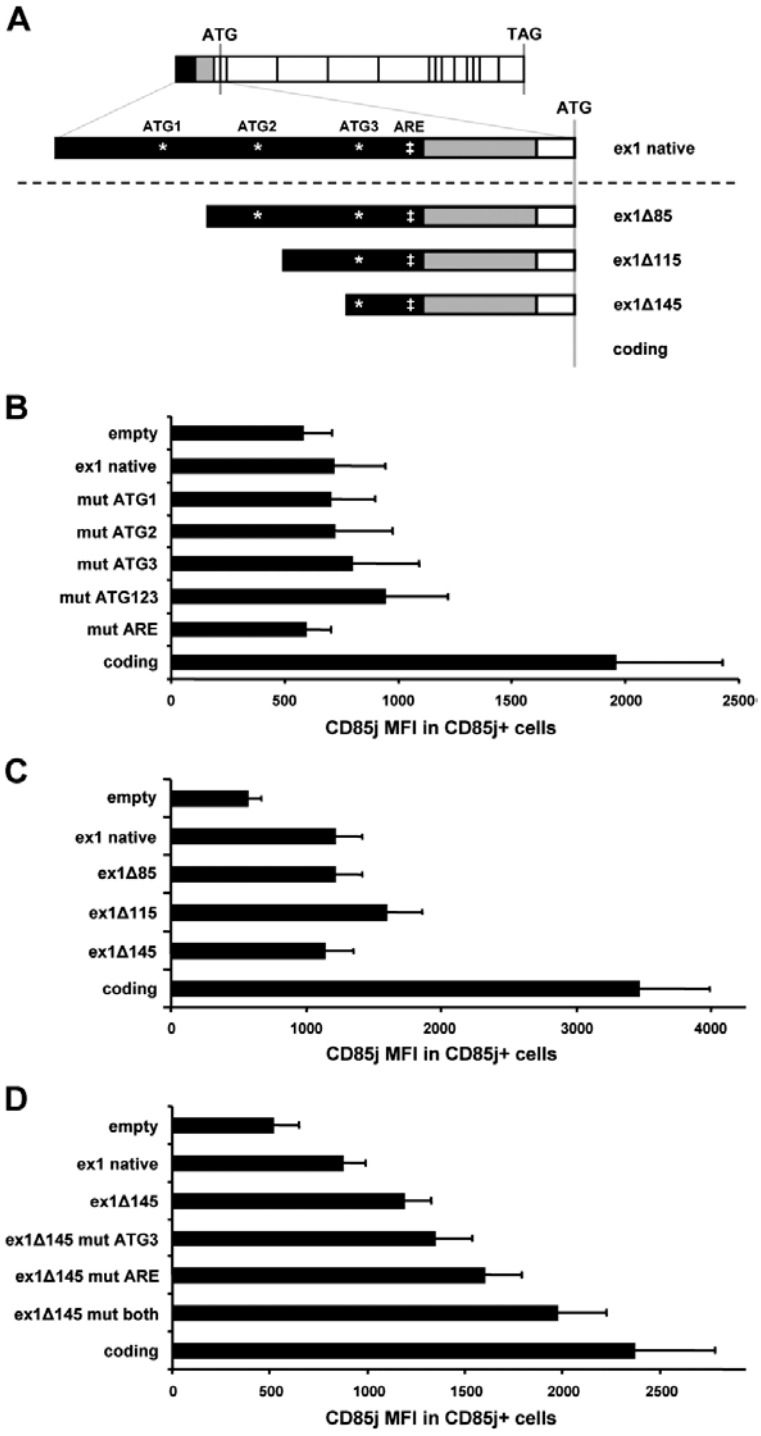

Figure 2.3 The distal 30 nt of LILRB1 exon 1 accounts for the poor protein expression by LILRB1-exon 1-containing constructs. (A) Schematic of constructs used to isolate the region within *LILRB1* exon 1 responsible for poor protein expression. In panels B-D, contructs used for transfections contained the *LILRB1* coding region preceded by the portions of the *LILRB1* 5'UTR indicated here. Sites in exon 1 which, in some constructs in panels B and D, were mutated are indicated by an asterisk (*) for ATG sequences and a double-dagger (‡) for the ARE sequence. (B) Graph comparing *LILRB1* 5'UTR plus coding constructs. Mutations

were introduced into an *LILRB1* 5'UTR construct containing the full-length exon 1. For mut ATG1, 2, 3, and 123 the three potential start codons found in *LILRB1* exon 1 were changed to AAG. For mut ARE, the ATTTA sequence was changed to ATCTA. n=4 transfections. (C) Graph comparing *LILRB1* 5'UTR plus coding constructs. Progressively-truncated *LILRB1* exon 1 constructs were compared to an *LILRB1* coding region construct. n=3 transfections. (D) Graph comparing *LILRB1* 5'UTR plus coding constructs. The ATG and ARE sites in the ex1Δ145 construct were mutated and and CD85j expression was compared to the unmutated construct and an *LILRB1* coding region construct. n=5 transfections. All bars represent mean CD85j MFI $\pm$ S.D.

Chapter 3: Age-dependent acquisition of CD85j on CD8 T cells[‡]

CD85j is an age-dependent cell-surface marker on CD8 T cells

Our interest in CD85j began with an investigation of possible cell-surface markers of aging. It is well known that the fraction of CD28+ T cells declines with age.[51] This change is regulated at the transcriptional level[52] and is a phenomenon that is much more prevalent in CD8 T cells than CD4 T cells.[53] CD28 loss is also a feature of conditions of immune dysfunction, such as rheumatoid arthritis[54] and acute coronary syndrome.[55] In an attempt to identify other age-dependent genes in T cells, we performed a gene array comparing memory cells CD4 T cells from young and old donors, and comparing CD28+ and CD28- memory cells within old donors. Similar to findings from a study of CD28+ and CD28- CD8 memory T cells by Fann et al.,[56] among the genes we found to be increased in CD4+CD28- cells were members of the LRC on chromosome 19, included the genes *KIR2DL2*, *KIR2DL3*, and *KIR2DL4*. Fann et al. also found an increase in *KIR2DL2* expression in CD28- CD8 cells as well as the LRC members *KIR2DS2* and *NCR1*. Although neither our or Fann et al.'s gene array analysis identified increased *LILRB1* (CD85j) gene expression in CD28- T cells, other studies have reported that CD85j protein expression frequently occurs on CD28- T cells.[16,18] In addition, CD85j is found on very few T

[‡] Portions of this chapter have been published in the *Clinical Immunology*[19] and are included here with the permission of the publisher.

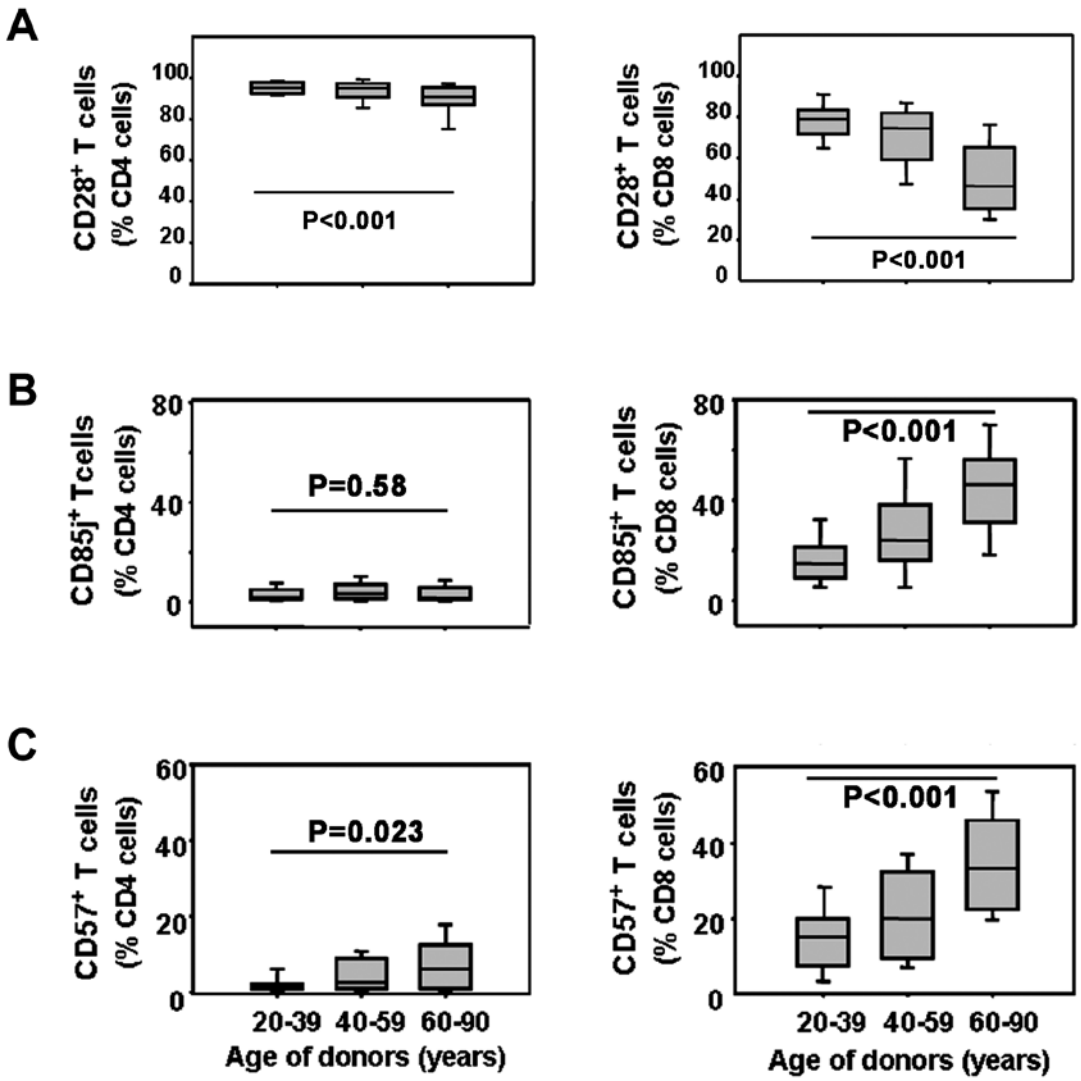

Figure 3.1 Influence of age on cell-surface receptor expression within CD4+ and CD8+ T cells. The frequency of (A) CD28, (B) CD85j, and (C) CD57 expression in CD4 (left panel) and CD8 (right panel) T cell subpopulations was determined by FACS in healthy individuals of different ages. Results are shown as box blots with medians, 25th and 75th percentiles as boxes and 10th and 90th percentile as whiskers for different age strata.

cells from umbilical cord blood[18] which suggests it accumulates with age. For these reasons, and because *LILRB1* is also located within the LRC,[57] we

included CD85j in the protein expression analysis we performed to verify our gene array findings.

In a cohort of dozens of donors divided into young (age 20-39), middle-aged (age 40-59), and elderly (age 60-90) age-groups, we found a clear and striking age-dependent increase in CD85j on CD8 T cells (Figure 3.1B). In fact, CD85j exhibited the steepest increase of any molecules we analyzed, including CD57 (Figure 3.1C) and HLA-DR (data not shown), and the magnitude of the change rivaled that of the loss of CD28 (Figure 3.1A). By linear regression analysis, we estimate the per year change in CD85j and CD28 expression on CD8 T cells is +0.7% and -0.6%, respectively. Of note, the cells expressing CD85j are largely the same cells that have lost CD28 (Figure 3.2A). This is true for CD4 T cells as well, although, as we have shown is the case for many age-dependent changes, the effect is less potent than in CD8 T cells. For example, in cells from an elderly donor, CD85j can be found on nearly all CD28- CD8 T cells but also on some CD28+ cells. By contrast, CD85j may or may not be expressed by CD28- CD4 T cells and is almost never seen on CD28+ cells (Figure 3.2B). As evidence that CD85j's age-dependent acquisition is not simply due to the accumulation of CD28- cells, the CD85j expression pattern on CD8 T cells from a young donor resembles how CD85j is expressed on CD4 T cells rather than the example given above for CD85j expression on CD8 T cells from the elderly (Figure 3.2B).

A detailed analysis of CD8 T cell subsets reveals a progressive increase in CD85j expression that parallels the differentiation steps from naïve cells to

terminally differentiated CD45RA effector cells (Figure 2.1C and reformatted in Figure 3.2C). This pattern applies to cells from donors of all ages but, as is the

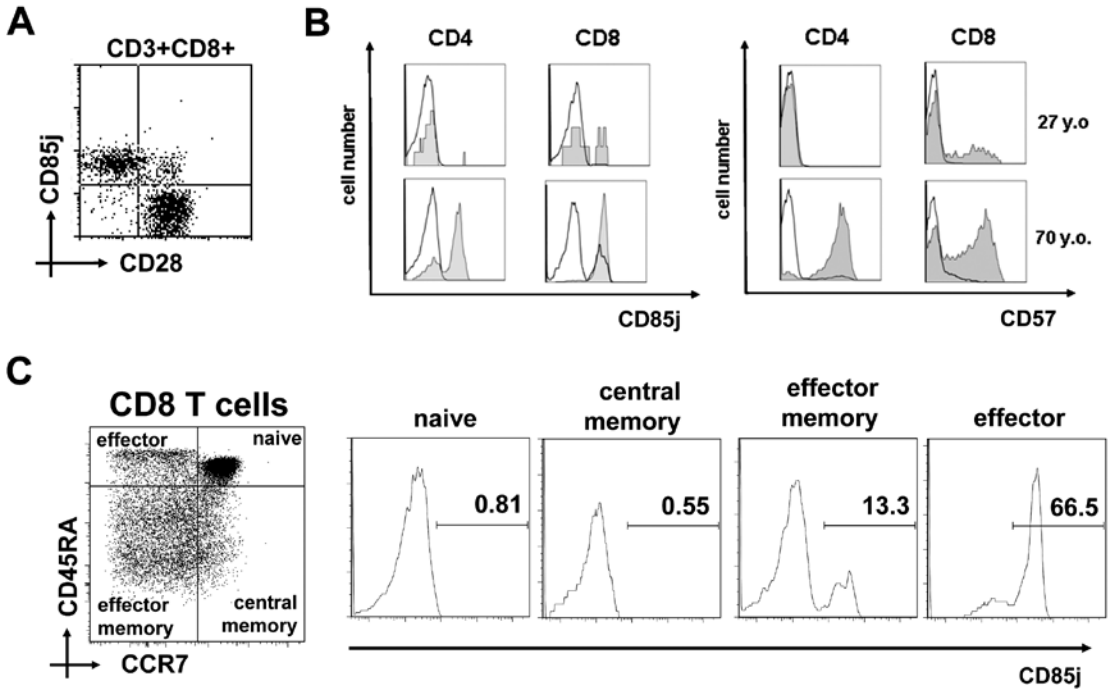

Figure 3.2 CD85j expression in T cells favors memory CD8 T cells that have lost CD28 and reverted to CD45RA+ phenotype. (A) Representative dot plot showing CD85j and CD28 expression on CD8 T cells. Lymphocytes from a young donor are gated on CD3+CD8+ T cells. (B) In both CD4 and CD8 T cells, CD85j and CD57 were preferentially expressed on CD28-negative (shaded areas) and less on CD28-positive cells (dark lines). (C) CD85j expression on CD8 T cell subsets. Histograms (right) show CD85j expression on the CD8 T cell subpopulations based on the expression of CCR7 and CD45RA as shown in the dot plot at left. Numbers in the histograms (bottom) indicate the percentage of cells expressing CD85j. (Panel C is identical to Figure 2.1C except for some reformatting and has been included here for clarity.)

case with CD85j expression relative to CD28 expression, cells from each subset are more likely to be CD85j-positive in the elderly.[19] Increasing CD85j expression in parallel with differentiation suggests that replicative history and/or repeated stimulation may promote CD85j acquisition in T cells. Consistent with this idea, we have shown that cultured T cells that were initially CD85j-negative, after several weeks of stimulation with OKT3, IL-2, and IL15, begin to express it (Figure 3.3A).[19] As expected,[51] CD28 expression is lost during this time as well.

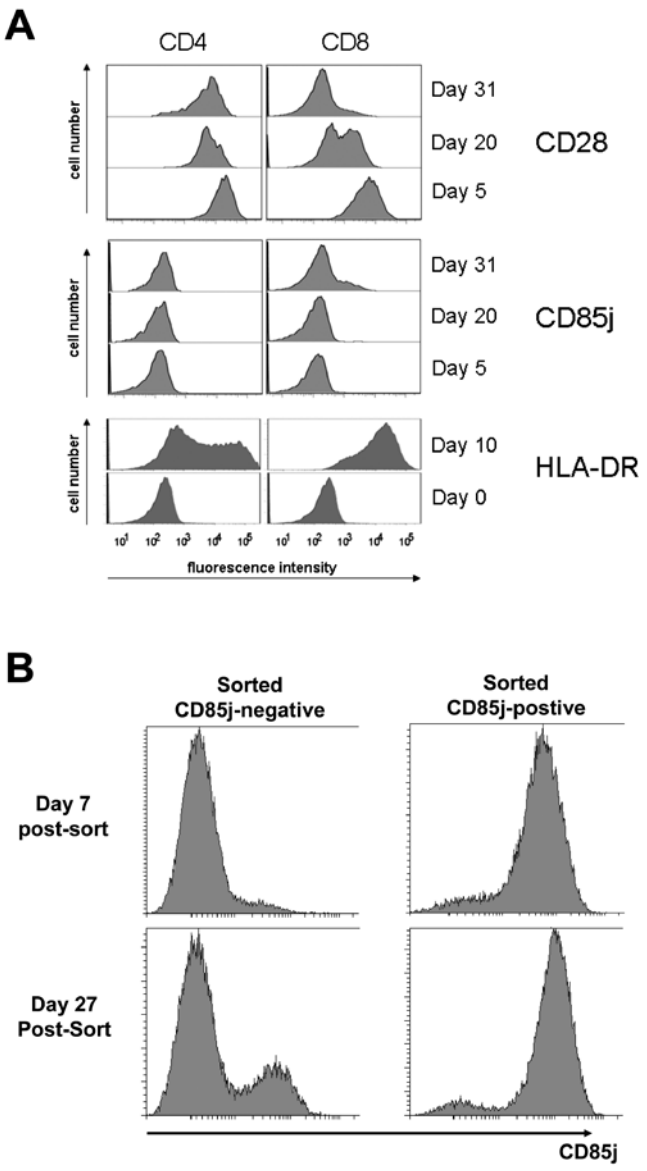

Figure 3.3 CD85j acquisition on highly-differentiated T cells can be recapitulated by repeated in vitro stimulations. (A) CD28 positive cells were sorted, labeled with CFSE, and stimulated with irradiated, EBV transformed PBMC and OKT3. FACS analysis was carried out every seventh day beginning from the first stimulation. CD28, HLA-DR, and CD85j molecules surface expression changes over time are shown as histograms. (B) An CD8 T cell line was established

against autologous EBV-transformed LCLs. CD85j-postive and -negative cells were sorted and restimulated. CD85j expression on days 7 and 27 after sorting is represented as a histogram.

We see similar results using another culture system: CD8 T cell lines generated against, and restimulated by, autologous EBV-transformed lymphoblastoid cell lines (LCLs). Using this system, we sorted CD85j-positive and -negative T cells from an established line and analyzed CD85j expression after restimulation (Figure 3.3B). We find that 27 days after sorting, a sizable fraction of the formerly CD85j-negative cells have begun expressing it. Of note, the sorted CD85j+ population remains so after restimulation.

Increased LILRB1 *transcription accounts CD85j surface expression on CD8 T cells*

In beginning to explore the mechanisms responsible for CD85j expression on T cells, we encountered reports from the Ciccone group in Italy claiming that all T cells express CD85j. To summarize their findings, Saverino et al.[17] detected CD85j-surface expression, like other groups before them,[3-4] on only a subset of T cells by flow cytometry. This was true for a number of different anti-CD85j mAbs (HP-F1, GHI/75, M402 and M405). Using HP-F1 and M402, they examined cytoplasmic staining for CD85j by flow cytometry and confocal microscopy. Surprisingly, all T cells were positive, whether PBMC-derived or clones and regardless of the presence of CD85j-surface staining. The authors also claimed

that every T cell clone tested, again regardless of CD85j-surface staining, contained detectable protein by western blotting and *LILRB1* mRNA detected by semi-quantitative RT-PCR. Protein and mRNA levels roughly correlated with CD85j surface-levels; in some clones that exhibited very low surface expression, such as many CD4 clones, a large amount of protein was required to detect a faint band by western blotting and mRNA was only detected after additional PCR cycles. We found these results surprising considering the uniform CD85j-staining intensity seen with intracellular flow cytometry; all examples shown by Saverino et al.[17,58] exhibited a unimodal intracellular CD85j-staining pattern at an MFI consistently higher than that seen in CD85j+ cells stained by standard surface staining. The same phenomenon was also found by a separate group.[22]

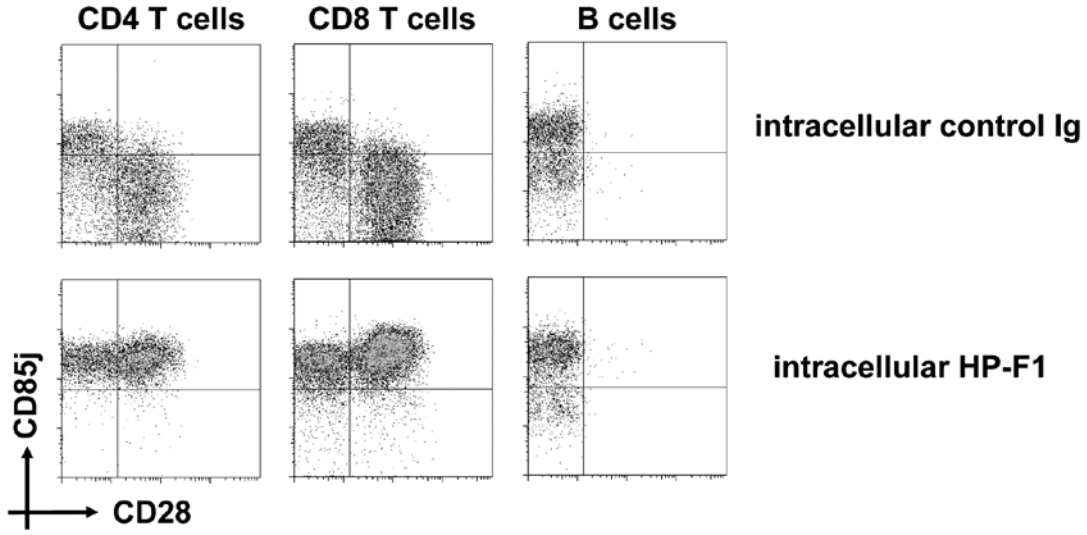

Figure 3.4 All lymphocytes stain positively for CD85j by intracellular flow cytometry. PBMCs were fixed and permeabilized and then stained with either an anti-CD85j mAb (HP-F1, bottom row) or a mouse IgG control antibody (top row). Prior to intracellular staining protocol, cells were stained for surface CD3, CD8, CD4

and CD19. Data is representative of experiments performed by W.W.L. and D.L.L. using several different donors.

Indeed, we repeated the intracellular flow cytometry experiments ourselves using HP-F1 and found ubiquitous staining among all T cells with an increased MFI compared to CD85j-surface staining (Figure 3.4). If all T cells express CD85j, its age-dependent emergence at the surface of CD8 T cells may simply result from translocation to the plasma membrane from intracellular stores, as occurs with CD152 following T cell activation. To address this possibility we examined CD85j protein expression by western blot in CD28+ and CD28- CD8 T cells lines which are CD85j-negative and CD85j-positive by surface staining, respectively. Importantly, both cell lines are CD85j-positive by intracellular staining (data not shown). As shown in Figure 3.5A, a clear band of ~115 kDa is present in protein from CD85j-surface positive CD28-negative cells. The same band is only faintly present in protein from CD28-positive cells. There is no other band present in the CD28-positive CD85j-surface-negative cells that could account for the strongly CD85j-positive intracellular staining found by flow cytometry. However, the anti-CD85j mAb used for western blotting was VMP55 which is commercially available in a purified form. At the time, there was no commercially-available purified, non-conjugated form of the clone HP-F1, which, in a PE-conjugated form from Beckman-Coulter, we used for flow cytometry. Neither was there a fluorophor-conjugated form of VMP55, and our attempts at detecting VMP55 expression by indierect immunofluorescence were unsuccessful (data not shown). Therefore, the possibility remained that an HP-

F1-reactive form of CD85j exists in the cytoplasm of all T cells that was not recognized by western blotting using VMP55.

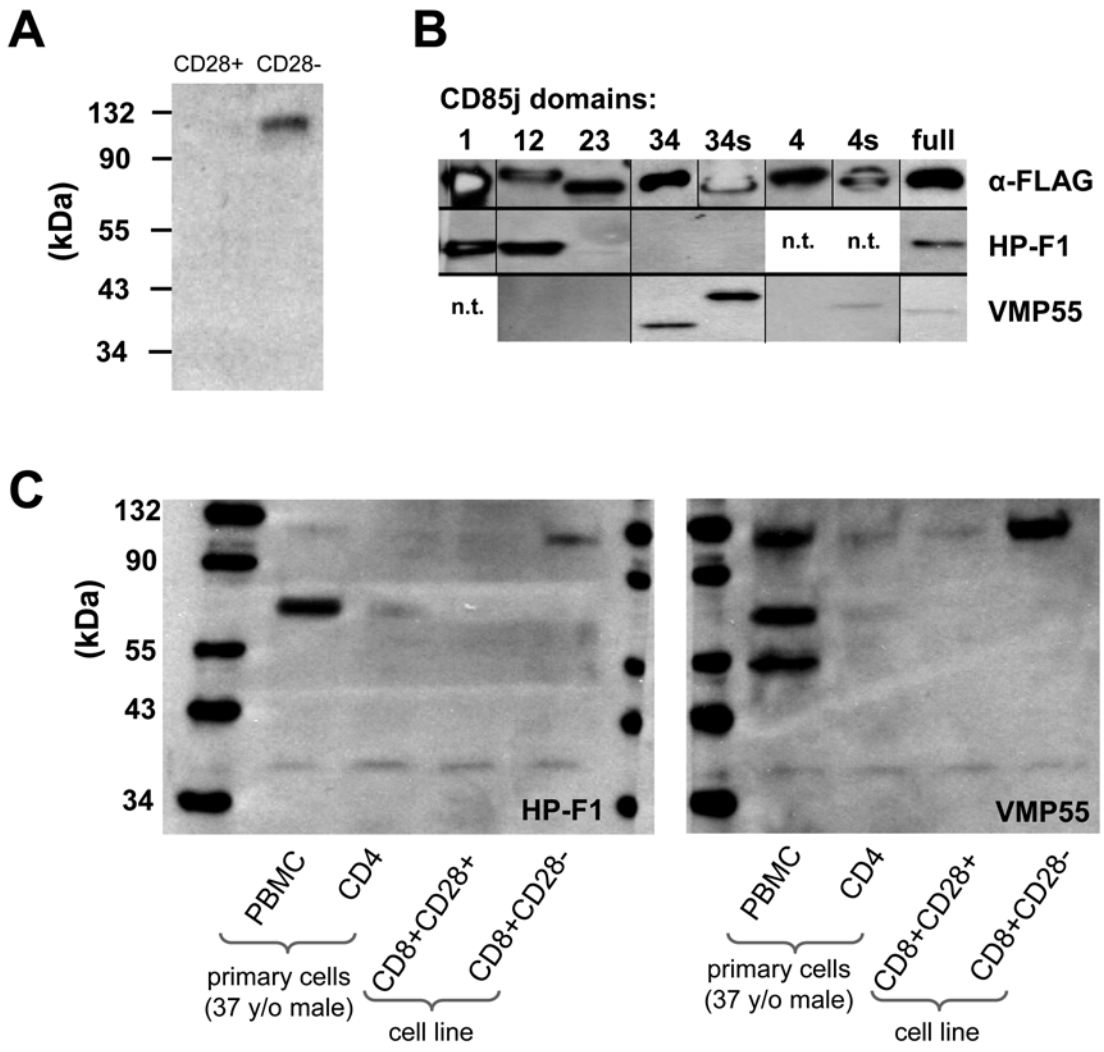

Figure 3.5 The anti-CD85j mAbs VMP55 and HP-F1 recognize distinct epitopes, both of which are present in the dominant CD85j species expressed by T cells. (A) Western blot of protein from a CD28-positive and a -negative T cell line probed with the anti-CD85j antibody VMP55. (B) Constructs fusing portions of the CD85j extracellular domain to a FLAG tag were generated and expressed in bacteria. Lysate from bacteria transformed with one construct was probed with anti-FLAG mAb and one or both anti-CD85j mAb (VMP55 and/or HP-F1).

Some constructs were not probed with both anti-CD85j mAbs ("n.t."). Numbers above lanes refer to the CD85j Ig-like domains included in the FLAG-tagged constructs. The "s" refers to the short spacer region that lies between Ig-like domain 4 and the transmembrane domain in native CD85j. Image is a composite of bands from several blots; lines separate portions taken from different blots. (C) Protein from primary PBMCs and CD4 T cells and the same cell lines used in panel A were probed, on two separate blots, with both VMP55 and HP-F1.

To address this possibility, we requested, and received as a gift, an aliquot of HP-F1 monoclonal supernatant from Miguel López-Botet of the Universitat Pompeu Fabra in Barcelona, Spain. Using flag-tagged constructs expressed in bacteria, we broadly mapped the CD85j epitopes recognized by HP-F1 and VMP55. As shown in Figure 3.5B, these two antibodies recognize distinct epitopes; CD85j Ig-like domain 1 (IgD1) is recognized by HP-F1 and an epitope requiring both IgD3 and IgD4 is recognized by VMP55. With this information in mind, we reanalyzed protein from CD28-positive and -negative CD8 T cell lines using both VMP55 and HP-F1 as probes. For both antibodies, a similarly-sized single band of ~115 kDa was present in protein from CD28-negative cells and, only faintly, in protein from CD28-postive cells. In neither blot was there a band that could account for the positive intracellular staining for CD85j in both cell lines (Figure 3.5C)

Protein from PBMCs and primary CD4 T cells was included on the same blots (Figure 3.5C). Interestingly, while the ~115 kDa band was present in protein from all cell types, if only faintly for CD4 T cells, two additional bands of

~70 kDa and ~55 kDa were evident in PBMCs probed with VMP55. All three bands in PBMCs probed with VMP55 were similarly intense. The ~70 kDa band was also recognized by HP-F1 and, in PBMCs, was considerably stronger than the ~115 kDa product. These data suggest that multiple CD85j species exist in PBMCs and that some may lack IgD1. Importantly, this domain contains many of the chief MHC class I-binding residues.

Based on our CD85j epitope mapping analysis and western blotting, it is our interpretation that detection of CD85j in all T cells does not represent meaningful quantities of full-length CD85j protein. Rather, intracellular staining may recognize degradation products that cannot be detected as species of discreet size by western blotting or a soluble form of CD85j that is washed away during cell lysate preparation. In support of this assertion, a recent study[59] reports that most cell-types expressing CD85j contain an alternatively spliced *LILRB1* mRNA transcript that results in a soluble protein of ~65 kDa that was detected in human monocyte-derived-DC supernatant. While the authors did not use HP-F1 or VMP55, the soluble CD85j protein is predicted to include each epitope we identified and therefore could represent the ~70 kDa product we found in PBMCs. We re-examined the data presented by Saverino et al.[17] and discovered that, in fact *all* PBMCs, not just all T cells as the authors suggest, exhibit intracellular CD85j staining. The authors performed two-color stains of fixed and permeabilized PBMCs using a CD85j-specific mAb and either anti-CD3, anti-CD4, or anti-CD8. While their discussion focused on double-positive cells, all cells negative for CD3, CD4, and CD8 were also positive for CD85j following

intracellular staining. In contrast, an epithelial cell line not known to express CD85j was clearly negative for both surface and intracellular CD85j. We re-examined our own data and found that we, too, see intracellular CD85j staining in all PBMCs (data not shown).

We feel that we can rule out translocation from intracellular stores to the plasma membrane as a likely mechanism leading to CD85j expression on CD8 T cells. Instead, we favor the hypothesis that increased transcriptional activity at the *LILRB1* promoter leads to increased surface expression. To address this hypothesis, we sorted CD85j-positive and -negative CD8 T cells from three middle-aged donors and performed qRT-PCR using primers binding to *LILRB1* sequences that code for a portion of the VMP55 epitope (IgD4). As Figure 3.6 shows, we find that *LILRB1* transcript levels are more than 6-fold higher in CD8 T cells expressing CD85j. While these data do not specifically address CD85j expression with age, and T cells from the elderly may behave differently than those from younger individuals, this is strong evidence that CD85j expression in CD8 T cells is regulated at the level of *LILRB1* gene transcription.

As described in Chapter 2, we have found that lymphocytes express CD85j from a unique promoter 13 kb upstream of the other *LILRB1* promoter. Use of the lymphocyte promoter results in an additional first exon that is absent in transcripts initiated from the downstream promoter. We compared *LILRB1* exon 1 and exon 8 mRNA levels in the sorted CD85j-positive and -negative CD8 T cells to determine which *LILRB1* promoter is used, CD85j-positive CD8 T cells

exhibit a ~1:1 ratio of exon 1 to exon 8 mRNA levels, suggesting that these cells transcript *LILRB1* from the upstream lymphocyte promoter (Figure 3.6C). In

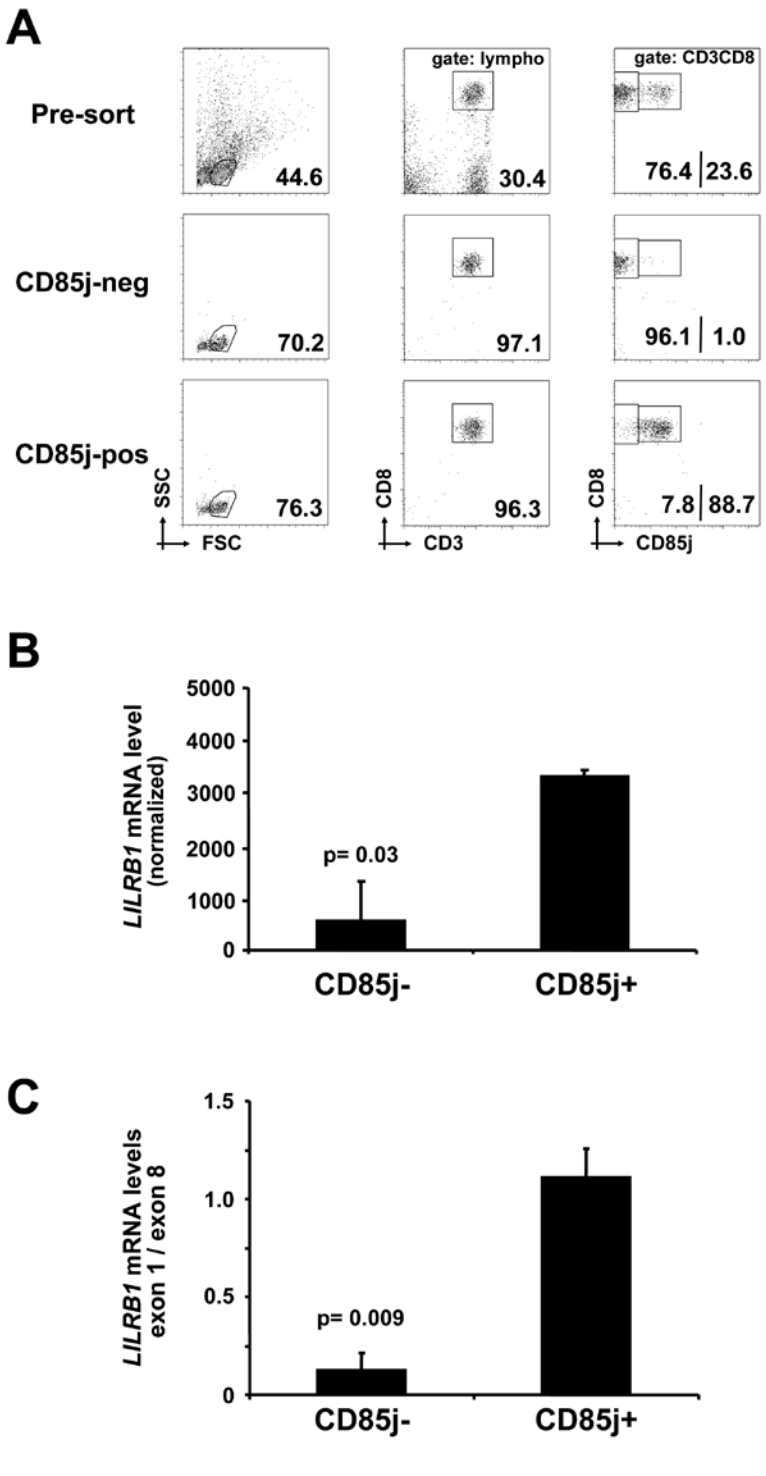

Figure 3.6 CD8 T cells that express CD85j do so by increased transcription from LILRB1 promoter 1. (A) Representative FACSort for CD85j expression on CD8 T cells. Plots showing gated cells are indicated; all other plots are ungated and show all events. Numbers are percentage of events shown that fall within drawn gates. (B) *LILRB1* transcripts were quantified by RT-qPCR in RNA from CD85j-positive and -negative CD8 T cells. Results for a primer

set within exon 8 are shown as mean transcript numbers (relative to $2x10^5$ beta-actin copies) ± S.D. of data from cells sorted from 3 donors. (C) *LILRB1* transcripts were compared for exon 1-3 and exon 8 sequences. Results are shown as mean ± S.D. of data from sample samples used in panel B.

contrast, the CD85j-negative CD8 T cells have a low exon 1: exon 8 ratio resembling that seen in monocytes (see Chapter 2, Figure 2.2C), and suggesting that these cells use promoter 2 (Figure 3.6C). It is important to note that these cells have low *LILRB1* transcript levels and were sorted based on their lack of CD85j at the cell surface. Because promoter 2-generated LILRB1 transcripts are more efficiently translated (see Chapter 2), it is possible that, despite low transcript levels, these cells secrete soluble CD85j, as suggested by Jones et al.[60] but this possibility requires further study.

Chapter 4: Materials and Methods[††]

Isolation of human mononuclear cells (Chapter 2)

Healthy donors were recruited, with informed consent and per institutional review board protocols, to donate up to 50 ml of whole blood. In most cases, peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation using Lymphocyte Separation Medium (Lonza). Purified NK cells were isolated from whole blood using Human NK Cell Enrichment Cocktail (Stem Cell Technologies). Other purified cell subsets were obtained from PBMCs by magnetic bead-assisted sorting. Briefly, PBMCs were incubated with CD8, CD19, or CD14 microbeads as recommended by the manufacturer (Miltenyi). Desired cell populations were recovered by positive selection using AutoMACS (Miltenyi).

Study population (Chapter 3)

Peripheral blood was obtained from 140 individuals aged 20–90 years and immediately processed. The study cohort included 68 individuals age 20 to 39 years, 31 age 40 to 59, and 41 age 60 to 90 years. Exclusion criteria included the presence or a history of cancer, uncontrolled hypertension, diabetes mellitus, any chronic inflammatory or autoimmune disease, or any acute disease. Appropriate written informed consent was obtained, and the study was approved by the Emory Institutional Review.

[††]Portions of this chapter have been adapted from articles published in *Clinical Immunology*[19] in

Flow cytometry (Chapter 2)

Surface phenotyping of ex-vivo isolated and transfected PBMCs was performed on an LSRII flow cytometer (BD Biosciences). Briefly, cells were incubated with fluorophore-conjugated monoclonal antibodies (mAb) at 4°C for 15 minutes. Anti-human antibodies used were PE-Cy7-conjugated anti-CCR7; FITC-, PerCP-, APC-, and APC-Cy7-conjugated anti-CD3; PerCP-conjugated CD4; PerCP-, APC-, and PE-Cy7-conjugated anti-CD8; APC-conjugated anti-CD14; APC-Cy7-conjugated CD16; PerCP-conjugated anti-CD19; PerCP- and APC-Cy7-conjugated anti-CD20; PE-conjugated anti-CD24; APC-conjugated anti-CD27; PE-Cy7-conjugated anti-CD38; FITC-conjugated anti-CD45RA; FITC-conjugated anti-IgD (all from BD Biosciences); PE-conjugated anti-CD85j (clone HP-F1, Beckman Coulter) and APC-conjugated anti-CD85j (clone HP-F1, eBioscience). Following washing, cells were resuspended in 2% paraformaldehyde and analyzed by flow cytometry within 1-3 days of staining. Analyses were performed using FlowJo software.

Immunophenotyping and flow cytometry analysis (Chapter 3)

To confirm age-dependent differential expression of cell surface markers identified in the gene arrays, PBMC were stained with the following monoclonal antibodies in 5-color panels: FITC-conjugated anti-CD158b/j (GLI83; CH-L), PE-

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2008 and *Blood* in 2010. [46]

anti-CD85j (HP-F1), PerCP-Cy5.5-anti-CD28 (L293), APC-anti-CD8 (RPA-T8), APC-Cy7-anti-CD3 (SK7), FITC-anti-CD57 (HNK-1), PE-anti-CD26 (M-A261), PerCP-anti-CD4 (L200), APC-anti-CD3 (HIT3a); FITC-conjugated anti-HLA-DR (G46-6), PE-anti-CD3 (HIT3a), PerCP-anti-CD4 (L200), APC-anti-CD45R0 (4CHL1), PE-Cy7-anti-CD69 (FN 50). FITC- or APC-anti-CD45RA (RA5H9, HI100), PE- or APC-anti-CCR7 (150503), and PerCP-anti-CD8 (SK1) were used to define the functional subset distribution.

All samples were acquired with FACSort or LSRII (BD Biosciences), and data were analyzed by using CellQuest, FACS DIVA software (both BD Biosciences), or FlowJo (Tree Star, Inc., Ashland, OR). All antibodies were from BD Biosciences, except PE-, APC-anti-CCR7 (R&D Systems, Minneapolis, MN, USA), and anti-CD85j (Beckman Coulter, Fullerton, CA, USA).

RNA isolation and cDNA synthesis

Total RNA was isolated by Trizol extraction (Invitrogen) from 2-4 million cells. RNA pellets were washed with 75% ethanol and dried prior to cDNA synthesis for real-time PCR or 5'RACE analysis. cDNA for real-time PCR was synthesized with AMV RT enzyme and random hexamer primers (Roche).

Quantitative real-time PCR

LILRB1 cDNA levels were quantified using Sybr-Green fluorescence (Invitrogen) analyzed on the MXP3000P real-time PCR machine (Stratagene). *LILRB1* levels

are represented as copy numbers relative to $2x10^5$ copies of β-actin, both determined using standard curves. Prior to quantification of *LILRB1* transcript levels from transfected cells, cDNA was treated with DpnI for 1 hour at 37°C to digest plasmid DNA.

Western blotting

Whole cell extracts were obtained from washed and pelleted cells. Sodium-dodecyl-sulfate-denatured protein was separated by polyacrylamide gel electrophoresis using Ready-Gels (BioRad) and transferred to Hybond-P PVDF membrane (Amersham). Following blocking with 5% blocking solution (Bio-Rad), blots were probed by overnight incubation at 4°C with 1:200 dilution of anti-CD85j mouse mAb (clone VMP55, Santa Cruz) or 1 hour incubation at room temperature with a 1:5000 dilution of anti-actin mouse mAb (Santa Cruz). Primary antibody staining was followed by washing and 1 hour incubation at room temperature with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse Ig secondary antibody (Santa Cruz) followed by washing and horseradish peroxidase detection with Immobilon (Millipore). CD85j-probed blots were detected and stripped prior to β-actin probing and detection.

Plasmids

Transfection studies were performed using plasmids containing the pcDNA3 vector backbone (Invitrogen) and a variety of cDNA sequences amplified from

the *LILRB1* cDNA clone BC015731[50] using platinumTaq polymerase (Invitrogen) and cloned using KpnI and NotI sites included in the sense and antisense primers, respectively. The green-fluorescent protein (GFP) control transfections were performed with a plasmid consisting of the XhoI-XbaI fragment from mCD8-GFP[61] cloned into pcDNA3. *LILRB1* 5'UTR-GFP fusion constructs were made by cloning the 5'UTRs from BC015731 and AF283985 upstream of GFP in pmaxGFP (Lonza) using KpnI and NheI sites. Mutation of sequences within *LILRB1* exon 1 was performed on pcDNA3-*LILRB1* full-length plasmid using the QuikChange II XL site-directed mutagenesis kit, as described by the manufacturer (Stratagene). Luciferase reporter constructs were generated by cloning *LILRB1* promoter sequences into NheI and XhoI sites of pGL4.10 (Promega). All plasmids were confirmed by sequencing (Agencourt) and doubly purified from bacterial culture by HiSpeed Plasmid Maxi Kit (Qiagen) followed by QIAquick PCR Purification Kit (Qiagen) prior to transfection.

Transfection of human PBMCs

Freshly isolated PBMCs or AutoMACS-purified monocytes were transfected using the Nucleofector II (Lonza) as described by the manufacturer. The transfection program used for T cells was V-24 and for monocytes was Y-01. Cells were stained for analysis by flow cytometry, or processed for luciferase reporter assays, 24 hours after transfection. 2 µg of plasmid DNA was used for all transfections except for GFP co-transfections for which 2 µg of *LILRB1* cDNA

plasmid were combined with 1 µg of GFP plasmid, and luciferase reporter assays for which 2.5 ug of DNA was used.

5'-rapid amplification of cDNA ends (5'RACE)

5'RACE analysis was performed on total RNA from 4 million cells using the Invitrogen system as described by the manufacturer (catalog no. 18374-058). Briefly, cDNA was synthesized from total RNA using an *LILRB1*-specific antisense primer (GSP1). A poly-cytidine tag was added to the 3'-end of cDNA with the enzyme TdT. PCR was performed on tagged and non-tagged cDNA using a tag-specific primer and a second nested *LILRB1* primer (GSP2). Amplified products were re-amplified in a second nested PCR using a third *LILRB1* primer (GSP3). Products from the second nested PCR were cloned into pCRII-TOPO (Invitrogen) and sequenced (Agencourt). GSP3 and a fourth *LILRB1* primer binding within exon 3 (GSP4) was used for control PCR for total *LILRB1* cDNA.

Luciferase reporter assay

Primary cells were transfected, as describe above, with a DNA mixture containing 0.5 ug of pRL-SV40 vector and 2.0 ug of either the basic pGL4.10 vector or an *LILRB1*-pGL4.10 construct. 24 hours post-transfection, cells were processed and analyzed using the Dual-Reporter Assay System (Promega) read on a TD-20/20 luminometer (Turner Designs).

In vitro cultures

CD28 positive cells were sorted from PBMC with biotin-labeled anti-CD28 antibody (BD Biosciences), and anti-biotin labeled microbeads (Miltenyi). Isolated cells were labeled with CFSE and stimulated with anti-CD3 (OKT3, 30 ng/ml) in the presence of irradiated EBV transformed cells, rhIl-2 (50 U/ml) and rhIl-15 (100 ng/ml). Cultures were split every 4 days. Cells were analyzed at 6- to 8-day intervals for the expression of CD85j, CD28 and HLA-DR on CD4 and CD8 T cells that had undergone the same number of divisions as determined by CSFE dilution.

Statistics (Chapter 2)

For all comparisons, an ANOVA with post-hoc Tukey's test was performed using SigmaStat 3.0 software.

Statistical analysis (Chapter 3)

Results are expressed as medians, 25th/75th percentiles as boxes and 10th/90th percentiles as whiskers. Groups were compared using ANOVA or non-parametric Mann–Whitney *U* test. p values < 0.05 were considered statistically significant.

Primer Sequences

Quantitative real-time PCR

LILRB1 exon 1-3

-sense GGCGCCTCTACTTTCTGGAGTTT

-antisense CCCACTGCCCTGCTCTGTGGAT

LILRB1 exon 8

-sense GATCAACGTACCAATCTCAAA

-antisense TCAGGGCCTGCTGAGACCACGAGCT

β-actin

-sense ATGGCCACGGCTGCTTCCAGC

-antisense CATGGTGGTGCCGCCAGACAG

Plasmids

LILRB1 promoter 1, -2000 bp

-sense GGGGTACCAGAGCCAAATCAGGAATGCAAT

-antisense TTACTCGAGTAAAGGAGGAAGTGAGCTGTGGG

LILRB1 promoter 1, -500 bp

-sense GGGGTACCAAGGATCCTACTTCTAGTTGGGA

-antisense TTACTCGAGTAAAGGAGGAAGTGAGCTGTGGG

LILRB1 full-length

-sense GGGGTACCGAGGAGGAACAGAAAAGAAAAGAAAA

-antisense GAGCGGCCGCAATTTGAGATGGAGTCTCCCTC

LILRB1 5’UTR+coding region

-sense GGGGTACCGAGGAGGAACAGAAAAGAAAAGAAAA

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

LILRB1 coding region

-sense GGGGTACCATGACCCCCATCCTCACGGTCCT

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

LILRB1 Δex1(AF84)+coding region

-sense GGGGTACCCCACACGCAGCTCAGCCTGGGCG

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

LILRB1 Δex1(AF85)+coding region

-sense GGGGTACCAGCATGGACCTGGGTCTTCCCTGAA

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

LILRB1 Δex1Δex2+coding region

-sense GGGGTACCCACCGAGGGCTCATCCATCCA

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

LILRB1 ex1Δ85+coding region

-sense GGGGTACCGTGGGCACTCCATTGGTTTTATGGC

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

LILRB1 ex1Δ115+coding region

-sense GGGGTACCTACTTTCTGGAGTTTGTGTAAAACAA

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

LILRB1 ex1Δ145+coding region

-sense GGGGTACCTACTTTCTGGAGTTTGTGTAAAACAA

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

LILRB1 ex1Δ145 mut ATG

-sense GGGGTACCATTAAGGTCTTTGTGCACATTTACA

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

LILRB1 ex1Δ145 mut ARE

-sense GGGGTACCATTAAGGTCTTTGTGCACATTTACA

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

LILRB1 ex1Δ145 mut both

-sense GGGGTACCATTAAGGTCTTTGTGCACATCTACA

-antisense GAGCGGCCGCCTAGTGGATGGCCAGAGTGGCGTA

BC015731 5'UTR-GFP fusion

-sense GGGGTACCGAGGAGGAACAGAAAAGAAAAGAAAA

-antisense AATTCGCTAGCCTCTGTGGATGGATGAGCCCTCGG

AF283985 5'UTR-GFP fusion

-sense GGGGTACCAGCATGGACCTGGGTCTTCCCTGAA

-antisense AATTCGCTAGCCTCTGTGGATGGATGAGCCCTCGG

Site-directed mutatgenesis primers

mut ATG1

-sense GGAAACAAATAATCTAAGAAGGAGGAGAAAGCAAGAAGAGTGACC

-antisense GGTCACTCTTCTTGCTTTCTCCTCCTTCTTAGATTATTTGTTTCC

mut ATG2

-sense GGCACTCCATTGGTTTTAGGGCGCCTCTACTTTCTGG

-antisense CCAGAAAGTAGAGGCGCCCTAAAACCAATGGAGTGCC

mut ATG3

-sense GTAAAACAAAAATATTAGGGTCTTTGTGCACATTTAC

-antisense GTAAATGTGCACAAAGACCCTAATATTTTTGTTTTAC

mut ARE

-sense

ATATTATGGTCTTTGTGCACATCTACATCAAGCTCAGCCTGGGCG

-antisense

CGCCCAGGCTGAGCTTGATGTAGATGTGCACAAAGACCATAATAT

5’RACE primers

GSP1: GGCCTGCAGTGTCGCTACCATAGTA

GSP2: CTGCATGTTCCCAGGTGATGGATG

GSP3: GGGGAACTGGCCCTTCTTCACAA

GSP4: GGGCTCATCCATCCACAGAGCA

Chapter 5: Discussion°

Within the hematopoietic system, CD85j is expressed to varying degrees by most cell-types. In this report, we provide evidence that CD85j expression is regulated in a lineage-specific manner, and we identify a novel promoter, used by lymphocytes but not monocytes, that lies 13 kb upstream of the monocyte promoter and the main *LILRB1* exon cluster on human chromosome 19. Use of the lymphocyte promoter results in an additional exon within the 5'UTR that is absent in transcripts originating from the monocyte promoter. We show that transcripts containing this first exon do not efficiently translate CD85j protein compared to transcripts beginning with exon 2. Promoter choice, combined with translational repression, accounts for cell-specific differences in CD85j expression that are responsible for the context-dependent differences in CD85j function.

Our report suggests CD85j expression is regulated in a lineage-specific manner whereby lymphocytes strongly favor CD85j expression from the upstream promoter and monocytes exclusively utilize the downstream promoter. For CD8 T cells, CD85j is well positioned to interfere with TCR activation by competing for CD8 binding to MHC class I[25] and recruiting phosphatases to the synapse. For NK cells, CD85j is one of many inhibitory receptors that may be expressed to detect MHC class I on other cells.[62] In these settings, the upstream promoter may be more amenable to the transcriptional apparatus needed to

°Portions of this chapter have been adapted from an article published in *Blood* in 2010. [46]

restrict CD85j expression to a subset of cells and the translational inefficiency of the resulting transcript may allow tighter regulation of CD85j protein levels

Although B cells, CD8 T, and NK cells share a lineage and use the same *LILRB1* promoter, B cells and monocytes employ CD85j in a more similar functional context. First, whereas only a tightly defined subset of CD8 T cells expresses CD85j, it is ubiquitous on mature B cells and monocytes. Secondly, the defining operations of neither B cells nor monocytes directly involve MHC class I-interactions. Naïve B cells survey their surroundings by expressing many copies of a single rearranged surface Ig and are activated through combined signals delivered by (a) the antigen-crosslinked BCR (surface Ig plus Igα and Igβ signaling domains) and (b) a CD4 T cell engaging MHC class II:antigen peptide complexes on the B cell surface.[63] Peripheral blood monocytes use surface receptors to sense chemokines and endothelial cell changes signaling inflammation[64] and, following extravasation into the tissue, additional interactions and chemical mediators drive their differentiation into phagocytic effector cells such as macrophages and DCs.[65] B cell- and monocyte-activating signals require kinase activity that can be influenced by CD85j-recruited phosphatases.[14,66-69] The MHC class I that CD85j encounters during these events exists either on the B cell or monocyte itself (*cis*) or on the surface of surrounding cells (*trans*), but not within the primary activation interface. CD85j functioning in a dispersed distribution on B cells and monocytes likely increases the threshold required to deliver activating signals by shifting the intracellular kinase:phosphase balance rather than directly interfering with activating signals.

Indeed, CD85j ligation on monocytes during in vitro DC-generation dramatically affects the phenotype of resulting DCs which lack many characteristic surface markers and respond poorly to LPS stimulation.[40]

While all mature B cells and monocytes express CD85j, a consequence of distinct promoter usage by B cells and monocytes is higher CD85j protein levels in monocytes. It is likely that the distinct roles these cell-types play are best served by different CD85j levels. Monocytes are innate immune cells and lack the antigen-specificity that defines B cells. When activated in the periphery, their effector functions act broadly and destructively toward surrounding tissues. A lowered activation threshold provided by high CD85j expression reserves highly damaging responses to all but the strongest inflammatory scenarios. On the other hand, mature B cells result from a meticulous process of receptor gene rearrangement and negative selection to ensure each B cell is functional and self-tolerant.[70] These cells continually circulate, awaiting antigen encounter. CD85j levels on B cells may help to establish a balance such that responses to self-antigens are avoided while allowing the subtle survival signals necessary to continue circulating.[71] Interestingly, our finding that circulating transitional B cells lack CD85j expression suggests CD85j does not interfere with the signals required to establish the naïve B cell repertoire but becomes available to influence survival and activation signals upon maturation.

Prior to this report, *LILRB1* was assumed to have a single promoter, upstream of the main exon cluster. Using reporter constructs in cell lines, Nakajima et al.[45] demonstrated that this exon 2-proximal *LILRB1* promoter is

highly active in the monocyte-like THP-1 cell line and is dependent on PU.1 and Sp1 transcription factors. However, promoter activity in Jurkat cells, a T cell-like line, was weak compared to THP-1 cells. These results are consistent with our findings that primary T cells and monocytes utilize distinct *LILRB1* promoters. Moreover, our findings from promoter reporter assays are reciprocal to those of Nakajima et al. Namely, exon 1-proximal promoter sequences are strongly active in T cells but not monocytes.

LILRB1 is distinguished as the only LILR family member that is expressed on T cells.[72] An examination of submitted transcripts from all other LILR genes reveals that *LILRB1* is also the only gene with transcripts beginning with a distant alternative first exon. Our finding that T cells predominately express *LILRB1* from the upstream promoter provides an explanation for its unique expression profile. In support of this claim, the *LILRB1* promoter found upstream of exon 2, which is used by monocytes, is nearly identical to the promoter *LILRB2*, which codes for CD85.[45] Despite the promoter similarity, CD85d is only expressed on myeloid cells. This implies that the *LILRB2* promoter, and by extension the *LILRB1* monocyte promoter, is insufficient to drive expression in T cells.

Analysis of potential transcription factor binding sites within the 500 bp preceding *LILRB1* exon 1 (using the same algorithm used by Nakajima et al.[45]: http://mbs.cbrc.jp/research/db/TFSEARCH.html) reveals several candidates molecules that are known to be important in T cells, and in particular cytotoxic CD8 T cells (Figure 4.1). While most of these transcription factors function in a

wide range of cell-types, each has a well established role in T cells. For example, REL has been shown in mice to be required for proliferation of CD8 T cells.[73]

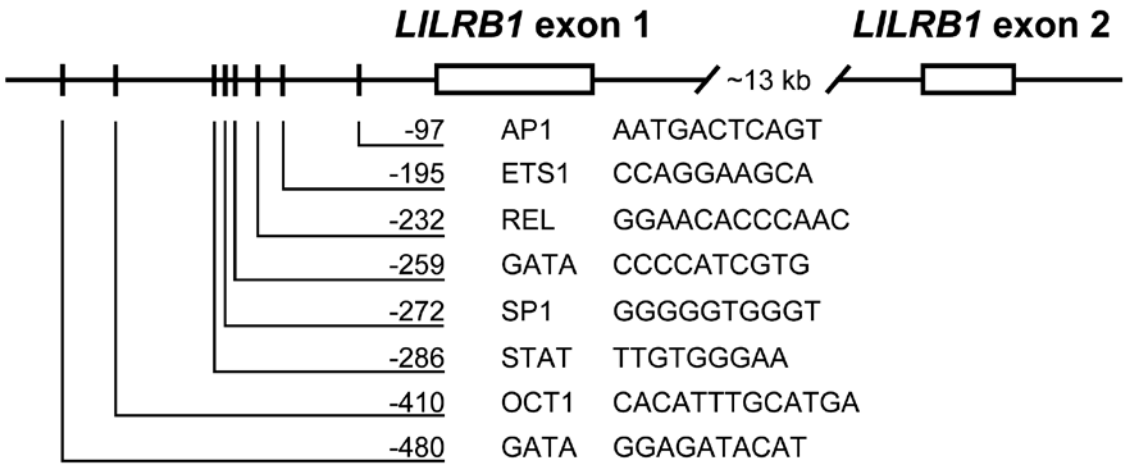

Figure 4.1 Predicted transcription factor binding sites within the LILRB1 upstream promoter used by lymphocytes. The 500 bp preceding *LILRB1* exon 1 was analyzed for possible transcription factor binding sites using TFSEARCH version 1.3 (http://mbs.cbrc.jp/research/db/TFSEARCH.html). All predicted human molecules are shown. The position and sequence of each site is indicated. The numbers refer to the position of the first nt of the transcription factor binding motif relative to the transcriptional start site for *LILRB1* exon 1.

ETS1, AP1, and SP1 each have binding sites within the promoter regions of the cytotoxicty genes perforin, granzyme B, and FasL (reviewed by Glimcher et al.[74]). GATA-family member transcription factors, most famously GATA-3 for its role in Th2 differentiation,[75] are key players in T cell development and function.[76] STAT family members are critical for cytokine signaling in many cell-types and have been especially well studied in lymphocytes.[77]

Several of these transcription factors are also known to play key roles in NK cells and B cells, the other two cell-types that we have found use *LILRB1* promoter 1. ETS1 is important for NK cell and NK-T cell development and function.[78-79] Oct1 is ubiquitously expressed but an Oct1 cofactor, OBF-1, is highly specific for B cells and inducible in T cells.[80-81] In fact, OBF-1 controls Btk transcription during B cell development,[82] As Btk is required for BCR signaling,[83] its expression precedes the transition from pro-B cell to pre-B cell, which is also the earliest precursor stage where CD85j expression is found in bone marrow.[9] Further analysis specifically addressing the role and importance of each of these transcription factors in CD85j expression by lymphocytes is warranted.

The upstream *LILRB1* promoter used by lymphocytes serves as an alternative to the promoter used by monocytes. It is estimated that 30-50% of all human genes have alternative, and often distant, promoters.[84-85] This phenomenon is thought to provide a mechanism for flexible and diverse regulation of single genes by many cell types and during various developmental stages and activation states. In some cases, this is achieved at the level of transcriptional activity. For example, some alternative promoters may be more active than others in a certain cell-type and the hierarchy of promoter activities may differ among cell-types.[86-87] In other cases, distinct promoters yield unique 5'UTRs that affect the character and/or quantity of the translated protein. When additional exons transcribed from alternative promoters contain an ATG sequence, it is possible the resulting protein will contain an altered N-terminus or even a completely new protein.[88] Conversely, distinct 5'UTRs may not alter the

protein product but, rather, may affect transcript stability or translational efficiency.[89] Our findings suggest use of the distant upstream *LILRB1* promoter is cell-type-specific and results in inefficient CD85j protein expression without altering the resulting amino acid sequence. Similar to genes such as *CDKN2C*,[90] we find the upstream *LILRB1* promoter yields a 5'UTR that profoundly effects protein expression in primary cells. Cells utilizing the upstream *LILRB1* promoter (such as B cells) express far less CD85j protein compared to cells using the downstream promoter (such as monocytes) despite similar mRNA levels.

We isolated the region responsible for poor CD85j protein expression by lymphocytes to the last 30 nt of exon 1. This region contains the last of three ATGs found in exon 1 and an ARE motif, which are known to mediate translational repression by recruitment of RNA-binding proteins.[91] Although AREs typically contain several repeats of this motif, they can function as a single pentamer.[92] We mutated the ATG and ARE motif within the 30nt sequence and found an improvement in CD85j expression. Expression was strongest when both mutations were present in the ex1Δ145 construct. Mutation of these elements in the context of the full exon 1 had no or only a modest effect on CD85j levels, suggesting that no single element is responsible for exon 1-mediated translational repression of CD85j expression and that the truncated exon 1 construct provides a context, such as a unique mRNA conformation, in which mutations are more potent than when the full exon 1 is present.

This study addressed steady state CD85j expression in primary cells. Future studies might focus on how CD85j is acquired or lost during activation and

differentiation states. For example, CD85j levels are known to increase during in vitro differentiation from monocytes into DCs.[40] Conversely, DCs are known to downregulate CD85j upon activation.[93] As suggested by the results in Figure 2.1, B cells acquire CD85j expression during the differentiation steps leading from transitional to mature B cells. Perhaps the most interesting scenario is the de novo acquisition of CD85j expression by CD8 T cells with advancing age, a phenomenon that can be mimicked by repeated stimulation cycles in vitro (see Chapter 3).[21] We show that CD8 T cells achieve CD85j expression by transcription from the *LILRB1* lymphocyte promoter, but it this needs to be examine directly in cells from elderly donors. This has important implications given the pivotal role CD8 T cells play in infections, cancer, and autoimmunity, each of which disproportionately affect the elderly. It remains to be seen whether a given cell-type can simultaneously activate and/or switch between the two *LILRB1* promoters or if expression is chiefly controlled by increased transcriptional activity at a single promoter. The disparity between translational efficiencies between the two promoters implies that even a small shift could profoundly affect CD85j protein levels. Potential therapeutic interventions, such as turning CD85j expression on in tumor cells or off in T cells, will require a thorough understanding of the mechanisms governing *LILRB1* promoter choice and activity in a variety of cell-types and settings.

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References

1. Cahalan MD, Chandy KG. The functional network of ion channels in T lymphocytes. Immunol Rev. 2009;231(1):59-87.

2. Volz A, Wende H, Laun K, Ziegler A. Genesis of the ILT/LIR/MIR clusters within the human leukocyte receptor complex. Immunol Rev. 2001;181(39-51.

3. Cosman D, Fanger N, Borges L, et al. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. Immunity. 1997;7(2):273-282.

4. Colonna M, Navarro F, Bellon T, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. J Exp Med. 1997;186(11):1809-1818.

5. Samaridis J, Colonna M. Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways. Eur J Immunol. 1997;27(3):660-665.

6. Mori Y, Tsuji S, Inui M, et al. Inhibitory immunoglobulin-like receptors LILRB and PIR-B negatively regulate osteoclast development. J Immunol. 2008;181(7):4742-4751.

7. Wagtmann N, Rojo S, Eichler E, Mohrenweiser H, Long EO. A new human gene complex encoding the killer cell inhibitory receptors and related monocyte/macrophage receptors. Curr Biol. 1997;7(8):615-618.

8. Pulford K, Micklem K, Thomas J, Jones M, Mason DY. A 72-kD B cell-associated surface glycoprotein expressed at high levels in hairy cell leukaemia and plasma cell neoplasms. Clin Exp Immunol. 1991;85(3):429-435.

9. Banham AH, Colonna M, Cella M, et al. Identification of the CD85 antigen as ILT2, an inhibitory MHC class I receptor of the immunoglobulin superfamily. J Leukoc Biol. 1999;65(6):841-845.

10. Andre P, Biassoni R, Colonna M, et al. New nomenclature for MHC receptors. Nat Immunol. 2001;2(8):661.

11. Uhrberg M, Valiante NM, Shum BP, et al. Human diversity in killer cell inhibitory receptor genes. Immunity. 1997;7(6):753-763.

12. Witt CS, Dewing C, Sayer DC, Uhrberg M, Parham P, Christiansen FT. Population frequencies and putative haplotypes of the killer cell immunoglobulin-like receptor sequences and evidence for recombination. Transplantation. 1999;68(11):1784-1789.

13. Young NT, Canavez F, Uhrberg M, Shum BP, Parham P. Conserved organization of the ILT/LIR gene family within the polymorphic human leukocyte receptor complex. Immunogenetics. 2001;53(4):270-278.

14. Fanger NA, Cosman D, Peterson L, Braddy SC, Maliszewski CR, Borges L. The MHC class I binding proteins LIR-1 and LIR-2 inhibit Fc receptor-mediated signaling in monocytes. Eur J Immunol. 1998;28(11):3423-3434.

15. Galiani MD, Aguado E, Tarazona R, et al. Expression of killer inhibitory receptors on cytotoxic cells from HIV-1-infected individuals. Clin Exp Immunol. 1999;115(3):472-476.

16. Speiser DE, Valmori D, Rimoldi D, et al. CD28-negative cytolytic effector T cells frequently express NK receptors and are present at variable proportions in

circulating lymphocytes from healthy donors and melanoma patients. Eur J Immunol. 1999;29(6):1990-1999.

17. Saverino D, Fabbi M, Ghiotto F, et al. The CD85/LIR-1/ILT2 inhibitory receptor is expressed by all human T lymphocytes and down-regulates their functions. J Immunol. 2000;165(7):3742-3755.

18. Young NT, Uhrberg M, Phillips JH, Lanier LL, Parham P. Differential expression of leukocyte receptor complex-encoded Ig-like receptors correlates with the transition from effector to memory CTL. J Immunol. 2001;166(6):3933-3941.

19. Czesnikiewicz-Guzik M, Lee WW, Cui D, et al. T cell subset-specific susceptibility to aging. Clin Immunol. 2008;127(1):107-118.

20. Vitale M, Castriconi R, Parolini S, et al. The leukocyte Ig-like receptor (LIR)-1 for the cytomegalovirus UL18 protein displays a broad specificity for different HLA class I alleles: analysis of LIR-1 + NK cell clones. Int Immunol. 1999;11(1):29-35.

21. Czesnikiewicz-Guzik M, Lorkowska B, Zapala J, et al. NADPH oxidase and uncoupled nitric oxide synthase are major sources of reactive oxygen species in oral squamous cell carcinoma. Potential implications for immune regulation in high oxidative stress conditions. J Physiol Pharmacol. 2008;59(1):139-152.

22. Nikolova M, Musette P, Bagot M, Boumsell L, Bensussan A. Engagement of ILT2/CD85j in Sezary syndrome cells inhibits their CD3/TCR signaling. Blood. 2002;100(3):1019-1025.

23. Merlo A, Tenca C, Fais F, et al. Inhibitory receptors CD85j, LAIR-1, and CD152 down-regulate immunoglobulin and cytokine production by human B lymphocytes. Clin Diagn Lab Immunol. 2005;12(6):705-712.

24. Chapman TL, Heikeman AP, Bjorkman PJ. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. Immunity. 1999;11(5):603-613.

25. Shiroishi M, Tsumoto K, Amano K, et al. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. Proc Natl Acad Sci U S A. 2003;100(15):8856-8861.

26. Lanier LL, Corliss B, Phillips JH. Arousal and inhibition of human NK cells. Immunol Rev. 1997;155(145-154.

27. Chapman TL, Heikema AP, West AP, Jr., Bjorkman PJ. Crystal structure and ligand binding properties of the D1D2 region of the inhibitory receptor LIR-1 (ILT2). Immunity. 2000;13(5):727-736.

28. Borges L, Hsu ML, Fanger N, Kubin M, Cosman D. A family of human lymphoid and myeloid Ig-like receptors, some of which bind to MHC class I molecules. J Immunol. 1997;159(11):5192-5196.

29. Hunt JS, Petroff MG, McIntire RH, Ober C. HLA-G and immune tolerance in pregnancy. Faseb J. 2005;19(7):681-693.

30. Willcox BE, Thomas LM, Bjorkman PJ. Crystal structure of HLA-A2 bound to LIR-1, a host and viral major histocompatibility complex receptor. Nat Immunol. 2003;4(9):913-919.

31. Dietrich J, Cella M, Colonna M. Ig-like transcript 2 (ILT2)/leukocyte Ig-like receptor 1 (LIR1) inhibits TCR signaling and actin cytoskeleton reorganization. J Immunol. 2001;166(4):2514-2521.

32. Sayos J, Martinez-Barriocanal A, Kitzig F, Bellon T, Lopez-Botet M. Recruitment of C-terminal Src kinase by the leukocyte inhibitory receptor CD85j. Biochem Biophys Res Commun. 2004;324(2):640-647.

33. Bellon T, Kitzig F, Sayos J, Lopez-Botet M. Mutational analysis of immunoreceptor tyrosine-based inhibition motifs of the Ig-like transcript 2 (CD85j) leukocyte receptor. J Immunol. 2002;168(7):3351-3359.

34. Latour S, Veillette A. Proximal protein tyrosine kinases in immunoreceptor signaling. Curr Opin Immunol. 2001;13(3):299-306.

35. Navarro F, Llano M, Bellon T, Colonna M, Geraghty DE, Lopez-Botet M. The ILT2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. Eur J Immunol. 1999;29(1):277-283.

36. Kirwan SE, Burshtyn DN. Killer cell Ig-like receptor-dependent signaling by Ig-like transcript 2 (ILT2/CD85j/LILRB1/LIR-1). J Immunol. 2005;175(8):5006-5015.

37. Binstadt BA, Brumbaugh KM, Dick CJ, et al. Sequential involvement of Lck and SHP-1 with MHC-recognizing receptors on NK cells inhibits FcR-initiated tyrosine kinase activation. Immunity. 1996;5(6):629-638.

38. Maeda A, Scharenberg AM, Tsukada S, Bolen JB, Kinet JP, Kurosaki T. Paired immunoglobulin-like receptor B (PIR-B) inhibits BCR-induced activation of Syk and Btk by SHP-1. Oncogene. 1999;18(14):2291-2297.

39. Saverino D, Merlo A, Bruno S, Pistoia V, Grossi CE, Ciccone E. Dual effect of CD85/leukocyte Ig-like receptor-1/Ig-like transcript 2 and CD152 (CTLA-4) on cytokine production by antigen-stimulated human T cells. J Immunol. 2002;168(1):207-215.

40. Young NT, Waller EC, Patel R, Roghanian A, Austyn JM, Trowsdale J. The inhibitory receptor LILRB1 modulates the differentiation and regulatory potential of human dendritic cells. Blood. 2008;111(6):3090-3096.

41. Agarwal A, Salem P, Robbins KC. Involvement of p72syk, a protein-tyrosine kinase, in Fc gamma receptor signaling. J Biol Chem. 1993;268(21):15900-15905.

42. Kiener PA, Rankin BM, Burkhardt AL, et al. Cross-linking of Fc gamma receptor I (Fc gamma RI) and receptor II (Fc gamma RII) on monocytic cells activates a signal transduction pathway common to both Fc receptors that involves the stimulation of p72 Syk protein tyrosine kinase. J Biol Chem. 1993;268(32):24442-24448.

43. Dustin ML. T-cell activation through immunological synapses and kinapses. Immunol Rev. 2008;221(77-89.

44. Orange JS. Formation and function of the lytic NK-cell immunological synapse. Nat Rev Immunol. 2008;8(9):713-725.

45. Nakajima H, Asai A, Okada A, et al. Transcriptional regulation of ILT family receptors. J Immunol. 2003;171(12):6611-6620.

46. Lamar DL, Weyand CM, Goronzy JJ. Promoter choice and translational repression determine cell type specific cell surface density of the inhibitory receptor CD85j expressed on different hematopoietic lineages. Blood. 2010.

47. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. Blood. 2005;105(11):4390-4398.

48. Palanichamy A, Barnard J, Zheng B, et al. Novel human transitional B cell populations revealed by B cell depletion therapy. J Immunol. 2009;182(10):5982-5993.

49. Sanz I, Wei C, Lee FE, Anolik J. Phenotypic and functional heterogeneity of human memory B cells. Semin Immunol. 2008;20(1):67-82.

50. Strausberg RL, Feingold EA, Grouse LH, et al. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci U S A. 2002;99(26):16899-16903.

51. Effros RB, Boucher N, Porter V, et al. Decline in CD28+ T cells in centenarians and in long-term T cell cultures: a possible cause for both in vivo and in vitro immunosenescence. Exp Gerontol. 1994;29(6):601-609.

52. Vallejo AN, Bryl E, Klarskov K, Naylor S, Weyand CM, Goronzy JJ. Molecular basis for the loss of CD28 expression in senescent T cells. J Biol Chem. 2002;277(49):46940-46949.

53. Goronzy JJ, Lee WW, Weyand CM. Aging and T-cell diversity. Exp Gerontol. 2007;42(5):400-406.

54. Warrington KJ, Takemura S, Goronzy JJ, Weyand CM. CD4+,CD28- T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. Arthritis Rheum. 2001;44(1):13-20.

55. Nakajima T, Goek O, Zhang X, et al. De novo expression of killer immunoglobulin-like receptors and signaling proteins regulates the cytotoxic function of CD4 T cells in acute coronary syndromes. Circ Res. 2003;93(2):106-113.

56. Fann M, Chiu WK, Wood WH, 3rd, Levine BL, Becker KG, Weng NP. Gene expression characteristics of CD28null memory phenotype CD8+ T cells and its implication in T-cell aging. Immunol Rev. 2005;205(190-206.

57. Liu WR, Kim J, Nwankwo C, Ashworth LK, Arm JP. Genomic organization of the human leukocyte immunoglobulin-like receptors within the leukocyte receptor complex on chromosome 19q13.4. Immunogenetics. 2000;51(8-9):659-669.

58. Merlo A, Saverino D, Tenca C, Grossi CE, Bruno S, Ciccone E. CD85/LIR-1/ILT2 and CD152 (cytotoxic T lymphocyte antigen 4) inhibitory molecules down-regulate the cytolytic activity of human CD4+ T-cell clones specific for Mycobacterium tuberculosis. Infect Immun. 2001;69(10):6022-6029.

59. Jones DC, Roghanian A, Brown DP, et al. Alternative mRNA splicing creates transcripts encoding soluble proteins from most LILR genes. Eur J Immunol. 2009;39(11):3195-3206.

60. Jones DC, Roghanian A, Brown DP, et al. Alternative mRNA Splicing Creates Transcripts Encoding Soluble Proteins From Most LILR Genes. Eur J Immunol. 2009.

61. Lee T, Luo L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron. 1999;22(3):451-461.

62. Valiante NM, Uhrberg M, Shilling HG, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. Immunity. 1997;7(6):739-751.

63. Mills DM, Cambier JC. B lymphocyte activation during cognate interactions with CD4+ T lymphocytes: molecular dynamics and immunologic consequences. Semin Immunol. 2003;15(6):325-329.

64. Martin J, Collot-Teixeira S, McGregor L, McGregor JL. The dialogue between endothelial cells and monocytes/macrophages in vascular syndromes. Curr Pharm Des. 2007;13(17):1751-1759.

65. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol. 2008;26(421-452.

66. Tenca C, Merlo A, Merck E, et al. CD85j (leukocyte Ig-like receptor-1/Ig-like transcript 2) inhibits human osteoclast-associated receptor-mediated activation of human dendritic cells. J Immunol. 2005;174(11):6757-6763.

67. Harwood NE, Batista FD. New insights into the early molecular events underlying B cell activation. Immunity. 2008;28(5):609-619.

68. Schulze-Luehrmann J, Ghosh S. Antigen-receptor signaling to nuclear factor kappa B. Immunity. 2006;25(5):701-715.

69. Kurosaki T, Hikida M. Tyrosine kinases and their substrates in B lymphocytes. Immunol Rev. 2009;228(1):132-148.

70. Hardy RR, Hayakawa K. B cell development pathways. Annu Rev Immunol. 2001;19(595-621.

71. Crowley JE, Scholz JL, Quinn WJ, 3rd, et al. Homeostatic control of B lymphocyte subsets. Immunol Res. 2008;42(1-3):75-83.

72. Brown D, Trowsdale J, Allen R. The LILR family: modulators of innate and adaptive immune pathways in health and disease. Tissue Antigens. 2004;64(3):215-225.

73. Liou HC, Jin Z, Tumang J, Andjelic S, Smith KA, Liou ML. c-Rel is crucial for lymphocyte proliferation but dispensable for T cell effector function. Int Immunol. 1999;11(3):361-371.

74. Glimcher LH, Townsend MJ, Sullivan BM, Lord GM. Recent developments in the transcriptional regulation of cytolytic effector cells. Nat Rev Immunol. 2004;4(11):900-911.

75. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell. 1997;89(4):587-596.

76. Ho IC, Pai SY. GATA-3 - not just for Th2 cells anymore. Cell Mol Immunol. 2007;4(1):15-29.

77. Adamson AS, Collins K, Laurence A, O'Shea JJ. The Current STATus of lymphocyte signaling: new roles for old players. Curr Opin Immunol. 2009;21(2):161-166.

78. Barton K, Muthusamy N, Fischer C, et al. The Ets-1 transcription factor is required for the development of natural killer cells in mice. Immunity. 1998;9(4):555-563.

79. Lacorazza HD, Miyazaki Y, Di Cristofano A, et al. The ETS protein MEF plays a critical role in perforin gene expression and the development of natural killer and NK-T cells. Immunity. 2002;17(4):437-449.

80. Strubin M, Newell JW, Matthias P. OBF-1, a novel B cell-specific coactivator that stimulates immunoglobulin promoter activity through association with octamer-binding proteins. Cell. 1995;80(3):497-506.

81. Sauter P, Matthias P. The B cell-specific coactivator OBF-1 (OCA-B, Bob-1) is inducible in T cells and its expression is dispensable for IL-2 gene induction. Immunobiology. 1997;198(1-3):207-216.

82. Brunner C, Wirth T. Btk expression is controlled by Oct and BOB.1/OBF.1. Nucleic Acids Res. 2006;34(6):1807-1815.

83. Conley ME, Dobbs AK, Farmer DM, et al. Primary B cell immunodeficiencies: comparisons and contrasts. Annu Rev Immunol. 2009;27(199-227.

84. Davuluri RV, Suzuki Y, Sugano S, Plass C, Huang TH. The functional consequences of alternative promoter use in mammalian genomes. Trends Genet. 2008;24(4):167-177.

85. Landry JR, Mager DL, Wilhelm BT. Complex controls: the role of alternative promoters in mammalian genomes. Trends Genet. 2003;19(11):640-648.

86. Rigault C, Le Borgne F, Demarquoy J. Genomic structure, alternative maturation and tissue expression of the human BBOX1 gene. Biochim Biophys Acta. 2006;1761(12):1469-1481.

87. Shephard EA, Chandan P, Stevanovic-Walker M, Edwards M, Phillips IR. Alternative promoters and repetitive DNA elements define the species-dependent tissue-specific expression of the FMO1 genes of human and mouse. Biochem J. 2007;406(3):491-499.

88. Arce L, Yokoyama NN, Waterman ML. Diversity of LEF/TCF action in development and disease. Oncogene. 2006;25(57):7492-7504.

89. Larsen LK, Amri EZ, Mandrup S, Pacot C, Kristiansen K. Genomic organization of the mouse peroxisome proliferator-activated receptor beta/delta gene: alternative promoter usage and splicing yield transcripts exhibiting differential translational efficiency. Biochem J. 2002;366(Pt 3):767-775.

90. Phelps DE, Hsiao KM, Li Y, et al. Coupled transcriptional and translational control of cyclin-dependent kinase inhibitor p18INK4c expression during myogenesis. Mol Cell Biol. 1998;18(4):2334-2343.

91. Barreau C, Paillard L, Osborne HB. AU-rich elements and associated factors: are there unifying principles? Nucleic Acids Res. 2005;33(22):7138-7150.

92. Soustelle L, Roy N, Ragone G, Giangrande A. Control of gcm RNA stability is necessary for proper glial cell fate acquisition. Mol Cell Neurosci. 2008;37(4):657-662.

93. Ju XS, Hacker C, Scherer B, et al. Immunoglobulin-like transcripts ILT2, ILT3 and ILT7 are expressed by human dendritic cells and down-regulated following activation. Gene. 2004;331(159-164.