FCRL as a Target for Immunotherapy of B-cell Lymphoma

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FCRL as a Target for Immunotherapy of B-cell Lymphoma

By

Mollie Capone

A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree Masters of Science in the College of Graduate Studies.

Department of Microbiology and Immunology

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Abstract

Current treatments for B-cell lymphomas are largely non-specific and survival rates among those diagnosed with aggressive subtypes of non-Hodgkin’s lymphoma (NHL) remain poor. Thus, there is a demand for immune therapies that could reduce cyto-toxicity and improve outcomes. While B-cell lymphoma tumor cells express HLA class II molecules, there are multiple defects in functional HLA class II antigen (Ag) presentation to CD4+ T-cells. Studies in our laboratory suggest that in a subtype of non-Hodgkin’s lymphoma, Burkitt’s Lymphoma (BL) there are associated molecules that may disrupt CD4+ T cell recognition of malignant B-cells. To dissect the role of BL-associated molecules in HLA class II Ag presentation, BL shed molecules from multiple BL cell lines were isolated. Furthermore, the functions of these proteins in biochemical and immune evasion by B-cell lymphoma were characterized. This study suggests that many B-cell lymphomas shed a 49 kDa FCRL (Fc Receptor Like)-like molecule that inhibit functional HLA class II Ag presentation to CD4+ T-cells. Chromatography, Western blotting and other biochemical analyses showed that the secreted 49 kDa molecule is FCRLA protein which is primarily expressed in B-cells, and B cell malignancies. Immunofluorescence and confocal microscopy demonstrated that FCRLA protein may co-localize with HLA-DR molecules, which could suggest that FCRLA may block functional Ag presentation via the MHC class II pathway. Co-immunoprecipitation and functional Ag presentation assays supported this finding that indeed FCRLA binds to class II proteins and disrupts CD4+ T cell recognition of BL cells. Furthermore, we initiated studies to isolate human monoclonal antibody against a 49 kDa FCRLA molecule that will block inhibitory function of
FCRLA and restore class II-mediated immune recognition of BL cells. For these experiments, a sensitive FCRLA screening ELISA was developed, and preliminary library screening validated this approach. These studies suggested that a fully human monoclonal antibody against a 49 kDa FCRLA protein can be isolated for further study. Overall, my data suggest that BL cells secrete a 49 kDa FCRLA protein that inhibits functional HLA class II Ag presentation to CD4+ T-cells. This study also suggests that FCRLA could be a viable protein target for immunotherapy of BL and other B-cell lymphomas.
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CHAPTER 1: INTRODUCTION

B-cell Lymphomas

Antigen (Ag) recognition of malignant cells is crucial for host defense, as well as potentiating tumor destruction and clearance. However, lymphoid malignancies such as lymphoma and leukemia often have poor anti-tumor responses. The transformation of these cells compromises host defense and many of these tumors evolve mechanisms to evade immune recognition [1, 2]. Lymphoid neoplasms vary greatly in the ways that they behave as well as in their clinical presentation. Leukemias, for instance, typically involve the bone marrow and peripheral blood, whereas, lymphomas generally produce masses in the lymph nodes or other tissues. Also, plasma cell tumors typically originate within the bones and can cause systemic symptoms due to the production of immunoglobulins [3]. All of the neoplasms have the ability to spread to various tissues within the body.

There are two different groups of lymphomas, Hodgkin’s lymphoma (HL) and Non-Hodgkin’s lymphoma (NHL) where HL is distinguished from NHL by the presence of Reed- Sternberg giant cells [3]. Lymphomas commonly stem from germinal center and post-germinal center B-cells due to the high risk for transforming mutations during class switching and somatic hypermutation. This could potentially influence immune recognition, because B-cell lymphomas arise at distinct stages of B-lymphocyte development and maturation. However, the functional interactions of these cells with other components of the immune system remains unclear and is complicated by the complexity of how each lymphoma behaves and presents clinically.

In the United States, B-cell lymphomas comprise about 85% of NHL [4]. Although about 71% of patients diagnosed with NHL live five years after diagnosis,
those with more aggressive subtypes have particularly poor outcomes [5]. For example, diffuse large B-cell lymphoma (DLBCL) has an event-free survival rate of 17-45% [5]. Unfortunately, there are currently very few therapies aimed at these types of lymphomas, and the survival rates among these aggressive types remain poor [4, 5]. Therefore, new specific therapies for these types of lymphomas could potentially increase the survival rate for patients who have been diagnosed with DLBCL.

Another aggressive subtypes of NHL is Burkitt’s lymphoma (BL) which is caused by the translocation of the c-myc oncogene with the immunoglobulin heavy chain enhancer [6]. The overexpression of c-myc as a consequence of these genetic alterations contributes to a number of both human and animal neoplasias [7] and overexpression of c-myc gene production BL has been shown to alter antigen (Ag) presentation by HLA class I molecules [9-11]. Our laboratory has recently shown that overexpression of c-Myc disrupts functional HLA class II Ag presentation by BL [12]. In order to maintain long-lasting immunity against tumors, CD8+ as well as CD4+ T-cells play vital roles in the tumor killing and recruitment of host defenses [13]. Therefore, improving CD4+ T cell responses would help to maintain a sustained immune response against lymphoma cells.

There are two classifications of BL. The first is endemic BL, which occurs in African populations, mainly in children, and is associated with the Epstein-Barr virus (EBV). The second type is sporadic BL, which occurs mainly in the United States and other Western countries, and whose etiology is unknown [5]. Interestingly, although both endemic and sporadic types of BL express Ag presenting HLA class I/II molecules, immune recognition in these cell types remains poor.
Current Treatments for Burkitt Lymphoma

Treatments for B-cell lymphomas typically consist of chemotherapy and radiation which are largely non-specific, causing cell death to normal, healthy tissues surrounding the targeted cancer cells. BL is a type of lymphoma that typically is treated with a very aggressive regimen of chemotherapy drugs which is not always tolerated by the patients, and it can also have some serious side effects such as tumor lysis syndrome [14]. Tumor lysis syndrome is very serious and occurs as the body struggles to clear chemicals released from the tumor into the blood stream. This can cause both the heart and kidneys to function improperly. Because, the mainline regimen is so aggressive and can cause very serious side effects, most patients require a hospital stay during their treatment[14]. Therefore, there is a need for a new treatment that is less harmful to the patients and that can also increase the survival rates of patients with BL and similar types of lymphomas. Monoclonal antibody-based immunotherapies that target tumor antigens or potentiate tumor immunity tend to be less toxic and make viable treatment options either on their own or in combination with the mainline treatments. One example of antibody immunotherapy is called Rituximab, which has been popularly used as a supplemental treatment with chemotherapies, and has led to improved outcomes for these patients [14]. This supports that a new immunotherapy might make a strong contribution to improved outcomes through a targeted treatment. It is also important to note in developing treatments against Burkitt’s lymphoma, that CD8+ cytotoxic T-cell function is augmented by HLA-class II-restricted CD4+ T-cells. This makes the function of CD4+ T-cell function crucial in maintaining a sustained and long-lasting immune response against these tumor cells.
HLA Class II Antigen Presentation

The Histocompatibility Locus (HLA) class II pathway presents Ag to CD4+ T-cells via the class II Major Histocompatibility Complex (MHCII) (Figure 1A). MHC class II molecules differ from MHC class I in that they are primarily expressed by professional antigen presenting cells (APCs) while MHC class I molecules are expressed ubiquitously by almost all cell types [15]. For this thesis, it is important to note that B-cells can present Ags as APCs. The class II pathway works with the synthesis of the MHC II molecule which is comprised of an α and β chain in the rough endoplasmic reticulum (RER) (Figure 1B). Also synthesized in the RER is the invariant chain (Ii) which then associates with the MHC II molecule. In this complex, the invariant chain prevents proteins from binding in the MHC II binding groove, and also acts as a chaperone by bringing the complex out of the RER through the Golgi apparatus, to a late endosomal/lysosomal compartments which have been termed the MHC class II compartments (MIIC) [16]. The Ii is then digested in the MIIC leaving behind a fragment termed the class II-associated Ii peptide (CLIP) which occupies the binding groove of the MHC II molecule. At this stage the human leukocyte antigen DM (HLA-DM) molecule facilitates an exchange of CLIP with antigenic peptides which were derived from a protein broken down in the endolysosomal pathway. The complete class II MHC-peptide complex is then transported to the cell surface by a vesicle, where it is recognized by a CD4+ T cell receptor. Importantly, in B-cells, there is also an HLA-DO molecule whose role is to restrict HLA-DM activity. Therefore, peptide binding to MHC II can be regulated via HLA-DO.
HLA Class II Pathway Defects in BL

The function of CD8+ cytotoxic T-cells is augmented by HLA-class II-restricted CD4+ T-cells making the function of these CD4+ T-cells crucial for continued cytotoxic
immune responses to tumor cells. Previous studies have shown that there is a failure to stimulate CD8+ T-cells through the HLA class I pathway by some B-cell lymphomas, including BL [10, 17]. Thus, there is a possibility of using tumor specific CD4+ T-cells to eliminate malignant B-cells. This is supported by recent studies which have developed cytotoxic CD4+ T cell clones that are class II-restricted and specific to BL and non-Hodgkins follicular lymphoma (FL) [18, 19]. It has also been shown that a greater influx of activated helper CD4+ T-cells is associated with a better prognosis for those with large B-cell lymphoma [20, 21]. Furthermore, the majority of B-cell tumors express HLA class II molecules, supporting that these tumors are prospective antigen presenting cells for CD4+ T-cells. Previous studies have shown that BL and B-lymphoblastoid cell lines (B-LCL) express measurable levels of HLA class II molecules. Despite this, class II-peptide complexes on BL cells failed to be recognized by CD4+ T-cells [22]. In recent studies, HLA-DR4 restricted peptide Ag presentation assays were performed comparing IL-2 secretion of CD4+ T Cell hybridomas after incubation with either a BL cell line (Nalm-6.DR4 or Ramos.DR4) or a B-LCL cell line (6.16.DR4) each stably transfected with HLA-DR4. Results showed that both BL cell lines failed to stimulate the CD4+ T-cells when compared with the B-LCL cell line. This shows that there are defects in Ag processing/presentation through the HLA class II pathway, as well as poor stimulation of CD4+ T-cells, in BL and FL [22, 23].

Recent studies suggest that BL-associated inhibitory molecules disrupt CD4+ T cell recognition of malignant B-cells [24]. Previous studies have also shown that BL cells either lack, or express at a reduced level, a 17 kDa peptidlyprolyl-like protein [23], and that bryostatin-1 can upregulate this 17 kDa molecule and enhance class II
presentation. The HLA class II defect also appears broader and results from expression of BL-associated molecules, which impairs the presentation of Ag to CD4+ T-cells via the HLA class II pathway [25]. Thus, targeting multiple pathway(s) of immune recognition may help to develop novel strategies for immune-mediated elimination of malignant B-cells. Previous unpublished studies suggest that B-cell lymphoma produces a distinct 49 kDa Fc receptor like (FCRL) molecule that may disrupt CD4+ T cell recognition of B-cell tumors (God and Haque, personal communications). Burkitt Lymphoma shed 49 kDa FCRL extract was isolated by differential fractionation, gel filtration and ion exchange and size exclusion chromatography. The 49 kDa fraction was tested for Ag presentation using B-LCL and DC cell lines, and data showed a significant inhibitory activity of this 49 kDa FCRL molecule in HLA class II Ag presentation by 6.16.DR4.DM (B-LCL line) and FSDC.DR4 (DC line) (God and Haque, personal communications). These data suggest that BL-shed 49 kDa FCRL-like molecules impair Ag presentation and immune recognition in the context of HLA class II molecules. A sustained CD4+ T cell response could serve to augment generation of a robust CD8+ T cell response to the tumors.

Therefore, investigation of the role of this secreted 49 kDa FCRL molecule in immune disruption of B-cell tumors may lead to development of novel immunotherapies which could lessen or eliminate the need for toxic chemotherapies.

**FCRL Nomenclature**

Due to the discovery of the FCRL protein family occurring by multiple groups in the same timeframe, these proteins were described with multiple different names. Therefore, a unified nomenclature for the FCRL protein family was developed in 2006 [26]. The first FCRL protein to be identified was actually a rat ortholog of FCRL6. It was
a glycosylphosphatidylinositol (GPI)-anchored membrane protein and was thus named gp42. Gp42 was also found to be upregulated on rat NK cells after exposure to IL-2 [27-29]. Furthermore, it had two Ig-like domains and was thought to be an activation marker due to stimulation of intracellular calcium influx via antibodies directed against gp42 on the surface of NK cells [28]. It wasn’t until ten years later, in 2001, when the first human FCRL genes were identified by the Dalla-Favera group [30]. While identifying the genes found at a chromosomal translocation breakpoint t(1;14)(q21;q32) in multiple myeloma cells, Hatzivassiliou et al. detected the gene corresponding to the FCRL4 protein, which they referred to as IgSF receptor translocation associated gene 1 (IRTA1) [30, 31]. Further investigation by the group revealed a total of five FCRL genes on chromosome 1q21-q23, which they named IRTA1-IRTA5 [30, 31]. The 2006 nomenclature that is related to these genes can be seen in Figure 2. Davis et al. also discovered six members of the FCRL gene family by searching the human genome database with a 32 amino acid consensus motif shared by FcγR1, FcγRII, FcγRIII and the polymeric Ig receptor utilizing a bioinformatics approach [32]. They referred to the genes as Fc receptor homologs (FcRH) FcRH1-FcRH6 and their associated FCRL nomenclature can be seen in Figure 2. Simultaneously, Xu et al. discovered two members of the FCRL family of proteins by utilizing in silico strategies to search for molecules that had characteristics shared by the IgSF, as well as, Fc receptor and gp42 proteins [33]. They designated these two new proteins as Src homology (SH)-2 domain-containing phosphatase anchoring proteins SPAP1 and SPAP2, which have since been referred to as FCRL2 and FCRL3, respectively (Figure 2). Also at the same time, Nakayama et al. used subtractive hybridization methods and uncovered four of the genes for these proteins. They termed
them B-cell cross-linked by anti-IgM activation sequence (BXMAS) genes [34]. Their correlating FCRL nomenclature can be seen in Figure 2. Guselnikov et al. again identified the family based upon an expressed sequence tag (EST) database search after probing with a consensus sequence corresponding to the unique extracellular domain of FCγR1 [35]. They called the resulting genes IFGPs for their homology to IgSF, FcR, and Gp42. The corresponding FCRL names for the IFGP proteins are also listed in Figure 2. The same group also identified two additional homologs that had no obvious transmembrane sequences, which they named FCRL1 and FCRL2 [36, 37]; these proteins were later renamed FCRLA and FCRLB, respectively [26, 38]. These same two proteins were also described simultaneously by the Colonna group as Fc receptor expressed in B-cells FREB [39] and FREB2 [40], and again by the Burrows group as Fc related proteins FcRX [41] and FcRY [42]. In total, 8 different human FCRL family members have been discovered, and in 2006, a unifying nomenclature was proposed, designating the 6 membrane bound human FCRLs as FCRL 1-6, and the two intracellular proteins as FCRLA and FCRLB [26, 38].

The unified nomenclature identifies each FCRL based upon its domain structure [26]. FCRL1 corresponds to FcRH1, IRTA5, IFGP1, or BXMAS1. FCRL2 replaces previous names FcRH2, IRTA4, IFGP4, BXMAS2, or SPAP1. FCRL3 was formerly identified as FcRH3, IRTA3, IFGP3, BXMAS3, or SPAP2. FCRL4 was previously referred to as IRTA1, FcRH4, or IFGP2. FCRL5 coincides with IRTA2, FcRH5, IFGP5, or BXMAS. FCRL6 was previously named FcRH6 or IFGP6. FCRLA was adopted for the intracellular protein previously named FCRL, FREB, or FcRX; and FCRLB was adopted for the intracellular protein earlier referred to as FCRL2, FREB2, or FcRY.
These changes in nomenclature are summarized in **Figure 2**. Additionally, to unify the naming of FCRL splice variants, they proposed adding the suffix “\_v” followed by the number of the variant to the gene name, e.g., FCRL1\_v1 for splice variant 1 of FCRL1 gene [26].

**Family member structure**

FCRL1-FCRL6 are all type 1 transmembrane glycoproteins that contain

![Diagram of human Fc receptor-like proteins](image)

**Figure 2:** **Human Fc receptor-like proteins.** The structures for FCRL1-6 as well as FCRLA and FCRLB are represented. Boxes indicate immunoglobulin domains while circles indicate ITAM and ITIM sequences. Mucin-rich regions are also denoted by triangles. The smaller D5 domain on FCRLA refers to the truncated version of the D5 domain found on this protein. Previous nomenclatures for each protein are listed beneath the FCRL name.
immunoglobulin-like domains in their extracellular regions. Their summarized domain structures can be seen in Figure 2. They also contain, unlike FCRs, both cytoplasmic ITAM-like and/or ITIM sequences. This could suggest that these proteins are capable of dual-modulation. FCRL1 is the only FCRL family member that contains two ITAM-like regions and is also the only FCRL that contains a charged residue in its transmembrane region where FCRLs 2-6 are hydrophobic and uncharged. FCRL1 also contains three extracellular domains D1, D2, and D3 [35, 43]. FCRL2 contains an additional D4 domain and has one ITAM and one ITIM sequence in its cytoplasmic tail. FCRL3 has two D1 domains followed by one of each D2-D5 domains, and like FCRL2, has one ITAM and one ITIM sequence in its cytoplasmic tail. FCRL4, on the other hand, only has one ITIM sequence in its intracellular region and has domains 2, 3, 4, and 5. FCRL5 is the largest of the FCRL proteins with six D1 domains, a D3, D4, and D5 domain. It also has two ITIM sequences and one ITAM sequence in its intracellular region. Finally, FCRL6 has domains D1, D3, and D4 as well as one ITIM sequence in its cytoplasmic tail. FCRLA and FCRLB differ from the 6 main isotypes of FCRL in that they are intracellular proteins and they each contain two to three Ig-like domains. They also lack ITAM or ITIM sequences, and instead have C-terminal mucin-like regions.

**Isoforms**

Although not many isoforms have been identified, each of the six membrane-bound FCRL molecules has the potential to be expressed as different isoforms. FCRL5, FCRL6, and FCRLA are some of the FCRLs with known isoforms. Alternate splicing of FCRL5 generated both a secreted and GPI-anchored isoform along with the typical transmembrane form, and the secreted isoform has been detected at high levels in patients
with MM, CLL, and MCL [30]. Through gene expression analysis and cloning of liver fragments, 4 alternative transcripts were discovered for FCRL6, designated FCRL6v1-v4 [44]. The v1 isoform corresponds to the typically recognized FCRL6 protein whereas v2 had only one extracellular D5 domain. FCRL6v3, on the other hand, had a D3 and D5 domain as well as a cytoplasmic tail with an altered cytoplasmic tail in the C-end resulting in an additional tyrosine residue. FCRL6v4 had the three extracellular domains, but a shortened cytoplasmic tail. These studies also found that expression of v2 and v3 transcripts were low in comparison with v1 and v4 [44].

FCRLA has been shown to have seven different isoforms, including a secreted isoform. FCRLA contains only a single signal peptide (SSP) exon, whereas the other FCRLS have previously been shown to contain two exons for a signal peptide (SP) [45, 46]. However, the EST databases contain some FCRLA cDNAs with 21 base pairs inserted between domain 1 and the SP sequence. This exon appears to be an SP2 exon and therefore could allow for the production of an FCRLA isoform with a longer signal peptide (LSP) as suggested by Kulemzin’s group [45]. They also investigated FCRLA transcripts encoding SSP and LSP isoforms by using RT-PCR using forward primers that matched the SP1 and SP2 exons, which produced a number of different fragments. These fragments were then cloned and the seven alternative FCRLA transcripts were found. Five of these isoforms were SSP containing isoforms, while the remaining two were LSP containing isoforms. The five SSP isoforms were designated FCRLA4d (containing domains 1, 2, 3, and 4), FCRLA3d (domains 1, 3, and 4), FCRLA2d (domains 3 and 4), FCRLA2d’ (domains 1 and 4), and FCRLA1d (domain 4). The LSP containing isoforms were then designated LSP-FCRLA4d and LSP-FCRLA2d. Of these isoforms the four
domain isoform was found to be much more abundant than the other isoforms, and SSP isoforms were about 10 times more abundant than LSP isoforms. Further investigation showed that LSP-FCRLA2d is a secreted isoform of the FCRLA protein [45].

**Cellular Expression of FCRLs**

FCRL gene expression is seen primarily, although not exclusively, in B-lymphocytes. Expression of FCRL1 can be seen at low levels in pro- and pre-B-cells and expression increases on naïve and memory B-cell populations. However, expression is also down regulated in germinal center (GC) B-cells [47, 48]. FCRL2 expression is primarily seen on peripheral memory B-cells, but was also seen, after staining of tonsil sections, in intra- and sub-epithelial areas that were adjacent to the mantle zone (MZ) [49-51]. One of the only FCRLs to show expression on cells other than B-cells was FCRL3, whose expression can be seen in natural killer (NK) and CD8+ T-cells as well as T-regulatory (Treg) cells [48]. FCRL3 expression also showed low expression on memory cells and GC B cells [48]. Studies have shown expression of FCRL4 on a unique subset of B-cells which localize primarily in subepithelial region. Otherwise, FCRL4 expression can be detected on a number of different types of B cells including memory and naïve B cells, but only on a small percentage of these cells [52-54]. FCRL5 expression can be seen on most B cells with low expression on pre-B cells, but full expression on naïve and memory B cells. Expression was much lower on GC B-cells, but unlike other FCRLs had high expression among plasma calls from the tonsil, spleen, and bone marrow [48]. Similarly to FCRL3, FCRL6 expression can be seen in cytolytic lymphocytes as it has been found primarily on the surface of NK cells and has also been seen on CD8+ T-cells specifically in the blood and spleen [44, 55, 56]. Expression of
FCRL1-FCRL5 in B-cells has also been shown to be increased during B-cell differentiation and peaks in circulating cells as well as cells localized in the secondary lymphoid tissues as found by employing transcript analyses from tissues or sorted cells using Northern blot, PCR, as well as in situ hybridization [31, 32]. As for FCRLA and FCRLB, FCRLA expression is predominantly expressed in GC B-cells, most notably in proliferating centroblasts[38], but also all B-cell subsets in the tonsil with the exception of plasma calls [57]. FCRLB expression typically is fairly low. Therefore, it is somewhat difficult to detect using Northern blot and RT-PCR, and expression could only be seen after amplification where it was found in the placenta, kidney and spleen [40]. Because BL arises in GC B-cells, this could make FCRLA and FCRLB possible candidates for the secreted FCRL-like molecule we have seen to inhibit class II Ag presentation.

Of the eight FCRL proteins, six of them are surface proteins with a transmembrane region, while two of them are exclusively intracellular proteins. As previously described, the surface FCRLs (FCRL1-FCRL6) express Ig-like domains on the surface of the cells; and ITIM or ITAM-like sequences in their cytoplasmic tails. This suggests their involvement in immune regulation of their respective cell types. FCRLA and FCRLB, on the other hand, are expressed mainly intracellularly. Therefore, they do not contain the same ITIM or ITAM-like sequences. They do, however, have C terminus containing a proline-rich stalk region which precedes a leucine-rich coiled-coil motif [58]. Studies have shown association of FCRLA with IgG, IgM, and IgA occurs intracellularly suggesting its role as a possible chaperone protein in modulating cellular functions [58]. However, when tested, cell surface FCRLA failed to bind extracellular Ig [59], suggesting this protein may be unstable when expressed on the cell surface.
Furthermore, this could indicate that FCRLA could be involved in the development of B-cell lymphomas.

**FCRLs in Cellular Signaling**

Thus far, knowledge of ligands for FCRL family members has been minimal. There are no known ligands for FCRL1-FCRL3 or FCRLB; however, ligands for the other family members have been discovered. Due to their homology to the classical FcRs, FCRL4 and FCRL5 were tested to see if they could bind IgA and IgG. FCRL5 was seen to interact with IgG when stained with a preparation of mixed isotypes [48]. This was confirmed by using flow cytometry-based Ig-binding studies using FCRL1-FCRL6 transient transfectants where it was found that FCRL4 could bind heat-aggregated IgA, and FCRL5 could bind both heat-aggregated IgA and IgG [59]. This makes FCRL4 the only known receptor to inhibit IgA function. Wilson et al also found that FCRL5 had greater affinity towards IgG1 and IgG2 than IgG3 which also had greater binding affinity than IgG4 [59]. Additionally, of its nine domains, the three N-terminal domains were found to be sufficient for reactivity. This was tested using cDNA encoding different domains, which were then expressed in cells and analyzed for the ability to bind Ig. Further investigation showed that using receptor specific mAbs could block binding of FCRL4 and FCRL5 with Igs. This verified these domains as necessary for antibody binding and reinforces the roles of FCRL4 and FCRL5 as Ig receptors [59].

Schreeder et al. also found that FCRL6 can bind MHC molecules, specifically HLA class II protein [56, 60]. Due to its expression on NK cells and cytotoxic T-cells, this study was performed using an engineered NFAT driven GFP containing cell line co-
transduced with the human FCRL6 extracellular region fused to the ITAM-bearing mouse CD3ζ cytoplasmic tail of an [60]. Based on variable staining of FCRL6 in MHC class II expressing transductants, these studies also revealed that the binding affinities could differ between haplotypes of MHC II. As previously mentioned, MHC class II-expressing B-cells can influence helper T cell responses, and the interaction of FCRL6 with MHC class II could prove to be a critical pathway involved in regulating innate as well as adaptive immune responses in the host.

Similarly, there have also been ligands discovered for intracellular FCRLA. Despite the fact that early studies failed to show interactions with immunoglobulins, two of its domains bear a resemblance to two of the three subunits in the CD64/FcγR1. Furthermore, immunoprecipitation studies with endogenous FCRLA showed that it co-associates with IgM, IgG, and IgA in both cell lines and primary B-cells [58]. These findings, combined with its expression patterns in germinal center B-cells could suggest its involvement in Ig retention during affinity maturation as described [61]. Although, FCRLB is even more similar to CD64, there have been no similar results suggesting Ig binding [58].

**The Role of FCRLs in B-cell Associated Diseases**

Since the discovery of the FCRL proteins, their involvement in a number of different immune mediated malignancies has been reported for the different family members. As expected, the expression of these proteins is particularly prevalent in B-cell malignancies. Upon searching for FCRL sequences using the Lymphochip microarray database, FCRLs were found to be upregulated in DLBCL, FL, and CLL [62]. These results were later confirmed using mAbs for FCRL1-FCRL5, where protein expression
was found on the surface of the previously mentioned lymphomas as well as BL, Hairy Cell Leukemia (HCL), and Mantle Cell Lymphoma (MCL) [48]. Because of their differential expression on different malignancies, FCRL proteins are also being investigated as diagnostic markers as well as targets for immunotherapies. These proteins, therefore, play critical roles in the understanding and treatment of malignant diseases.

FCRL1 is expressed widely among B-cells and therefore could show promise as an immunotherapeutic target. Upon exploring this further, FCRL1 expression was found largely on CLL, FL, HCL, and MCL samples. These findings led to cytotoxicity studies where two recombinant immunotoxins E3(Fv)-PE38 and E9(Fv)-PE38 were constructed, bound to FCRL1, and were found to be cytotoxic to lymphoma cell lines [63]. This indicates that FCRL1 could be used in treatments for FCRL1-positive malignancies. The family member, FCRL2, has emerged as an important molecule in CLL. Somatic hypermutation in the heavy chain variable region (IGHV) gene differentiates the subtypes of the disease based on aggressiveness. FCRL2 also became an important factor when it was found to be 94% concordant with IGHV status [64]. This made it a better biomarker than those that have been previously used.

The FCRL3 protein has been found to be prevalent in autoimmune disorders [50]. When the region surrounding the FCRL1-FCRL5 locus was surveyed, there was a number of single nucleotide polymorphisms (SNP) uncovered. One of these SNPs was associated with 830 individuals with rheumatoid arthritis, systemic lupus erythematosus, Grave’s disease, as well as other autoimmune disorders [65]. Further investigation identified the principle variant was located in the FCRL3 promoter region in a possible
NF-κB binding motif. In more recent studies, there has been indication that FCRL3 modulation is associated with this SNP in T-cells and is also related to the clinical progression of rheumatoid arthritis [66]. A recent study has also shown that FCRL4-expressing B-cells express high levels of TNF-α and RANKL, and act as pro-inflammatory in Rheumatoid arthritis [67]. Because of FCRL3’s association with these autoimmune diseases, the gene has now been considered as a candidate susceptibility gene for autoimmunity [61].

Because FCRL4 expression marks a specific subset of B-cells that exist near the epithelium in MALT, it was investigated as a possible marker for MZL. Consequently, the protein was observed in 73% of nodal, and 93% in extranodal MZL making it an ideal histopathological marker for these malignancies [68]. However, it was not found to be in the specific subtype which stems from the spleen. FCRL4 also has a role in infectious diseases, where its expression has emerged in associated with diseases such as HIV and Hepatitis C [69, 70]. In addition, a population of FCRL4-expressing B-cells was found in patients with combined variable immunodeficiency. A unique subset of B-cells in viremic HIV patients was also found to co-express FCRL4. In addition, it has been shown that FCRL4 knockdown via siRNA could reestablish BCR-mediated proliferation, productions of cytokines and chemokines, and antibody responses specific to HIV [71]. However, it remains unclear how FCRL4 is involved with chronic viral infections.

FCRL5 has been shown to have a significant role in B-cell malignancies as well [61]. Patients with various B-cell malignancies were found to have soluble FCRL5 in their sera [72]. Importantly, FCRL5 is being studied as a possible target for MM immunotherapy. The expression of FCRL5 on MM plasma calls was established via
analysis of bone marrow aspirates from MM patients. Thus far, antibody-drug conjugates have been developed targeting FCRL5, and they have shown potential in preclinical efficacy for targeting MM in xenografts models [73]. FCRL6 has also been implicated in immune disorders as gene expression has been shown to be upregulated in the late stages of HIV-1 infection [44]. In a recent study, RT-PCR was utilized to analyze the expression levels of FCRL6 in peripheral blood lymphocytes of late-stage HIV-1 infected patients, where expression levels were significantly higher in stage 3 and 4 HIV-1 infected patients when compared with healthy individuals [44]. This study suggests that FCRL6 may influence HIV infection, and further studies may find ways to be able to utilize it in diagnosis or treatment of HIV infection.

**Rationale**

Previous studies have shown that there is a defect in class II antigen presentation by B cells in BL patients[22]. Antigen presentation assays have also shown that there is a BL secreted molecule that inhibits antigen presentation and that this molecule is a 49 kDa FCRL-like molecule. Molecules in the FCRL family are found almost exclusively on B cells and have been shown to be involved in cell signaling[46]. Therefore, it is likely that one of these FCRL molecules is being secreted by BL cells and could be inhibiting antigen presentation. For this reason, in this thesis, it was determined which FCRL molecule is being secreted by BL cells as well as its role in inhibiting antigen presentation. Also, a screening ELISA was created for this protein and an attempt was made to create a fully human monoclonal antibody against the protein. Consequently, this molecule could be used as a target for immunotherapy against BL and other B cell lymphomas.
Hypothesis

I hypothesize that a secreted 49kDa form of FCRLA mediates immunosuppression against BL tumor cells and that this protein could be a viable target for a monoclonal antibody-based immunotherapy of Burkitt’s lymphoma and other types of B cell lymphoma.

In order to test this hypothesis, I

(1) Determined the extent to which secreted FCRLA protein impairs HLA class II protein functions and CD4+ T cell recognition; and

(2) Determined the extent to which depletion of 49 kDa secreted FCRLA restores HLA class II antigen presentation and CD4+ T cell recognition of B-cell lymphoma.
CHAPTER 2: A Secreted 49 kDa FCRLA Protein Impairs HLA Class II Protein Functions and CD4+ T-cell Recognition

BL Cells Secrete FCRLA Protein

Because there are many FCRL proteins with each protein having multiple possible transcripts, I first set out to identify which FCRL protein was secreted by the BL tumor cells. This was done by Western blotting of concentrated supernatants and cell lysates for Nalm-6 and Frev BL and B-LCL cell lines were used (a more detailed description can be found in the methods section). The supernatants were concentrated using a 30k Amcon Ultra Centrifugal Filter, protein amount were then estimated and 50µg of protein was loaded into each lane. The blots were then probed for expression of each of the eight FCRL proteins using mAbs specific for each protein. It was found that FCRLA was differentially expressed at 49kDa in the supernatants of the BL cell lines (Figure 3). This can be supported by the fact that that there are 7 different possible transcripts of FCRLA including one that would yield a secreted isoform of FCRLA[45]. These data suggest that FCRLA was a candidate for inhibiting HLA class II Ag presentation by B-LCLs. Furthermore, as previously mentioned, FCRL6 has been shown to bind MHC molecules [60], specifically HLA class II. Seeing as the proteins all have similar structure, it would not be unlikely that another FCRL, specifically FCRLA, would have the ability to bind MHC molecules thereby inhibiting Ag presentation by B-LCLs.
Figure 3: B-cell Lymphomas secrete 49 kDa FCRLA into culture medium. Human B-cell lymphoma cells (Nalm-6.DR4) and a B-LCL (Frev) were grown in serum-free AIM-V medium (Gibco) for 48 hrs. Supernatants were then collected and concentrated using a 30k Amicon Ultra Centrifugal Filter. Cell supernatants and cell lysates (50ug/lane) were then analyzed by Western blotting for FCRL 1-6 as well as FCRLA and FCRLB. These data suggest that a 49 kDa form of FCRLA is secreted into B-cell lymphoma supernatants while other FCRL proteins were restricted to the cell lysates.
Isolation of FCRLA via Size Exclusion and Ion Exchange Chromatography

In order to test the ability of the secreted FCRLA (49 kDa) to impair class II Ag presentation, chromatography methods were used to attempt to isolate FCRLA from the BL supernatants using size exclusion chromatography as well as ion exchange chromatography. This was done using 150ml of supernatant from Nalm-6.DR4 cells grown in AIMV serum-free media for 48 hrs. Ammonium sulfate was then used to precipitate the protein from the supernatant and analyzed both the precipitated pellet and the supernatant.

Figure 4: FCRLA Isolated After Column Chromatography. Supernatant (150 ml) was collected after growing B-cell lymphoma cells (Nalm-6) in AIM-V serum-free media for 48 hrs. Ammonium sulfate was then used to precipitate the protein from the supernatant. Both the pellet and supernatant after precipitation were analyzed by Western blotting for FCRLA (lanes 2 and 3). These data show that FCRLA protein was precipitated using ammonium sulfate. Following this, the pellet was reconstituted and run through a size exclusion column. The fraction containing 49 kDa protein was then further isolated using an ion exchange column. Fractions were then analyzed by Western blot and two fractions are shown in lanes 1 and 4. Lane 1 shows a fraction that was negative for FCRLA protein while lane 4 shows a fraction that was positive for FCRLA protein.
the supernatant after precipitation by Western blotting for FCRLA, and it was found that the protein was precipitated in this case (Figure 4). The sample was then reconstituted and it was run through a size exclusion column. The fraction containing 49 kDa proteins was analyzed by Western blot, and was found to be positive for FCRLA (data not shown). An attempt was then made to further isolate FCRLA using an ion exchange column and 22 fractions were analyzed by Western blotting for FCRLA. Following this, an FCRLA positive fraction and an FCRLA negative fraction were selected for use in further experiments (Figure 4).

**BL Secreted Proteins Inhibit Class II Antigen Presentation by B-LCL**

We next set out to test the extent to which the BL secreted proteins inhibit Ag presentation by the B-LCL Frev. This was done using a functional Ag presentation assay, as described in the methods section, Frev B-LCL cells were treated with 50µg of BSA, or concentrated media as controls, as well as concentrated sups from Frev (B-LCL), Ramos.DR4 (BL), or Nalm-6.DR4 (BL) cells cultured for 48 hrs in AIMV serum-free media. The B-LCL was treated for 3 hrs before being incubated with 10 µg of IgG Kappa188-203 (K1) peptide at 37°C overnight. The cells were then washed, resuspended, and then cocultured in a 96-well plate with an HLA-DR4 restricted T cell hybridoma (2.18a) specific for the K1 peptide. There were also wells that had only the T cell hybridoma (2.18a) as a negative control. IL-2 secretion in the co-culture supernatants was then measured by ELISA as a function of T cell activation. The results show that BL supernatants reduced IL-2 secretion by the T cell hybridoma when compared to untreated cells suggesting that they inhibited functional Ag presentation by B-LCL (Figure 5). Furthermore, there was no inhibition in cells treated with either BSA or the concentrated
media. These data were not surprising, as these supernatants have been previously tested using the 6.16 (B-LCL) and Nalm-6 cell lines and HSA peptide in our laboratory, and found to be inhibitory [74].

Figure 5: BL Secreted Proteins Inhibit Antigen Presentation by B-LCL. Two BL cell lines (Nalm-6.DR4 and Ramos.DR4) or a B-LCL (FREV) were cultured for 48hrs in AIM-V serum-free media and culture supernatants were collected and concentrated as previously described in Figure 3. B-LCL were then incubated for 3hrs with Nalm-6.DR4 supernatant, Ramos.DR4 supernatant, Frev supernatant, concentrated media, or BSA (50 µg). All supernatants and the media were measured for protein amounts and 50 µg amounts were used. IgG kappa^{188-203} peptide (10 µM) was then added, and the cells were incubated for overnight in a shaking incubator at 37 °C. Cells were then washed and co-cultured with the peptide specific T cell hybridoma (2.18a) for 24 hrs. T cell production of IL-2 was then measured by ELISA. Data are representative of at least six separate experiments. Statistical analyses were performed by student’s t test. NS=Not significant.
After establishing that the BL supernatants inhibited Ag presentation, further testing was done to test the extent to which the FCRLA positive and negative fractions inhibited Ag presentation by Frev B-LCL cells following the treatment of the FCRLA positive and negative fractions that were obtained from the chromatography fractions. For

**Figure 6: FCRLA positive Chromatography Fraction Inhibits Antigen Presentation by B-LCL.** Protein amounts were measured for both the FCRLA positive and FCRLA negative fractions obtained from the chromatography experiments. B-LCL (Frev) cells were incubated for 3 hrs with 10 µg amounts of each fraction or 2.5 mM Tris buffer. IgG kappa peptide (10 µM) was then added, and the cells were incubated for overnight in a shaking incubator at 37 °C. Cells were then washed and co-cultured with the peptide specific T cell hybridoma (2.18a) for 24 hrs. IL-2 secretion by T cells was then measured by ELISA. Data are representative of seven separate experiments. Statistical analyses were performed by student’s t test. NS=Not significant.
this experiment the amount of protein in the fractions was measured and the B-LCL was treated with either the FCRLA positive or negative fraction in amounts that corresponded with 10 µg of protein for 3hrs at 37ºC while rocking. Following this K1 peptide was added (10µg) and incubated overnight at 37ºC. The B-LCL was also treated with the 2.5 M tris buffer that was used in the chromatography experiments as a negative control. The

Figure 7: FCRLA Blocks Peptide Presentation, but Not Anti-CD3

**Stimulation.** B-LCL (Frev) cells were incubated for 3hrs with 1ug of BSA, vehicle, 0.5 µg rFCRLA, or 1.0 µg rFCRLA. IgG kappa_{188-203} peptide (10 µM) was then added and the cells were incubated overnight. Cells were then washed and co-cultured with the peptide specific T cell hybridoma (2.18a) for 24 hrs. IL-2 secretion was then measured by ELISA. These data are representative of four separate experiments. Statistical analyses were performed by student’s t test. NS=Not significant.
cells were then washed and cocultured with a T cell hybridoma (2.18a) in a 96-well plate for 24 hrs. An ELISA for IL-2 secretion was then performed and it showed that the FCRLA positive fraction significantly inhibited Ag presentation by B-LCL when compared to the untreated and buffer only treated cells (p=0.0004). By contrast, the FCRLA negative fraction did not significantly inhibit Ag presentation (p=0.325) (Figure 6). Together, these data suggest that a secreted 49 kDa form of FCRLA inhibits functional HLA class II Ag presentation.

**FCRLA Blocks Peptide Presentation, but not Anti-CD3 Stimulation of T-Cells.**

After showing that FCRLA inhibited Ag presentation by B-LCL, further testing was done to determine whether FCRLA was, in fact, inhibiting Ag presentation, or if it instead inhibited T cell stimulation. To test this, an Ag presentation assay was done in which the Frev (B-LCL) cells were treated with either the BL-eluted FCRLA positive or FCRLA negative fractions. I also used a T cell hybrid only control as well as a control in which the cells were not incubated with peptide. However, cells were co-cultured on uncoated plates or plates that were coated with anti-CD3 antibody (Santa Cruz, Clone no.145-2C11) with T cell hybridoma line (2.18a) If FCRLA directly affected T cell stimulation, I would expect to see reduced levels of IL-2 in the anti-CD3 coated wells when the cells were treated with the FCRLA positive fraction. The data from this experiment indicated that the FCRLA positive fraction inhibited Ag presentation, but did not affect anti-CD3 T cell stimulation (Figure 7). Furthermore, the FCRLA negative fraction did not inhibit Ag presentation or anti-CD3 T cell stimulation, as expected. These data suggest that FCRLA acts through inhibiting functional Ag presentation by B-LCL and does not directly affect T cell stimulation.
rFCRLA Protein Impairs Functional Class II Presentation by B-LCL

In order to confirm that FCRLA was the secreted protein that inhibited Ag presentation, we repeated the Ag presentation assay utilizing a commercially available recombinant FCRLA protein. The B-LCL Frev was treated with either 0.5 or 1.0 µg of rFCRLA (Origene), a vehicle control, or 1µg of BSA specificity control for 3hrs, followed by addition of antigenic peptide overnight at 37ºC. The cells were then washed

Figure 8: rFCRLA Protein Inhibits Functional Antigen Presentation by B-LCL. B-LCL (Frev) cells were incubated for 3 hrs with 1 µg of BSA, vehicle, 0.5 µg rFCRLA, or 1.0 µg rFCRLA. IgG kappa188-203 peptide (10 μM) was then added and the cells were incubated overnight. Cells were then washed and co-cultured with the peptide specific T cell hybridoma (2.18a) for 24 hrs. IL-2 secretion was then measured by ELISA. These data are representative of 4 separate experiments. Statistical analyses were performed by student’s t test.

NS=Not significant.
and co-cultured with the peptide specific T cell hybridoma for 24hrs in a 96 well plate. An ELISA was then done to measure the amount of IL-2 that was secreted as a function of T cell activation. Results obtained from the analysis of T cell production of IL-2 showed that 1.0 µg of rFCRLA significantly reduced the amount of IL-2 that was secreted into the supernatants inhibited functional Ag presentation by B-LCL (p=0.002). It was also found that treatment of B-LCL with 0.5 µg of rFCRLA inhibited Ag presentation less than 1.0 µg of rFCRLA when compared with the controls (p=0.035) (Figure 8). This finding suggests that rFCRLA acted in a dose dependent manner to inhibit MHC class II-mediated Ag presentation by B-LCL.
I next set out to determine whether it depleting the BL supernatants of FCRLA would reduce the inhibition of Ag presentation. Depletion was done by first incubating the supernatants with either anti-FCRLA antibody or an NN4 isotype control, and then

**Figure 9. Depletion of FCRLA from BL Supernatants Restores Antigen Presentation by B-LCL.** FCRLA was depleted from supernatants collect from BL cell lines Ramos.DR4 and Nalm-6.DR4 using antibodies against FCRLA and protein A/G beads. (A) Western blot analysis using an antibody against FCRLA; FCRLM1 28.1 antibody (Santa Cruz) revealed that FCRLA was successfully depleted from BL supernatants. When compared with pre-depleted supernatants and NN4 isotype control depletion supernatants. (B) An Ag presentation assay in which a B-LCL (Frev) was treated with depleted supernatants revealed that depleting FCRLA from both BL supernatants could restore Ag presentation by B-LCL.

**BL Supernatants Depleted of FCRLA No Longer Inhibited Antigen Presentation by B-LCL.**
incubating with protein A/G beads and pelleting to remove from the supernatants. The supernatants were then analyzed by Western blotting (as described in the methods section) which showed that FCRLA had, in fact, been depleted from the supernatants when compared to the NN4 depleted supernatants (Figure 9A). Following this, an Ag
presentation assay was done in which the FCRLA depleted supernatant, NN4 depleted supernatants, and the pre-depletion supernatants were used to treat a B-LCL (Frev). The cells were then incubated with the K1 peptide and cocultured with the 2.18a T cell hybridoma as previously described. It was found that the pre-depletion supernatants as well as the NN4 depleted supernatants both inhibited Ag presentation by B-LCL as expected, while the FCRLA depleted supernatants significantly restored Ag presentation (Figure 9B). These data suggests that the secreted 49kDa form of FCRLA does inhibit Ag presentation and blocking this form of FCRLA could restore Ag presentation in BL cell lines.

**FCRLA May Inhibit Antigen Presentation by HLA-DR B binding**

I then set out to test if FCRLA inhibited Ag presentation by binding with HLA-DR molecules, therefore blocking peptide binding and/or presentation. Co-immunoprecipitation was done as described in the methods section. Cell lysates and supernatants from both the Ramos.DR4 (BL) and Frev (B-LCL) cell lines were used and an anti-FCRLA antibody with Protein A/G beads were used to pull down FCRLA and any bound protein. I then analyzed these samples by Western blotting for FCRLA using an anti FCRLA antibody (anti-FCRLM1 (N28.1) antibody Santa Cruz). I also did Western blotting for HLA-DR using an anti-HLA-DR antibody (Santa Cruz), and an isotype control (NN4). These data show that FCRLA was precipitated from the supernatants (Figure 10B). Furthermore, HLA-DR was able to be pulled down using FCRLA in the BL cell line lysate, suggesting that FCRLA may be binding HLA-DR in BL cell lines (Figure 10C). However, these data are inconclusive due to all of the background bands that were detected, and further testing is needed.
Figure 11. FCRLA Colocalizes with HLA-DR Molecules in BL Cells. A) A BL cell line (Ramos.DR4) and a B-LCL (Frev) were stained for FCRLA (DyLight 488- green) and HLA-DR (DyLight 594–Red). The cells were then mounted onto slides and visualized using a confocal microscope at a magnification of 630x. B) Merged panels were enlarged to better visualize colocalization. There appears to be no colocalization of FCRLA and HLA-DR in the B-LCL while there does appear to be colocalization of FCRLA and HLA-DR in the BL cells (as designated by arrows). These data are representative of three separate experiments.
FCRLA Co-localizes with HLA-DR Molecules in BL Cells

Therefore, I stained for both FCRLA using a goat anti-FCRLA antibody (Santa Cruz) and HLA-DR using a mouse anti HLA-DR antibody as described in the methods section, on both Frev (B-LCL) and Ramos.DR4 (BL) cell lines. They were then stained using a Dylight 488 horse anti-goat secondary antibody and a DyLight 594 horse anti-mouse secondary antibody. The cells were also stained for DAPI, and they were visualized by confocal microscopy (Figure 11). These images show that both cell lines were positive for FCRLA and HLA-DR. However, when the images are merged, FCRLA and HLA-DR appear to colocalize in the Ramos.DR4 cells but not in the Frev (B-LCL) cell line. This supports the data collected from my immunoprecipitation experiments suggesting that FCRLA might interact or bind to HLA-DR to inhibit functional Ag presentation by the class II pathway; however the experiments are merely correlative and do not provide conclusive proof.

To further investigate whether FCRLA binds with HLA-DR on the surface of the BL cells, I performed a surface staining experiment analyzed by flow cytometry (as described in the methods section). Two BL cell lines and two B-LCLs were stained with an anti-FCRLA PE antibody, and analyzed by flow cytometry for Median Fluorescence Intensity (MFI). The MFI results indicated that the two BL cell lines had low levels of PE staining on their cell surface while the B-LCLs did not compared with the unstained cells (Figure 12). Because the FCRKA levels were very low, double staining for FCRLA and HLA.DR was impossible to detect inconclusively.
Figure 12. FCRLA Surface Staining on BL and B-LCLs. Two BL cell lines (Nalm-6.DR4 and Ramos.DR4) and two B-LCLs (Frev and 6.16.DR4) were stained using anti-FCRLA PE antibody (Santa Cruz) before being analyzed by flow cytometry. Comparison of MFI values of results show that there was FCRLA staining in BL cell lines but not B-LCLs.
Chapter 3: FCRLA Targeted Immunotherapy

Development of ELISA for Detection of FCRLA Protein.

In order to develop a monoclonal antibody against the secreted 49kDa form of FCRLA, I needed to create a sensitive FCRLA detection method. Therefore, I set out to create an FCRLA specific ELISA for screening cell supernatants.

Figure 13. FCRLA ELISA for Screening B-cell Libraries. An ELISA was created for screening anti-FCRLA antibodies. (A) This was done by coating with 1µg/ml of rFCRLA using either a neutral or pH9 coating buffer. A mouse anti-FCRLA antibody (FCRLM1 N28.1 Santa Cruz) was then added at a 1:100 dilution followed by a secondary anti-mouse antibody conjugated with AP at a 1:2500 dilution. (B) Results of this ELISA showed that detection was possible using this technique, but only when using a pH 9.0 coating buffer.
This was done by first coating wells in a 96-well ELISA plate with the commercially available rFCRLA at 1µg/ml. to test for the optimal binding buffer, used both a neutral

![Figure 14](image1)

**Figure 14. B-cell Libraries Were Screened for FCRLA Reactive Clones.**

(A) Supernatants pooled from 10 plates were added to a 96 well plate and screen using an FCRLA ELISA. Wells C7 and D11 were positive for anti-FCRLA. (B) Wells C7 and D11 from 10 plates were added to a 96 well plates and screened using an FCRLA ELISA. Well C7 on plate 9 appeared to have nonspecific binding, while well D11 on plate 9 was positive for anti-FCRLA. This well was cultured and expanded for 30 days. (C) The expanded cells were then screened again and yielded a number of positive wells for anti-FCRLA.
coating buffer and a pH 9.0 coating buffer. Following this, I added commercially available mouse anti-FCRLA antibody at the manufacturer’s suggested dilution of 1:100. An AP conjugated goat anti-mouse secondary was then added to the wells at a 1:2500 dilution which was previously determined to be optimal (Figure 13A). The absorbance of the wells was then read at 405 nm. I found that with the neutral coating buffer the rFCRLA was unable to bind to the plate. However, with the pH 9.0 coating buffer I was able to detect rFCRLA when both primary and secondary antibody was added but not when either the primary and secondary antibody were added alone (Figure 13B). A detailed description of this assay is found in the methods section of this thesis.

**Screening B-cell Libraries for anti-FCRLA Antibodies.**

I next attempted to isolate a fully human monoclonal antibody against 49 kDa FCRLAI tested antibody containing supernatants from a B cell library that was created in the Sutkowski laboratory using her patented technology (US patent no. 8,715,743). Briefly, B-cells were isolated from peripheral blood samples. The B-cells were then transformed with Epstein-Barr virus to immortalize the cells and class switching from IgM to IgG was induced using recombinant cytokines and agents that mimic antigen binding and T-cell help. Ten-14 days later, the screening ELISA that I developed was used to screen supernatants pooled from ten plates for FCRLA reactive clones. Due to the fact that these supernatants were pooled from ten plates, I only expected to see a weak positive signal in any positive well because it would have been diluted ten-fold. As can be seen in Fig 15A two wells had values that were significantly above background, C7 and D11 (Figure 14A). I, therefore, chose to further screen these individual wells from each of the ten plates. As a specificity control, we compared the supernatants on
rFCRLA-coated ELISA plates to uncoated ELISA plates. These results were graphed and
the two positive plates were identified. The first was well C7 on plate 9, however, the
uncoated well also showed a positive signal in this case, and indicating that there was
non-specific binding (Figure 14B). The second positive well was D11 from plate 9
(Figure 14B), which showed a significant difference between the FCRLA coated and
uncoated plates. Therefore, this well was chosen to subclone and expand in a 96–well
culture plate. After 30 days, this plate was screened, and there were a number of wells
that appeared to be positive which were subcloned at this point (Figure 14C).
Unfortunately, after subcloning we lost the cells due to bacterial contamination. New
libraries are now being screened by Dr. Wei Sun in the Sutkowski lab as the project
continues to move forward.
# Chapter 4: Materials and Methods

### Cell Lines

**Table 1. Cell Line Descriptions.** Description of Cell lines used in experiments.

<table>
<thead>
<tr>
<th>Cell Line:</th>
<th>Description:</th>
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</tr>
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<tbody>
<tr>
<td><strong>Ramos.DR4[12, 74]</strong></td>
<td>BL cells that has been retrovirally transduced with DR4 in the Haque laboratory and has EBV genes.</td>
<td>Dr. Mike Hollers, University of Colorado, Denver</td>
</tr>
<tr>
<td><strong>Nalm-6.DR4 [12, 74]</strong></td>
<td>BL-type (Acute Lymphoblastic Leukemia), peripheral blood pre-B cell line. Retrovirally transduced with DR4 in the Haque laboratory.</td>
<td>Dr. Mike Hollers, University of Colorado, Denver</td>
</tr>
<tr>
<td><strong>Frev [12, 74]</strong></td>
<td>B-LCL that has been EBV-immortalized.</td>
<td>Janice Blum, Indiana University School of Medicine, Indianapolis</td>
</tr>
<tr>
<td><strong>6.16.DR4 [12, 74]</strong></td>
<td>B-LCL type cells that have been EBV immortalized and retrovirally transduced with DR4α and DR4β.</td>
<td>Janice Blum, Indiana University School of Medicine, Indianapolis</td>
</tr>
<tr>
<td><strong>2.18a</strong></td>
<td>Mouse T cell hybridoma specific to K188-203 peptide. HLA-DR4 Restricted cells.</td>
<td>Janice Blum, Indiana University School of Medicine, Indianapolis</td>
</tr>
</tbody>
</table>
The BL cell lines 6.16.DR4, Ramos.DR4 and Nalm6.DR4, stored in vials in liquid nitrogen, were thawed and cultured at 2x10^6 cells in nonpyrogenic sterile 25cm² flasks (T25) (Corning) in 10ml of RPMI 1640 medium (Gibco) plus 10% heat- inactivated FBS (HyClone) plus 5000 IU/mL penicillin/5000 μg/mL streptomycin, and L- glutamate (Mediatech Inc.). The B-LCL, Frev (PA), was cultured at 2x10^6 cells in 10ml of IMDM; Iscove’s DMEM (Cellgro) plus 10% heat- inactivated BGS (HyClone) plus 5000 IU/mL penicillin/5000 μg/mL streptomycin, and L- glutamate (Mediatech Inc.).

**Chromatography**

Serum free supernatants obtained from Nalm-6 culture were subjected to AmSO4 precipitation using saturated AmSO₄. Supernatant and AmSO₄ were then cooled to 4°C and AmSO₄ was slowly added to the supernatant to 50% while stirring. The solution was then stirred for 1hr at 4°C and then centrifuged for 1hr at 12,000g. After centrifugation, both supernatants and reconstituted AmSO₄ precipitate were tested by Western blotting for FCRLA. Reconstituted AmSO₄-precipitate was positive for FCRLA. This reconstituted precipitate was run through superdex 200 10/30 GL size exclusion column (GE Healthcare)., The fraction containing 49kDa molecule was further fractionated by using a pre-equilibrated HiTrap DEAE FF 1ml column (GE Healthcare) using a linear elution from 0 to 100 mM NaCl and a control step elution at 1 M NaCl. Fractions eluted at 0.1M NaCl, 0.5 M NaCl, 1M NaCl were tested for FCRLA by Western blotting. After screening, both FCRLA positive and FCRLA negative fractions were tested in functional Ag presentation assay. Detailed method will be included in the revised version of this thesis.
Collection and Concentration of Cell Supernatants

Cells (2x10^6) were incubated in 1X AIMV 10 ml serum-free media (Gibco) for 48hrs. The cells were then centrifuged at 1200 rpm for 5 minutes at 4 °C. Supernatants were then collected and filtered using a 30k Amicon Ultra Centrifugal Filter (Milipore) where they were centrifuged at 2500 rpm for 20 minutes at 4°C. Supernatants containing proteins >30k were then collected from the top of the filter and moved to a labeled 1.5 ml microcentrifuge tube and stored at 4 °C.

Preparation of Cellular Lysate

Cells were incubated at 37 °C in a 5% CO2 incubator for 24 hrs, after which the cells were collected and washed with Hank’s buffered salt solution (HBSS, Cellgro), pelleted, and stored at -20 °C. Next, 10 μl each of the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK) were added per 1 ml of stock lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, and 1% Triton X-100) to make complete lysis buffer (CLB). 100μL of CLB was added to each cell pellet and incubated 5 minutes on ice before vortexing the pellet and incubation for an additional 15 minutes on ice. The cells were then centrifuged at 6000 rpm for 2 minutes and the supernatant was transferred to a second set of microcentrifuge tubes. These tubes were then centrifuged for 8.5 minutes at 10,000 rpm. The lysate was aliquoted into microcentrifuge tubes (30 μl/ aliquot) and 15 μl of each lysate was saved for protein determination.
**Protein Determination**

Bovine serum albumin (BSA) standard (Thermo) was prepared by diluting the 2 mg/ml stock to concentrations of 0 μg/μl, 0.2 μg/μl, 0.4 μg/μl, 0.6 μg/μl, 0.8 μg/μl, and 1.0 μg/μl. Standard concentrations were added to wells of a 96-well EIA/RIA flat bottom medium binding plate (Costar) in triplicate with 25 μl per well. 5 μl of lysate or supernatant was added to 20 μl of double distilled water (dd H2O). These samples were added in triplicate to the sample wells for a total of 25 μl per well. DC Protein Assay Reagent A (BioRad) was made by adding 20 μl of DC Protein Assay Reagent S (BioRad) to each mL of DC Protein Assay Reagent A (BioRad), and the 25μL of Reagent A was added to each well. Then, 200 μl of DC Protein Assay Reagent B (BioRad) was added to each well. The plate was then agitated for 5 seconds, before incubated for 15 minutes at room temperature. The plate was read at 630 nm and a standard curve was generated from the BSA standard.

**Peptide**

The human IgG immunodominant peptide \( \kappa_{188-203} \) (sequence: KHKVYACEVTHQGLSS) was produced using Fmoc technology and an Applied Biosystems Synthesizer (Applied Biosystems, Foster City, CA) as described previously [75]. Peptide purity (> 99%) and sequence were analyzed by reverse-phase HPLC purification and mass spectroscopy. Peptides were dissolved in PBS and stored at -20 °C until used.

**Western blotting**

Lysate samples, dithiothreitol (DTT), and the MagicMark XP® Western protein standard (Invitrogen) were thawed on ice. The lysate samples were centrifuged at 10,000
rpm for 2 minutes. Samples buffer was prepared by adding 5% 2-mercaptoethanol (Gibco) to 2x Laemmli sample buffer. Then, 50 μg of protein for each sample was added to a labeled microcentrifuge tube containing 15μl of sample buffer, 1.0 μl of 1M DTT, and ddH2O to bring the total volume to 35 μl. The prepared samples were then boiled on a heat block at 70 °C for 10 minutes. Running buffer was then prepared using 760 ml ddH2O and 40 ml of 20X NuPage MES SDS running buffer (Invitrogen). 600 ml of the buffer was poured into the outer chamber of the Western running unit and the remaining buffer was poured into the inner chamber. 250 μl of NUPAGE antioxidant (Invitrogen) was then added to the inner chamber. Using a 10 or 12 well NUPAGE 4-12% Bis-Tris gel (Invitrogen) 5 μl of marker was added to the first well and the samples were added to each well after that. The gel was then run for 50 minutes at 200 volts. Transfer buffer was prepared by adding 225 ml ddH2O, 15 mL NUPAGE 20X transfer buffer (Invitrogen), 60 mL of methanol (Fisher Scientific), and 250 μl of NUPAGE antioxidant (Invitrogen).

After the gel was run, it was moved to the transfer unit with a nitrocellulose membrane (BioRad). The transfer unit was closed and placed into the gel box, and the remaining buffer was added to the inner chamber while ddH2O was added to the outer chamber. The gel was then transferred at 30 volts for 1 hr. Next, TBS-Tween was prepared by combining 960 ml of ddH2O, 10 ml of 1M Tris, 30 ml of 5M NaCl, and 1ml Tween 20 (Fisher Scientific). The nitrocellulose membrane was blocked by placing in 5% milk made with non-fat milk and TBS-Tween overnight at 4 °C. Primary antibody was added at the appropriate dilution in 2.5% nonfat milk in TBS-Tween and incubated while rotating at room temperature for 3 hrs or at 4 °C overnight. Following this, the membrane was washed five times for five minutes each with TBS-Tween. Next, secondary antibody
was added at the appropriate concentration in 5% nonfat milk in TBS-Tween and incubated while rotating at room temperature for 1 hr. The membrane was once again washing the membrane five times for 5 minutes each with TBS-Tween. The membrane was then imaged using BioRad Geldoc XRS imager and adding 250 μl each of Clarity Western ECL Substrate peroxide solution and Clarity Western ECL substrate Luminol/Enhancer Solution (Thermo).

**Immunoprecipitation**

Protein A/G PLUS-Agarose beads (Santa Cruz) were first equilibrated by adding 50 μl per sample to an Eppendorf tube. They were then centrifuged at 10,000 rpm for 5-10 seconds. The supernatant was aspirated and 1ml of dulbecco’s phosphate buffered saline (DPBS) was added. The beads were spun again at 10,000 rpm for 5-10 seconds. Supernatant was then aspirated and 1-2 ml of DPBS was added.

To pre-clear the samples 50ul of beads was then added to each 1ml sample and incubated for 1 hr at 4 °C on a shaker. The samples were then spun at 10,000 rpm for 5-10 seconds. The supernatant was then collected and stores at 4 °C. FCRLM1 (N28.1) antibody (Santa Cruz cat #SC-53583) was then added at 1μg/ml and the samples were incubated overnight at 4 °C on a shaker.

Protein A/G beads were once again equilibrated as previously discussed. 20ul of beads was then added to each sample and incubated for 1 hr at 4 °C on a shaker. The samples were then spun at 10,000 rpm for 5-10 seconds and the supernatant was collected and stored at 4 °C. The pellet was then washed 3 times using DPBS and centrifuging at 10,000 rpm for 5-10 seconds. A 29 gauge ½ ml insulin syringe (Exel) was also used to
remove remaining liquid in each wash. Following this, 50 µl of sample buffer and 1.5µl of DTT was then added and samples were prepared for Western blotting.

**Antigen Presentation Assay**

The B-LCL, Frev, was cultured for 24 hrs in cIMDM media as previously described. Cells were then counted and resuspended at a volume of 2x10^6/ml and 100 µl (2x10^5 cells) was added to a microcentrifuge tube. Next, 10µg of Kappa_{188-203} (K1) peptide was added. If treated, 10µg of K1 peptide was added after 3 hr treatments at 37 °C. The microcentrifuge tubes were then placed on a mutator at 37 °C overnight. The T cell hybridoma line 2.18a was then thawed and cultured in cRPMI 24hrs. T-cells were then counted and resuspended to a volume of 5x10^5 cells/ml and 100ul was added to each well of a 96-well culture cluster round bottom plate (Corning). Following this, 1 ml cRPMI was used to wash the Frev cells with K1 peptide and the cells were centrifuged at 7,000 rpm for 2 minutes twice. Next, 650 µl of cRPMI was added to the Frev cells and 100ul of each treatment was added to the 2.18a cells in the 96-well culture plate in triplicate. The plate was then incubated at 37 °C with 5% CO₂ for 24 hrs before the plate was frozen at -80 °C.

**Detection of IL-2 via ELISA**

EIA/RIA High Binding 96-well plates were coated using 100 µl per well with purified rat anti-mouse IL-2 (BD Pharmingen) mixed with 0.1M NaHCO₃ pH9.5 (coating buffer) using 1µg of anti-IL2 per ml of coating buffer. The plates were then covered with saran wrap and allowed to coat overnight at 4 °C. The next day the plate was washed one time with 1X PBS-Tween (pH7.4) using 200ul/well, and the T cell assay plate was moved to 37 °C 5% CO₂ incubator. The ELISA plates were then blocked using 2% BSA
(Sigma) solution in PBS and incubated for 30 minutes. Serial dilutions of recombinant mouse IL-2 (R&D Cat# 402-MC) in 1% BSA solution were then done to yield standard IL-2 concentrations (0-200 pg/ml). The T cell assay plates were then removed from the incubator and centrifuged at 2000 rpm for 5min at 4 °C. The ELISA plates were then washed three times with PBS-T using 200 µl/well. Standard dilutions and samples were then added to the ELISA plates in triplicate using 100µl/well. The plates were then covered with saran wrap and allowed to incubate for 2 hrs at room temperature. Plates were then washed three times using 200ul of PBS-T per well. Then, 1µg of biotinylated rat anti-mouse IL2 (BD Pharmingen) was added to each ml of 1%BSA solution and was then added to the plates at 100 µl/well. Plates were then covered and incubated for 1hr at room temperature. The plates were once again washed four times with PBS-T using 200 µl/well. NeutrAvidin Alkaline Phosphatase Conjugated (Pierce) was then prepared using 1µl/10 ml in 1%BSA solution and 100 µl were added to each well. The plates were then covered and incubated for 30 minutes at room temperature. Plates were washed four times again using 200 µl/well of PBS-T. PNPP phosphatase substrate (Thermo Scientific) was then prepared dissolving a p-nitrophenyl phosphate disodium salt with a balance of inert filler (PNPP) tablet in 1ml of 5X diethanolamine (DEA) substrate buffer (pH 9.8) and 4ml of ddH2O. The substrate was then added using 100 µl/well and the plates were covered and incubated at room temperature. The plates were then read at 405nm using an ELISA plate reader at 30minutes, 1hr, and overnight.

**Immunofluorescence and Confocal Microscopy**

Cells were counted and washed two times with DPBS. They were then resuspended to 6x10^6 cells per ml. Then, 50ul (3x10^5 cells) of each cell type was pipetted
onto a 22x22-2 microscope coverglass (Fisherbrand) in a 6-well cell culture plate (Corning). The cells were then spun at 900 rpm for 5 minutes. Next, 4% PFA solution was made by diluting 16% methanol-free paraformaldehyde (Alfa Aesar) in DPBS to 4% PFA. The cells were then fixed by adding 4% PFA solutions to cover the slides. PFA was removed after 10 minutes and the coverslips were washed twice with PBS for five minutes. Blocking was then done by adding 3% BSA + 0.1% Triton for 30 minutes. Primary antibody (HLA-DR or FCRLM1) was then added using a 1:100 dilution. This was allowed to incubate overnight at 4 °C with mild rocking. The following day the wells were aspirated and then washed three times with PBS for five minutes. Secondary antibody (DyLight 488 or DyLight 594) was added to 3% BSA at a 1:100 dilution. Next 1ml of this solution was added to each well and allowed to incubate for 1 hr with mild rocking. The coverslips were then washed twice with PBS for 15 minutes and once with ddH₂O for 15 minutes. The ddH₂O was then aspirated and the coverslips were allowed to dry. A drop of Vectashield (cat # H-1500) mounting media, including DAPI, was then added to the slides and the coverslip was mounted to the slides and allowed to dry for 30 minutes. The slides were then visualized at 630x on a confocal microscope.

Surface Staining and Flow Cytometry

Both BL cell line (Ramos.DR4 and Nalm-6.DR40 as well as the two B-LCLs (Frev and 6.16.DR4.DM) were cultured for 24 hrs. The cells were then counted and resuspended in FACs Buffer (DPBS + 1% FBS) at 5x10^6 cells per ml. Next, 100ul (5x10^5 cells) were added to a 1.5 ml Eppendorf tube. anti-FCRLM1-PE N28.1 (Santa Cruz) antibody was then added at a 1:100 dilution to each sample and HLA-DR FITC was added at a 1:20 dilution. The cells were then incubated on ice for 1 hr before being
washed twice with 1ml of FACs buffer centrifuged at 1200 rpm for 5 minutes each wash. The FACs buffer was then aspirated and 500 µl of 1% PFA was added to each sample to fix the cells. The samples were then analyzed using flow cytometry on a BD LSRFortessa flow cytometer and then analyzed using FlowJo Software.

**FCRLA Depletion from BL Supernatants**

Protein A/G beads (Santa Cruz) were first equilibrated by adding 50 µl per sample to an Eppendorf tube. They were then centrifuged at 10,000 rpm for 5-10 seconds. The supernatant was aspirated and 1ml of PBS was added. The beads were spun again at 10,000 rpm for 5-10 seconds. Supernatant was then aspirated and 1-2 ml of PBS was added.

To pre-clear the samples 50 µl of equilibrated beads was added to each 1ml supernatant sample and incubated for 1hr at 4 °C on while rocking. The samples were then spun at 10,000 rpm for 5-10 seconds. The supernatant was then transferred to a new tube. FCRLM1 (N28.1) antibody (Santa Cruz cat #SC-53583) was then added at 3µg/ml and the samples were incubated overnight at 4 °C while rocking.

Protein A/G beads (Santa Cruz) were once again equilibrated as previously discussed. 20 µl of beads was then added to each sample and incubated for 1hr at 4 °C on a rocker. The samples were then spun at 10,000 rpm for 5-10 seconds and the supernatant was transferred to a new centrifuge tube. The samples were then centrifuged a second time at 10,000 rpm for 5-10 seconds. The supernatants were once again transferred to a new Eppendorf tube. The supernatants were then prepared for Western blotting by adding 25 µl of supernatant to 25 µl of samples buffer plus 1.5 µl of DTT. They were then used
for analyzed by Western blotting as previously described while the remainder of the supernatant was stored for later use in an Ag presentation assay.

**Creation of FCRLA ELISA**

Plates were coated with 1µg of rFCRLA protein in pH9 sodium bicarbonate coating buffer made using 10 ml of 0.1M Na₂CO₃ and 90ml of 0.1M NaHCO₃ or 1%BSA in PBS using 100µl/well. The plates were incubated overnight at 4 °C while rocking gently. The plate was then washed 3 times in PBS-Tween before blocking for 30 minutes using 100 µl of 1% BSA in PBS per well. The plates were washed again 3 times and 100 µl of anti FCRLM1 N28.1 antibody (Santa Cruz) using a 1:100 dilution in 1% BSA. The plates were incubated on shaker for 2 hours and the washed once again 3 times. 

Secondary anti-mouse antibody conjugated with AP was then added using a 1:2500 dilution in 1% BSA. The plate was then incubated for 1 hr while shaking and the plate was again washed 3 times. PNPP substrate was then added at 100 µl/well before the absorbance was read at 405 nm.

**Isolation of B-cells and Creation of B-cell Libraries (Patent Number: 8,715,74)**

Venous blood from healthy patients is first drawn into syringes containing heparin sodium to prevent coagulation. The 500 ml peripheral blood sample collected in this experiment was then diluted 1:1 by adding 500ml of DPBS after which the sample was centrifuged on a Ficoll cushion. The boundary layer containing the desired B-cells was then extracted and washed before being resuspended at 2x10⁷ cells per ml in Ca⁺⁺/Mg⁺⁺-free DPBS with 2% FBS in a round bottom polystyrene 5ml tube. Next, 1ml of B c-ell enrichment kit antibody cocktail was added per ml of cells and gently pipetted to mix. The cells were then allowed to incubate for 10mins at room temperature. Then, 0.1 ml of
EasySep magnetic beads per ml were added and mixed gently by pipetting. Once again the cells were incubated for 10 minutes at room temperature before adding 4 ml of DPBS/2% FBS to the mixture. The tube was then exposed to a magnet and incubated for 5 minutes. B-cells were then transferred to a 15 ml tube by gently pouring without disrupting the beads. Cells were counted and pelleted at 1600 rpm for 8 minutes. Next, 1 ml of 10X concentrated B95-8 EBV supernatant was added per $10^7$ cells and 1ml of the cells and virus mixture was added per well of a 6 well culture plate. Cells were then spininfected at 900g for 1-2 hr at 20 °C before being cultured overnight at 37 °C.

The next day, the cells were plated in 10 96-well plates at concentrations ranging from $10^4$ to $2\times10^5$ cells per well in complete RPMI media. In order to induce B-cell differentiation and immunoglobulin class switching, cytokines and other signaling agents are added to the cells. The agents added were recombinant human BAFF at 1ng/ml, recombinant human soluble CD40L at 5ng/ml, and goat anti-human IgM Fab’2 antibody at 1.4 µg/ml. Immunoglobulin isotype class switching is ongoing approximately 5-10 days after agents are added. Screening begins 10-14 days after Immortalization.

**FCRLA Antibody Screening ELISA**

The wells of a 96 well high binding culture plate were coated with 1µg/ml of rFCRLA using 100 µl/well in sodium bicarbonate (pH 9.0) coating buffer overnight at 4 °C. The plate was then washed 4 times with 200 µl/well of 1X PBS 0.05% Tween-20 before adding 200 µl/well of blocking buffer (1% BSA in 1X PBS) and blocking for 30 minutes. The wells were once again washed 3 times in PBS-Tween and B cell library supernatant sample well pools were added at 100 µl/well. There was also 3 wells without any sample added as well as 3 wells with 100 µl of anti-FCRLM1 28.1 (Santa Cruz) at a
dilution of 1:100 in blocking buffer using 100 µl/well. Plates were incubated for 1 hr at room temperature while shaking at 450 rpm. Plates were washed again 4 times and a detection anti-human IgG AP conjugate (Southern Biotech) was added at a dilution of 1:2500 using 100 µl/well. The plate was allowed to incubate for 1 hr at room temperature while shaking at 450 rpm. The plate was washed once again 4 times before adding AP substrate at 100 µl/well. Finally, the plate was read at OD 405.
CHAPTER 5: Summary and Future Directions

Until recently, there was little information available about the FCRL protein family, but the progress being made is beginning to show their critical roles in the human immune system. Their cellular expression and structural components highly suggest FCRLs show a strong potential for immune regulation primarily in B-cell populations, but also in other subsets of immune cells. Thus far the FCRL proteins have been implicated in both adaptive and innate immune functions; however, much more is still unknown about their functionality. Their roles in the immune system reveal themselves in their association with lymphoproliferative diseases, autoimmunity, cancer, as well as chronic viral diseases. This suggests that these proteins may be bio-markers and/or targets useful for diagnosis, prognosis, as well as treatment for these diseases. All of this supports FCRLA could play a role in the inhibition of HLA class II mediated activation of CD4+ T-cells.

While survival rates for NHL remains fairly high, aggressive subtypes still have poor outcomes. BL is one of the most aggressive subtypes of NHL and current treatments are extremely intensive. Chemicals from chemotherapy drugs often cause tumor lysis syndrome making it difficult to treat many patients. Therefore, a non-toxic treatment for these patients could greatly increase survival rates. FCRLA protein could be a viable protein target for immunotherapy of B-cell lymphomas which would provide a non-toxic treatment for these patients.

Results of Ag presentation assays in this thesis demonstrate that there is a secretory protein in BL supernatants that inhibits functional Ag presentation by normal B-LCLs. In this case, B-LCLs treated with BL supernatants significantly inhibited functional Ag presentation when compared to cells treated with a B-LCL supernatant.
(Figure 5). This suggests that there is a molecule secreted by BL cells that could inhibit antigen presentation by B-LCLs. Further investigation through Western blotting showed that a secreted 49 kDa form of FCRLA exists in BL cell lines but not in B-LCLs (Figure 3) which indicates that this FCRLA molecule could potentially be the inhibitory molecule secreted by BL cells. Furthermore, functional Ag presentation assays were done treating with secreted FCRLA that was isolated by chromatography or rFCRLA protein (obtained from commercially available vendor) (Figures 6 and 8). In both cases, the FCRLA positive fraction and the rFCRLA protein significantly inhibited Ag presentation by B-LCLs when compared with the FCRLA negative fraction or vehicle treated cells. These suggested that FCRLA was acting on BL cells to inhibit antigen presentation. Further investigation was done to determine whether FCRLA was acting on Ag presentation or if it was directly acting on T cell stimulation. This assay was performed in a way where some wells were coated with anti-CD3 before added cells treated with the FCRLA positive or negative fractions (Figure 7). This assay demonstrated that FCRLA did not inhibit T cell stimulation when they were stimulated by anti-CD3, but they continued to inhibit peptide Ag presentation when T-cells were not stimulated by anti-CD3. These data suggested that the secreted 49 kDa form of FCRLA found by Western blotting, was acting specifically on antigen presentation and not by directly affecting T cell stimulation.

To investigate whether FCRLA acts on Ag presentation by binding with HLA-DR, co-immunoprecipitation assay was performed in which FCRLA was pulled down followed by immunoblotting for HLA-DR (Figure 10). In this case, it was found that FCRLA was successfully pulled down from the supernatants. Furthermore, HLA-DR also
appeared to be pulled down from the BL lysate suggesting that FCRLA may bind with HLA-DR. However, there was also a large amount of background bands that were detected, particularly around the 49 kDa area, making it difficult to make any definitive conclusions. Furthermore, I was only able to see HLA-DR in BL lysates and not in the BL supernatants. Therefore these data support the hypothesis that FCRLA was binding with HLA-DR to inhibit antigen presentation by B-LCLs, but did not serve as conclusive proof. Further investigation was carried out by immunofluorescent staining for FCRLA and HLA-DR in B-LCL and BL cells (Figure 11). These cells were then visualized by confocal microscopy and it was found that FCRLA did appear to colocalize with HLA-DR in the BL cells while it did not appear to colocalize nearly as much in the B-LCL. These data were correlative with what we found in the co-immunoprecipitation experiments and also supported the idea that FCRLA could bind with HLA-DR. Surface staining was also performed on these cells and analyzed by flow cytometry. In this case we expected the results to show colocalization of HLA-DR and FCRLA on the surface of BL cells, but not on the B-LCL. However, staining with FCRLA appeared very weak while HLA staining was very bright. Therefore, with the flow cytometry, there was no convincing data for colocalization. There was, though, positive staining in the BL cells and not in the B-LCLs when comparing MFI values. This suggests that FCRLA is present on the surface of the BL cells and might potentially bind to HLA-DR (Figure 12).

Studies were also carried out to determine whether depleting this secreted form of 49 kDa FCRLA would restore Ag presentation. Depletion of FCRLA from BL supernatants using FCRLA antibodies and protein A/G beads was performed (Figure 1). When analyzed by Western blot, it was found that FCRLA was, in fact depleted from
these supernatants. These supernatants were then used to treat a B-LCL in an Ag presentation assay and it was found that Ag presentation was restored when treating with the depleted supernatants when compared with the pre-depletion supernatants. These data imply that the secreted 49 kDa form of FCRLA could be used as an effective target for an antibody based immunotherapy against BL because antibody depletion restored antigen presentation.

Attempts were made to isolate a fully human monoclonal antibody against the secreted 49 kDa form of FCRLA produced by BL (Figures 13 and 14). This was done by isolating B-cells from peripheral blood samples and transforming these cells with EBV to create immortalized B cell libraries. The cells were then subject to an “in vitro germinal” center which induces class switching from IgM to IgG. These libraries were then screened for anti-FCRLA antibodies using an FCRLA screening ELISA that was developed in Dr. Sutkowski’s laboratory. While FCRLA reactive antibody was initially detected, further study was curtailed due to a bacterial contamination. However, these studies are ongoing, and if an antibody against FCRLA could be produced, it could be used in immunotherapy against BL and other B cell lymphomas.

Going forward there is a number of studies that could be done using this secreted form of FCRLA. First, a knockdown experiment could be done to knockdown the FCRLA protein expression in BL cell lines. This could be used to determine if Ag presentation could be restored and also might provide a stable cell line for other studies. Additionally, a more in depth study of how this secreted form of FCRLA interacts with HLA-DR could be done.
Moreover, the FCRLA screening ELISA that was developed could be used in screening samples for FCRLA. Since, this 49 kDa secreted form of FCRLA is only present in B-cell lymphoma cells; it could be considered a biomarker for some of these cancers. Therefore, this ELISA screening method could be used as a diagnostic method for BL and other B-cell lymphomas. Immunocompromised or weak NHL patients often do not have many options for treatment. A secreted 49 kDa form of FCRLA is a protein that is secreted by BL cells and inhibits functional HLA class II Ag presentation. This prevents there from being a sustained response by the immune system against cancerous cells. Therefore, an immunotherapy targeting FCRLA could provide a nontoxic treatment that could be very effective in treating BL and other B-cell lymphomas.
CHAPTER 6: REFERENCES

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