Evaluation of Antibody-based Therapeutics in B cell Malignancies

DISSERTATION

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Abstract

Monoclonal antibodies therapy for B cell malignancies evolved vastly over the past few decades. Since its approval as the first therapeutic antibody for cancer, rituximab has improved outcome in virtually all CD20+ malignancies it was tested. However, despite its successes, most patients become resistant to rituximab therapy and novel therapeutic targets and agents are needed. Intelligently engineered antibodies and antibody-based protein therapeutics for the treatment of leukemia have newly emerged. These engineered variants enhance various mechanisms of action of antibodies. They include the next generation CD20-directed antibodies, as well as therapies directed at new therapeutic targets such as CD19 and CD37 for Acute Lymphoblastic Leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL). Understanding the benefits and properties of these engineered variants is vital before they can be moved to the clinic. XmAb-5574 is an amino acid engineered anti-CD19 antibody that mediates superior NK cell-dependent Antibody-dependent Cellular Cytotoxicity (ADCC) against ALL and CLL cells as compared to the currently utilized anti-CD20 and anti-CD52 therapies. SMIP-016\textsuperscript{GV} is a glyco-engineered novel mono-specific protein therapeutic against CD37 that mediates superior NK cell-mediated ADCC at low concentrations and low antigen density. It represents a second generation molecule that is more potent than the parent compound against both CLL and ALL patient cells. Finally, the next generation of anti-CD20 antibodies are coming in to the clinic and are engineered for specific functions. Ofatumumab has enhanced complement activation, while GA101 is a novel Type II antibody that is Fc engineered. GA101 shows enhanced activation of NK cells and ADCC. However, it shows diminished activation of monocytes, as well as decreased Antibody-dependent
Cellular Phagocytosis (ADCP) and monocyte/macrophage cytokine release. In addition, it shows decreased recruitment of FcγRIIa to signaling lipid rafts. These results question the importance of specific types of engineering and which cells are the most important for therapeutic antibody efficacy in vivo. The newly emerging therapies described above are adding to the armamentarium available to clinicians of specific biological therapies against B cell malignancies and giving insight into the mechanism of action of therapeutic antibodies.
Dedication

This thesis is dedicated to my parents.
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**Fields of Study**

Major Field: The Integrated Biomedical Science Graduate Program

Immunology, Cancer Biology
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Chapter 1: **Background and Introduction**

1.1. B cell Malignancies and Antibody-based Therapy

Monoclonal antibody therapy has revolutionized the treatment of cancer and represents one of the fastest growing classes of therapeutics [1]. There are numerous advantages to antibody-based therapy over traditional chemotherapy, one of the most important advantage being that antibodies can have more specific action against cancer cells with low “off target” effects to other cells in the body. In particular, antibody therapy has proven to be advantageous in B cell malignancies, where it has improved outcome in many patients.

1.1.1 Chronic Lymphocytic Leukemia

Chronic Lymphocytic Leukemia (CLL) is the most common type of adult leukemia, with about 15,000 new cases reported every year and an estimated 4,400 deaths per year [2]. Although predominantly seen in adults over the age of 70, it can also be found in younger adults with 10% of individuals being diagnosed under the age of 50 years [3]. CLL is highly variable in terms of stage at time of onset, disease progression, response to therapy, and outcome [4]. The disease is characterized by clonal proliferation of relatively mature, well-differentiated B cells that are non-functional [4]. These abnormal leukocytes are resistant to programmed cell death in the microenvironment where they accumulate in the bone marrow, lymph nodes, and periphery [5].
CLL cells are immunophenotyped as being CD20\(^+\), CD19\(^+\)/CD5\(^+\), CD23\(^-\), and FMC7\(^-\) and as having a weak-intensity surface immunoglobulin (Ig) \([6]\) [7]. Clinical diagnosis includes having >5,000 malignant B cells/\(\mu\)L \([8]\) with most patients not requiring treatment at diagnosis. Therapy is generally administered upon disease progression, since no survival advantages were observed with early intervention \([9]\). Symptoms of disease progression include hypogammaglobulinemia, organomegaly and severe anemia, thrombocytopenia, and neutropenia due to neoplastic cell infiltration into the bone marrow \([10]\) [11]. The life expectancy of individual CLL patients is variable due to the heterogeneous nature of the disease. Some patients have an aggressive disease that can lead to death in a few months, while others have a more indolent course with survival for over thirty years \([4]\). Although there are a variety of treatments for symptomatic CLL, currently there are no therapies that are curative outside of allogenic stem cell transplantation \([12]\) [13].

In the past decade monoclonal antibodies have begun to play an increasingly important role in CLL therapy. Immunotherapy with rituximab has been a major advance in the treatment of B cell malignancies. Rituximab is a chimeric, human-mouse monoclonal antibody that was approved for use in low grade non-Hodgkins lymphoma (NHL) in 1997. It is directed against CD20, a B cell marker found on both normal and malignant cells \([13]\). Rituximab is fairly effective in NHL \([14]\) and has also improved cure rate and overall survival benefits in patients with Diffuse Large B-Cell lymphoma (DBCL), follicular lymphoma (FL), and CLL. However, rituximab is less effective in CLL as a single agent, with patients having a partial or variable response to therapy and the majority of whom will eventually develop resistance and relapse \([14]\) [15] [16]. In addition to rituximab, alemtuzumab is another monoclonal antibody currently used in the clinic. Alemtuzumab is a humanized anti-CD52 monoclonal antibody that is approved for the treatment of CLL. CD52 is found on normal and malignant B and T-lymphocytes, NK cells, neutrophils, macrophages, and monocytes \([17]\). Alemtuzumab is effective in CLL patients, but
the widespread expression of CD52 on many immune cells makes therapy with alemtuzumab highly immunosuppressive [18] [19] [20].

Combination therapies of monoclonal antibodies along with purine analog based chemotherapeutics, such as Fludarabine (F), have been shown to improve both overall and complete response rates in CLL, giving patients longer progression free survival times [12] [21] [22]. Today, rituximab is routinely used as a part of FR (fludarabine, rituximab), FCR (fludarabine, cyclophosphamide, rituximab) or rarely R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone) combination immunochemotherapy regimens that are frontline therapy in many B-cell malignancies [23]. However, overall response to combination therapy is not curative and almost all patients will eventually develop resistance to treatment [24]. The majority of current therapies are not as effective in relapsed patients [25] [26].

While rituximab has validated CD20 as a successful target for therapy in CLL the mechanisms that cause resistance to rituximab are not well understood. Despite this, rituximab remains the prototypic therapeutic antibody and new anti-CD20 antibodies are emerging with enhanced mechanisms of action. In addition, alternative B cell antigens such as CD19 or CD37 may represent superior targets for B cell depletion.

1.1.2 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a common childhood malignancy, representing 80% of all childhood leukemias [2]. Childhood ALL has a 5-year event-free survival rate that ranges between 76-86% with protocol-based therapy [27]. ALL is much rarer in adults, constituting only 3% of all adult malignancies and has worse prognosis, with a 5 year survival rate of around 40% after intensive treatment [28]. While B-cell ALL represents the majority of cases seen, T-cell lineage ALL also occurs in 15-20% of patients [29]. Both of these lineage specific ALL subtypes
can be further stratified based on the stage of differentiation of the malignant cell [30]. ALL can be cured by either extensive combination chemotherapy or allogeneic stem cell transplantation.

While there are numerous chromosomal abnormalities seen in this disease, the most common cytogenetic abnormality in adult ALL is a translocation between the long arms of chromosome 9 and 22. This t(9;22) translocation, resulting in what is referred to as the Philadelphia chromosome (Ph), fuses the BCR gene on 22q11 to the ABL gene on 9q34 and results in a chimeric gene product that is oncogenic. The incidence of Ph positive ALL increases with age and these patients historically had the worst long term survival rate. Recently, kinase inhibitors targeting the BCR-ABL oncoprotein, when used alongside chemotherapeutics, have dramatically improved CR rates to >90% [31-35]. Imatinib was the first tyrosine kinase inhibitor (TKI) discovered to inhibit BCR-ABL. It works by competitively binding to the ATP binding site of the kinase and therefore blocks autoactivation of the oncoprotein as well as phosphorylation of downstream targets. As a single agent, imatinib has a response rate of only 20-30%. Most responses are short-lived and patients develop resistance to imatinib [30]. Although combination therapy of imatinib with chemotherapeutic has shown promising results [33] and second generation TKIs are available, there is still need for alternative therapeutic options for adult ALL. The TKI therapies only help the one-quarter of adult ALL patients that are Ph positive. Furthermore, there has been little improvement in therapy and survival in patients over the age of 60.

Anti-CD20 therapy with rituximab is being explored in ALL, especially given that 60-80% of B cell ALL patients express this antigen [36] and CD20 expression may be linked to worse prognosis [37]. Rituximab has mainly been studied in combination with chemotherapy, with modest benefits observed [38] [39]. The anti-CD52 antibody alemtuzumab is also being tested in ALL [40] [19]. However, given the relatively ubiquitous expression of this antigen on
immune cells, alemtuzumab therapy may increase the rate of infections in patients [41]. Therefore, more therapies and more specific therapeutic targets are needed in this disease. One such target may be CD19, which has shown promising results in CLL [42].

1.2. Antibody structure

The functional properties of Immunoglobulins (Ig), or antibodies, are a direct result of their structure. Antibodies are globular 150 kDA protein structures that are produced exclusively by B cells. They have two functional domains (Figure 1.1). The N-terminal domain is called the Fab domain. This is the domain that recognizes amino acid sequences on the target antigen and contains the variable (V) region of the antibody. The C-terminal domain consists of the Fragment crystalizable (Fc) or constant (C) region that interacts with effector cells and complement. The Fab domain and the Fc region are connected by the hinge region which gives the antibody flexibility.

1.2.1 Heavy and light chains

Immunoglobulins are structurally composed of two identical heavy chains and two identical light chains. Each of these chains is composed of structural domains called immunoglobulin (Ig) domains. Ig domains are 70-110 amino acid sequences that are designated V or C depending on whether they are variable or constant and are numbered from amino terminus to carboxyl terminus on the antibody molecule [43]. The light and heavy chains are linked together by disulfide bonds between cysteine residues on the carboxyl terminus of the light chain and the C_H1 domain on the heavy chain. The heavy chains are also linked to each other covalently by
disulfide bonds. There are two subtypes of light chain called lambda (\(\lambda\)) and kappa (\(\kappa\)). An antibody can only have two chains of one of these types and there are no known differential functions between \(\lambda\) or \(\kappa\)-containing immunoglobulins [44]. The variable (V) regions of both chains form the antigen-recognizing Fab domain and the Fc portion is created by the constant (C) region of the heavy chain. The variable region is further divided into hypervariable sequences or complementarity determining regions (CDR) and the framework regions. There are three CDR, about ten amino acid residues long, in the variable heavy chain and three in the variable light chain that form three loops at the outer edge of the \(\beta\)–sheet barrel created by the framework regions. This region is antigen-binding and variable so that different antibodies can bind to a vast number of diverse antigens. Furthermore, since each antibody has two light and two heavy chains, they contain two identical antigen-binding domains.

1.2.2. \(V(D)J\) Recombination

The genetic mechanism leading to amino acid variability in the hypervariable regions that bind antigen in the Fab is called \(V(D)J\) recombination [44]. \(V(D)J\) recombination is what creates the variable region of natural and therapeutic antibodies that recognizes antigen. The diversity seen in the immunoglobulin variable regions is generated by the rearrangement of different variable (V) region gene segments with diversity (D) and/or joining (J) gene segments within each individual B cell. The heavy chain, \(\lambda\) light chain and \(\kappa\) light chain are all located on separate loci. Each loci has clusters containing multiple copies of the V, and J gene segments, flanked by recombination signal sequences (RSS). The heavy chain loci also has copies of D gene segments that are not present on the light chain loci. The recombination process combines random V, (D), and J segments together to form a single exon that codes for a variable region. The first step in \(V(D)J\) recombination is synapsis. This is when two selected coding segments and their adjacent
RSS are brought in close proximity by a looping event and held in position by recombination-activating gene 1 and 2 (Rag-1 and Rag-2) proteins. Rag-1 and Rag-2 are complexed to form V(D)J recombinase and this complex enzymatically cleaves at the RSS, introducing double-strand breaks. The coding ends are hence removed from the RSS and are further modified by the addition or removal of bases to add greater diversity. The broken ends are ligated together by the normal cellular machinery involved in double-strand break, non-homologous end joining (NHEJ). This process results in a vast array of diverse immunoglobulins. The diversity of the hypervariable regions lends to the success of antibodies because they are specific and cause destruction of targeted cells.

1.2.3. Immunoglobulin Subclasses

There are five classes of immunoglobulin based on heavy chain constant regions in the Fc portion. The heavy chains are named after the Greek alphabet α, δ, ε, μ, and γ, which form the immunoglobulin isotypes IgA, IgD, IgE, IgM, and IgG, respectively. The heavy chain constant region of the antibodies belonging to each class has almost identical amino acid sequence, which are different than antibodies of another class. The C region of IgM and IgE in humans contains four Ig domains, while IgG, IgA, and IgD have only three Ig domains. Most antibodies are monomers but IgA is found as a dimer and IgM is a pentamer or hexamer. These immunoglobulins are connected by joining (J) chains that are covalently linked by disulfide bonds to the tail pieces of IgA or IgM. All of these antibody isotypes are excreted by B cells except for IgD which, along with surface IgM, is found bound to the cell surface. Each immunoglobulin isotype has differential and defined functions [44]. IgA is involved in mucosal immunity, while IgE is in defense against helminthic parasites. Both IgD and IgM function in the naïve B cell antigen receptor. IgM can also activate complement. IgG has the most diverse array
of functions including opsonization, antibody mediated cellular cytotoxicity (ADCC), complement activation, neonatal immunity, and feedback inhibition to B cells. It also has the longest serum half-life (23 days) of all the immunoglobulin isotypes. The number and location of interchain disulfide bonds as well as the length of the hinge region, results in several subclasses of IgGs: IgG1, IgG2, IgG3, and IgG4. These properties, along with others, are the reason why most therapeutic antibodies are of the IgG isotype.

1.2.4. Glycosylation of Antibodies

Immunoglobulins are glycoproteins and have a diverse array oligosaccharide structures attached to the protein core [45]. Ig glycoforms are varied in the number, type and location of their sugar structures, which can be located in both the Fc and Fab portions. These glycans can be N-linked or O-linked. N-linked glycans have oligosaccharides linked to the side of chain of Asparagine (Asn) while O-linked glycans have them linked to the hydroxyl group on the side chain of Serine (Ser) or Threonine (Thr) [45]. These glycosylations pay an important role in maintaining quaternary structure and function of the antibody, including modulating both antigen and Fc Receptor binding properties. IgM, IgD and IgE are all heavily glycosylated. On the other hand, IgG contains only one oligosaccharide chain that is an N-linked biantennary sugar found most often on the conserved Asn 297 in the C_{H}2 domain [46] [47]. On a single IgG molecule, the two Asn 297 sites may be differently glycosylated [45]. These structure are vital for many of IgGs’ functions and their removal results in inability to initiate complement, abrogated binding to Fc Receptors, and conformation issues [47] [48]. Furthermore, the exact sugar composition of this oligosaccharide chain also plays a role in antibody function. For example, decreasing the fucose content of this chain can enhance effector cell-mediated function by enhancing binding affinity [49], a property that is being taken advantage of in many new therapeutic antibodies.
1.3 Mechanisms of Action of Therapeutic Antibodies

The humoral response in humans is used to fight a variety of threats against the body. Natural antibodies play a vital role in this response with their ability to recognize foreign antigens, coat foreign invaders and recruit immune cells to mediate effector functions. Moreover, antibodies provide long-term defense against antigens to which the body has been exposed to. Recombinant therapeutic antibodies are most often of the IgG subclass, with the majority of them being of the IgG1 isotype. Due to the natural properties of IgG1, there are three main mechanisms of action of therapeutic antibody. These are complement-mediated cytotoxicity, Fcγ Receptor-mediated functions, and direct apoptotic signaling and are depicted in Figure 1.2. Given that it was the first monoclonal antibody approved for use, rituximab has been one of the most widely studied therapeutic antibodies. Therefore it will serve as an example in many of the descriptions below.

1.3.1. Complement-mediated cytotoxicity

The complement system is a conserved and natural defense against invading pathogens such as microbial intruders. It is composed of numerous plasma proteins and once initiated results in quick elimination of the trigger. There are three different complement pathways based on the mode of recognition of the target [44]. The Classical Pathway is triggered by clusters of immunoglobulins bound to antigen. The Alternative Pathway is a more ancient pathway that involves the direct recognition of foreign protein patterns. Finally, the Lectin Pathway is triggered by mannose-binding lectin in plasma that recognizes microbial glycoproteins and glycolipids. These separate pathways help complement discriminate between healthy cells, cellular debris, apoptotic cells and foreign pathogens and acts accordingly.
The most relevant arm of the complement system in antibody therapeutics is the classical complement cascade pathway which is antibody-initiated. This pathway can be initiated by IgM or clusters of IgG. It is initiated when the pattern recognition molecule C1q binds to the C_{H2} domain of these Ig molecules while bound to an antigen. For humans, C_{H2} domain residues D270, K322, P329, and P331 have been implicated to be essential in this interaction [50] [51]. C1q binding initiates a non-cellular complement cascade of over 20 tightly regulated proteins C1 through C9. The antibody-bound cells are covalently tagged with activated complement protein fragments and the cascade leads to an eventual formation of a transmembrane channel on the target cell. The channel is created by C5b, C6, C7, C8, and C9 which combine to form the Membrane Attack Complex (MAC) complex in the lipid bilayer. The MAC complex forms pores in the plasma membrane that allows the free movement of water and ions in and out of the cell causing lysis.

In addition to its non-cellular functions, complement is also involved with cellular activities [52]. Complement protein fragments bind to complement receptors (CR) that are found on numerous blood cells and have varied functions. For example, opsonic complement proteins C3b, iC3b, and C4b attached to target cells interact with CR on NK cells, neutrophils, and macrophages. These receptor interactions result CR-dependent cellular cytotoxicity (CDCC), phagocytosis of opsonized particles, or enhancement of Fcγ Receptor-mediated effector function. The synergy between IgG initiated complement and FcγRs includes enhancement of FcγR-mediated effector function. Furthermore, complement protein C5a is a potent recruiter and activator of leukocytes and regulates activating and inhibitory FcγRs [53] [54] leading to the destruction of microbes by leukocytes.

Complement-dependent or mediated cytotoxicity (CDC) has been shown to play a role in rituximab therapy [55]. Evidence for this can be found in murine models, where depletion of
complement leads to a decrease in rituximab efficacy [56] [57]. In vitro, human B cells that are resistant to rituximab upregulate complement regulatory proteins (CRP) such as CD59 and CD55 [58, 59]. These negative regulators of complement are found on normal healthy cells and block complement activation [60]. Finally, the consumption of complement in CLL patients following rituximab treatment further supports the role of complement in rituximab-mediated cytotoxicity [61].

Although there is evidence that rituximab’s mode of action involves complement activation, this is limited with rituximab for a number of reasons. First, the dependence of CD20 antigen density and complement activation with rituximab has been widely reported [62] [63, 64]. CLL B cells are known to express lower levels of CD20 than normal B cells [62] [63] [64] [65] and are susceptible to shaving of the CD20 antigen after rituximab treatment [66] [67] [68]. Furthermore, exhaustion of cytotoxic effector systems with the use of high concentrations of therapeutic antibodies in diseases with high tumor burden, such as advanced CLL, indicates that this mechanism is finite and saturable [69]. Finally, the role of complement in monoclonal antibody therapy is still controversial, with some suggesting it to be fundamental for response [56] [57], contributing to antibody activity [70] [71], unnecessary in vivo, or perhaps even detrimental [72] [73] [74].

1.3.2. Fcγ Receptors-mediated Antibody Mechanisms

The second mechanism of action of therapeutic antibodies is through the recognition of Ig Fc domains by receptors on effector cells. Each antibody isotype has its own Fc receptor that recognizes it and is named after its heavy chain. For example, IgE interacts with Fce Receptors that are found on mast cells, basophils and eosinophils. In the case of therapeutic antibodies, the IgG Fc region is recognized and bound by Fcγ Receptors (FcγR). FcγR are found on the majority
of effector cells of the immune system. These receptors are important in the activation of leukocytes, as well as antibody-mediated clearance of tumor cells [75] [76]. There are three classes of FcγR found in humans, FcγRIII, FcγRII, and FcγRI, each with a distinct cell expression, role and signaling mechanism (Figure 1.3).

FcγRIIIa (CD16) is a low affinity FcγR that is predominantly expressed on NK cells [44]. After binding to an IgG Fc region, it oligomerizes with a common γ-chain molecule on the cell membrane. The common γ-chain contains an Immunoreceptor Tyrosine-based Activating Motif (ITAM) which initiates an intracellular signal transduction cascade. An ITAM motif is a conserved sequence of amino acids, usually two repeats of YxxL representing a tyrosine (Y) separated from a leucine (L) by two other amino acids, found in the cytoplasmic tail of many immune receptors. After FcγR engagement and the common γ-chain association, the tyrosine residues in the ITAM motif are phosphorylated by the src family of kinases. This tyrosine phosphorylation recruits SH2 domain containing molecules that bind to the ITAM. SH2 domain containing molecules includes the syk family of kinases which act on additional molecules to mediate a downstream signaling cascade [44]. The specific kinases involved in these processes are dependent on cell type. A variety of downstream signaling is activated including the phosphatidylinositol-3 kinase (PI-3K) and MAPK/ERK pathways. FcγRIIIa mediates ADCC through signaling that ultimately results in effector functions such as the release of inflammatory cytokine and antibody-dependent cellular cytotoxicity (ADCC) via the release of pre-formed proteins and proteases, granzyme and perforin. These molecules aid in the destruction of the target cell. Macrophages, monocytes, neutrophils, and dendritic cells also express FcγRIIIa but at very low levels. However, this may be therapeutically relevant. Macrophages and dendritic cells can infiltrate tumors [77] [72] and monocytes are important in murine systems for antibody-dependent B cell depletion [72] [78].
Murine FcγRIV corresponds to human CD16 in the sense that this receptor also needs to associate with the common γ chain in order to activate the cell. FcγRIV has been shown to be the dominant receptor involved with in vivo efficacy of anti-CD20 antibodies in murine models [72] [78] [79]. Mouse studies using γ-chain knockout mice demonstrate that clinically relevant antibodies such as rituximab are ineffective in vivo [80]. However, the expression of FcγRIV in mice is on monocytes and macrophages and not on NK cells, unlike human FcγRIIIa which is predominantly on NK cells [75].

The clinical importance of the FcγRIIIa is made evident with a natural polymorphism in humans in this gene. Expression of a valine (V) at amino acid position 158 confers relatively higher affinity to IgG1 as compared to a phenylalanine (F) at this position. Expression of the high affinity receptor is indicative of greater response to antibody therapy in lymphoma [81, 82]. However, this phenomena is not observed in CLL patients with rixuimab therapy [13], perhaps due to lower levels of CD20 expression on cells or higher levels of soluble CD20 in plasma [83].

FcγRIIIb is a product of a different gene than FcγRIIIa, although they share some structural similarities [44]. It is decoy receptor only found on neutrophils and does not trigger activation or phagocytosis.

FcγRII (CD32) is a low affinity IgG receptor that has three isoforms, IIa, IIb, and IIc, resulting from the alternative RNA splicing of the same gene [44]. These isoforms have similar structures and binding affinities in their extracellular domains, but have separate cytoplasmic structures and functions. FcγRIIa and FcγRIIc are the only Fcγ Receptors that contain their own cytosolic ITAM domain. FcγRIIa is expressed on many cell types including monocytes, macrophages and neutrophils. FcγRIIa recognizes clusters of IgG1 or IgG3 bound to antigen and is phosphorylated at its ITAM. The upstream signals are similar to FcγRIII but FcγRIIa
downstream signals lead in particular to activation of phospholipase Cγ1 (PLCγ1) which is necessary to elicit calcium release, degranulation, ROS production and activation responses in cells expressing FcγIIa [84]. Furthermore, it leads to phosphorylation of ERK, which has been demonstrated to be critical in the induction of transcription factors such as NFκB and c-fos and subsequent expression of cytokines [85]. FcγRIIa is the dominant receptor used by macrophages to phagocytose opsonized particles [86] [87]. The importance of FcγRIIa in antibody-mediated tumor cell depletion is the reason monocytes and macrophage have been implicated as being vital for in vivo anti-CD20 response [86]. A functional polymorphism within this gene at site 131 where the histidine (H) isoforms binds binds IgG2 more tightly than the arginine (R) form [88].

FcγRIIc is the product of a gene that results of an unequal crossover of the FCGR2A and FCGR2B, the genes that encode FcγRIIa and FcγRIIb, respectively. It is a low affinity receptor that also has its own ITAM domain and is expressed on macrophages, neutrophils, and NK cells and capable of mediating ADCC [89]. A SNP in exon 3 of the FCGR2C gene results in either an open reading frame or, more commonly, a stop codon and hence determines the functional expression of this gene [89].

Contrary to FcγRIIa and FcγRIIc, FcγRIIb is the only FcγR to contain an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) that signals intracellularly to downregulate cell function [90]. Similar to the ITAM, an ITIM is a conserved sequence of amino acids, S/I/V/LxYxxI/V/L in this case, that is phosphorylated by src family kinases after ligand binding. This motif recruits phosphatases like SHP-1 and SHP-2 or SHIP-1, which dephosphorylate activated molecules in the cell. FcγRIIb is the only FcγR found on B cells and inhibits B cell activation by IgG complexes. Most other immune effectors cells express FcγRIIb, including macrophages and neutrophils, but it is not present in NK cells or T cells [91] [92]. Antibody antitumor activity and B cell depletion is improved in mice that are deficient of this receptor [80] [72]. Since FcγRIIa
and FcγRIIb are often expressed on the same cells, the ratio of activating to inhibitory receptors on monocytes, macrophages, and dendritic cells may play a role in antibody efficacy [93] [94]. Coengagement of both of these receptors sets the threshold and determines magnitude of response to IgG complexes. A polymorphism in the FcγRIIb gene in the transmembrane region translates to either a isoleucine or threonine at position 232 of the protein and alters receptor signaling through exclusion from lipid rafts rather than IgG binding affinity [95]

FcγRI (CD64) is a high affinity FcγR that is found mainly on cells of monocyte or macrophage lineage, as well as neutrophils [96]. Like FcγRIII, this receptor also associates with a signaling common γ-chain to activate the cell and has similar upstream signal transduction pathways. Downstream signals include phospholipase D1 (PLD1) and sphingosine kinase 1 that trigger calcium release. FcγRI activation results in higher levels of proinflammatory mediator such as TNF-α, IL-8, IL-6, IFN-γ, G-CSF, and MIP-1B [84].

1.3.3. Antibody-mediated Direct cytotoxicity

Recombinant therapeutic antibodies can mediate direct apoptotic signals in target cells by binding surface antigen. This antibody mechanism of action is both antibody and antigen dependent. Historically, antibodies were chosen based on their ability to bind one type of cell but not others. This method of choosing therapeutic targets is somewhat random because it does not take into account the natural properties or functions of the target protein. Binding of antibody to the surface marker can mimic receptor binding/interaction or inhibit association with functional partners. The signals sent into the cell upon antibody ligation are hence antigen dependent. In vivo, direct apoptotic signaling is augmented by the clustering of antigen and signaling molecules by Fcγ Receptors. [97].
For example, rituximab triggers a variety of direct inhibitory signals into CD20+ B cells. Rituximab inhibits the B cell receptor signaling cascade both proximally and distally in vitro, interfering with the activity of Lyn, Syk, PLCγ2, Akt, and ERK, as well as calcium mobilization [98]. Therefore, it targets the constitutive activation through the B cell Receptor (BCR) in the absence of receptor engagement, or “tonic” BCR signaling, that is a problem in many leukemias and lymphomas [99]. Rituximab is able to do this because its target antigen CD20 is known to associate [100] and cooperate [101] with BCR. Furthermore, rituximab treatment can lead to a variety of downstream effects in target cells such as altered gene expression leading to growth arrest, sensitizing cells to chemotherapy-induced death [102], or apoptosis through Bcl-2 family proteins and mitochondrial outer membrane permeabilisation. Rituximab loses efficacy in xenograft mouse models overexpressing the anti-apoptotic protein Bcl-2, indicating the importance of Bcl-2 in efficacy [103]. However these results were not confirmed in CD20/Bcl-2 transgenic mouse [84]. The detection of activated caspase-3 and caspase-9 in CLL patients after rituximab treatment verifies that programmed cell death activity is occurring in vivo [104].

All therapeutic antibodies do not signal in the same manner as the CD20-directed rituximab. The anti-CD52 antibody alemtuzumab shows more potent direct cytotoxicity against CLL B cells in vitro. Although its exact mechanism of action is poorly understood, it is known to directly signal apoptosis in cells independent of caspase activation and p53 status [105]. Similarly, the direct mechanism of anti-CD19 antibodies is not known but it may work through caspase activation [106] and inhibition of BCR signaling [107] . Therefore, selection of therapeutic target can play an important role in how successful an antibody will be in initiating programmed cell death.
1.4. Engineering antibodies to enhance their function

Over past decade, engineering therapeutic antibodies to enhance or decrease their natural effector functions has been an area of intense research. Major advances have been made in the knowledge of antibody structure and function and how to manipulate these to obtain more effective therapeutic antibodies.

1.4.1. Whole Molecule Engineering

Monoclonal antibodies were first produced and isolated using hybridoma technology. This involves injecting mice with the human antigen of choice and allowing the mouse to generate anti-human antibodies. Spleen cells from these mice are harvested and fused with a mutant myelomas line that does not produce antibody. After selecting for fused cells, the cells are cloned out and screened for clones producing a monoclonal antibody against the original antigen.

Using this method, the first antibodies generated for therapeutic purposes were mouse antibodies. These antibodies elicited the formation of human anti-mouse antibodies in patients. Therefore, chimeric antibodies, such as rituximab, were generated with mouse variable regions and human constant regions. These chimeric antibodies were still antigenic. Finally, highly humanized, recombinant antibodies were engineered by replacing the CDRs on human antibodies with the antigen specific mouse CDRs. These antibodies are mostly produced in mammalian cell lines and often have the same immunogenicity as entirely human antibodies (reviewed in [60]).

CD20-directed antibodies can also be made and classified by how they induce direct B-cell lysis. This distinction has led to the classification of antibodies as Type I or Type II antibodies. Type I antibodies are rituximab like in their mechanism of action. They mainly act through FcγR mechanisms and by stabilizing CD20 into lipid raft leading to stronger initiation of
complement [108]. On the other hand, Type II antibodies do not stabilize CD20 to lipid rafts, thus having reduced complement initiation, but instead work mainly through effector mechanisms and programmed cell death that is caspase independent. The most clinically relevant Type II antibody is the CD20-directed GA101 (obinutuzumab). During the humanization process of GA101, different hinge sequences in the variable region were considered to achieve enhanced cytotoxicity and Type II antibody properties [109].

Entirely engineered antibody-like proteins and derivatives are also emerging as new biological therapies. Fv fragments are noncovalent heterodimers of the variable light and heavy chains. They are stabilized by a hydrophilic flexible linker to generate a single chain (sc) Fv [110]. ScFv-Fc fusion proteins (scFv-Fc) are single chain peptides with Fc function. They are generated by fusing scFv fragments to the hinge-CH2-CH3 domain of different antibody isotypes to produce SMIPs (Monospecific Protein Therapeutic) [99] and other scFv-Fc molecules [111]. Due to their simple design, they can be produced and purified easily. Functionally, these proteins retain both functional domains of whole IgG molecules, with specific antigen binding and Fc-mediated properties and hence behave like complete IgG molecules in many ways. These protein therapeutics are often of lower molecular weight than IgG molecules and are therefore hypothesized to have better tissue and tumor penetrance, however there is currently no data supporting this.

1.4.2 Engineering to enhance complement.

Numerous different methods are being utilized to generate antibody variants with enhanced ability to initiate complement. One method takes advantage of the natural differences in CDC activity among human IgG isotypes, where complement binds IgG3> IgG1>>IgG2 = IgG4 [112], by swapping segments between isotypes to generate chimeric IgG molecules that have enhanced
complement recruitment [113] [114]. A second method is to screen antibody variants for C1q binding and then engineer 1-3 specific amino acid substitutions at the hinge region [115] or C\textsubscript{H}2 domain of IgG1 and look for enhanced C1q binding and CDC activity [50] [116]. Examples of this include substitutions at Fc position 326 and 333, as well as 267, 268, and 324 [117]. Finally, the complement-activation properties of antibodies can be enhanced by using site-directed mutagenesis to add amino acid substitutions known to have enhanced complement function. Studies in nonhuman primates have validated that antibodies enhanced for complement activity improve B cell depletion in vivo [114].

1.4.3. Engineering to enhance Fc function.

Glycoform perturbation is one method used to enhance Fc-mediated therapeutic antibody function. As described in section 1.2.4, the N-linked Fc glycosylations on IgG1 antibodies are important for effector function [118]. Sialylation, galactosylation, bisecting sugars, and fucosylation all affect binding and activity of IgG molecules [119] [120] [121] [122]. Removal of these biantennary sugars at amino acid 297 diminished Fc\textgamma R binding and ADCC [123] [124] [125]. However, altering the glycoforms present at this location can improve Fc-mediated antibody functions. For example, removal of fucose within the glycoforms present on IgG and scFv-Fc molecules has been shown to enhance Fc\textgamma RIIIa binding and NK cell-mediated ADCC [126] [127] [128]. Removal of the Fc fucose is hypothesized to enhance Fc\textgamma RIIIa binding by decreasing steric strain at the binding interface of the antibody and Fc receptor caused by the presence of a carbohydrate on Fc\textgamma RIIIa and the fucose on Fc region [129].

Controlling the glycosylation patterns on therapeutic antibodies can be done a number of different ways. The type of cell producing the recombinant antibody and its culture conditions affect glycosylation and activity of therapeutic antibodies [45]. Furthermore, bioreactor
conditions and downstream processing can also affect the glycan microheterogeneity. As mentioned in the previous paragraph, low or afucosylated antibodies have enhanced Fc-mediating properties. There are numerous ways to achieve this reduction of fucose levels by glycoengineering. One way is to manipulate the enzymes involved in the post-translational modification of antibodies. This can involve overexpression of glucosadases, such as β-1-4-N-acetylglucosaminyltransferase III [130], knocking out fucosyltransferases [131] [132], or using cell lines that are naturally fucose-deficient or have been mutated to express low fucosylation levels, i.e. Lec13 cells [133]. In addition, inhibitors of N-linked glucosadases such as castanospermine [134] have also been used to obtain low fucose bearing IgG molecules.

Alternatively, FcγR binding can also be enhanced by amino acid engineering. This is done by substitution of amino acids in the Fc region. Desirable mutations are determined by either alanine scanning [135] or rational design and library screening [136]. Using these technologies, many IgG variants have been identified with enhanced binding to FcRs and enhanced effector function [135] [133] [136] [79]. For example, the S239D/I332E double substitution has been shown to enhance binding to both isoforms of FcγRIIIa by 40-fold and functionally enhance ADCC/ADCP [117]. Amino acid engineered variants tend to have more broadly enhanced affinity for multiple FcγR, where glycoform engineered antibody are generally more specific for enhanced FcγRIIIa binding [137]. Amino acid engineering may also be changing the glycoforms attached to IgG [45]. Glycoforms interact with proximal amino acids on the Fc portion and replacement of the amino acid that come in contact with Ig oligosaccharides results in different glycoform structures attached to the Asn 297 [45]. Finally, scFv-Fc molecules with both glyco- and protein-engineering have been created and the combination of these Fc modifications results in synergistic enhanced binding affinity to FcγRs [128].
In vivo, there have been limited studies suggesting the success of these Fc engineered variants. This is predominantly because mouse FcγRs are not identical to human receptors and therefore these enhancements may not be seen with them. However, there have been studies done with FcγR transgenic mice showing inhibition of tumor growth with the variants [79] [138]. Furthermore, limited studies in non-human primates have shown that Fc modified antibodies mediated greater drops in B cell count than non-engineered antibodies [139] [136]. Recently a new mouse model has been described where all murine FcγRs have been deleted and replaced with functional human FcγR expression that are functional [140]. Detailed studies in this model may elucidate the in vivo benefits of Fc engineered antibody variants.

1.5 Significance

Antibody and antibody-based therapies are emerging as a billion dollar market not only for cancer therapy but also autoimmune disorders. Much effort has been spent in the past few years to engineer more potent and effective therapeutic antibodies. Understanding the benefits and properties of specific types of engineering is vital in moving forward with these therapies in the clinic.


49. Shinkawa, T., et al., *The absence of fucose but not the presence of galactose or bisecting N-acetylgalcosamine of human IgG1 complex-type oligosaccharides shows the critical..."


1.7 Figures

Figure 1.1. Immunoglobulin structure. From Fig. 34-18 of Voet & Voet, 2nd Edition
Figure 1.2. Mechanisms of Action of Therapeutic Antibodies. From Jaglowski et al. Blood 2011 vol.116 no.19 3705-3714
Figure 1.3. Human activating and inhibitory Fcγ Receptors.
Chapter 2: **Fc-Engineered anti-CD19 antibody XmAb-5574 demonstrates superior antibody-dependent cellular cytotoxicity as compared with CD52- and CD20-targeted antibodies in adult acute lymphoblastic leukemia cells**

2.1 Introduction

Acute lymphoblastic leukemia (ALL) has an age-adjusted incidence rate of 1.7 per 100,000 men and women per year with 40% of total cases occurring in adults[1]. ALL is risk stratified based upon genetic features and lineage (B-cell versus T-cell). It is traditionally treated based upon lineage (B versus T) and also in part based upon the cytogenetic or molecular features of the disease (reviewed in [2]). For virtually all types of ALL this treatment includes intensive chemotherapy sometimes accompanied by specific tyrosine kinase inhibitors (BCR-ABL+ ALL) and/or allogeneic stem cell transplant for ALL patients at highest risk of relapsing. Whereas these intensive treatment approaches have greatly improved outcome for children with ALL, adults with this diagnosis have a higher frequency of toxicity with therapy and significantly lower frequency of cure. ALL patients who relapse following receipt of intensive chemotherapy and/or allogeneic transplant have an extremely poor prognosis with survival measured in terms of months[3]. Despite these grim statistics, few new therapies have come forward that have greatly influenced outcome in this disease. Identifying such therapies represents a high priority.
One form of therapy that has greatly influenced outcome in other forms of B-cell malignancy has been CD20 therapeutic antibodies (reviewed in [4, 5]). Rituximab, a chimeric anti-C20 antibody, has been shown to prolong survival in virtually all mature B-cell malignancies in which it has been tested. Several recent small studies with CD52 (alemtuzumab) or CD20 (rituximab) directed therapy have been incorporated as single agents in relapsed patients or into traditional therapy for ALL, and have demonstrated some evidence of efficacy prompting several ongoing larger studies [6-8]. Both CD52 and CD20 are variably expressed on ALL cell blasts at modest copy numbers making these less ideal for tumor directed antibody therapy [9] [10] [11]. Contrasting with this, CD19 is expressed at a very early pro-B phase of B-cell development and is expressed uniformly on virtually all B-ALL cases. Recently, several anti-CD19 antibodies that are engineered either by directed mutagenesis of the Fc binding domain (XmAb-5574)[12, 13] or by afucosylation[14, 15] thereby enhancing their FcγRIIIa binding affinity and antibody dependent cytotoxicity, have been reported. We previously have shown that the Fc mutated antibody XmAb-5574 mediates superior ADCC in vitro against primary CLL cells [13]. Given the uniform expression of CD19 on B-ALL cells, we sought to compare this antibody to other CD20 and CD52 antibodies currently under study in this disease. Our data demonstrate that XmAb-5574 mediates robust ADCC and modest direct killing with a cross-linking antibody against primary ALL cells, as compared to all the CD20 and CD52 directed antibodies, making it an ideal candidate for future clinical trials in this disease.
2.2 Materials and Methods

2.2.1 Patient sample processing and cell culture

Blood was obtained from patients with informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the Institutional Review Board of The Ohio State University (OSU). All patients examined in this series had immunophenotypically defined B-ALL. ALL cells were isolated from freshly donated bone marrow with Ficoll density gradient centrifugation (Ficoll-Plaque Plus, Amersham Biosciences, Piscataway, NJ). Isolated cells were incubated in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 2mM L-glutamine (Invitrogen, Carlsbad, CA), and 56 U/mL penicillin/56 µg/mL streptomycin (Invitrogen) at 37°C in an atmosphere of 5% CO₂. Normal cells were obtained from Red Cross partial leukocyte preparations, and natural killer (NK) cells were negatively selected with Rosette-Sep kits (StemCell, Vancouver, BC, Canada) according to the manufacturer’s instructions. The purity of enriched populations of normal cells was routinely checked with the use of CD19 and CD56-PE staining by flow cytometry.

2.2.2 Antigen Quantification by flow cytometry

Quantitative analysis of CD19, CD20, and CD52 surface density was done using the Quantum Simply Cellular kit (Bangs Laboratories, Fishers, IN), according to the manufacturer's instructions. XmAb-5574, ofatumumab, and alemtuzumab were directly labeled with Alexa Fluor® 488 4-SDP Ester (Invitrogen) according to manufacturer’s instructions. FACS analysis was performed using a Beckman Coulter FC500 cytometer. Ten thousand events were collected for each sample. Data were acquired in list mode and analyzed using CXP Analysis Software (Beckman Coulter, Indianapolis, IN).
2.2.3 Assessment of apoptosis by flow cytometry

The apoptosis of cells was measured using annexin V-FITC/PI staining followed by FACS analysis according to the manufacturer’s protocol (BD Biosciences) as described previously [16]. Results are presented as percentage alive normalized, which is defined as (% annexinV⁻ and/or PI⁻ negative cells of treatment group) / (% annexin V⁻ and/or PI⁻ negative cells of media control) x 100. FACS analysis was performed using a Beckman Coulter FC500 cytometer (Beckman Coulter). Ten thousand events were collected for each sample and data were acquired in list mode.

2.2.4 Antibody-dependent cellular cytotoxicity (ADCC) assay

ADCC activity was determined by standard 4-hour ⁵¹Cr-release assay as previously described [13]. Briefly, ⁵¹Cr-labeled target cells (5x10³ cells/well of ALL cells) were incubated 30 minutes with 10 µg/ml of individual antibodies. Effector cells (NK cells from healthy donors) were then added at the indicated effector-to-target (E:T) ratios. After 4-hour incubation, supernatants were removed and counted on a Perkin Elmer Wizard gamma counter. The percentage of specific cell lysis was determined by: % lysis = 100 x (ER – SR)/(MR – SR), where ER, SR, and MR represent experimental, spontaneous, and maximum release, respectively.

2.2.5 Statistics

Data were analyzed by mixed-effect models, accounting for observational dependencies among various treatments. Holm’s method was used to adjust multiplicity for primary end points.
2.3 Results and Discussion

2.3.1 Surface antigen density on ALL cells and direct cytotoxicity

We first sought to determine the expression of target antigens CD19, CD52, and CD20 on primary adult ALL blast cells. As shown in Figure 2.1A, ALL patients express similar proportion of CD20 (80,000 average molecules/cell, ranging from 30-130,000) and CD19 (100,000 average molecules/cell, ranging from 60-150,000) on the cell surface with moderate variability among patients. In contrast, CD52 expression across all of the ALL cells was more varied, with an average of 400,000 molecules per cell, with a range of 40-500,000. However, the mean difference in surface antigen levels between CD19 and CD20 or CD52 is not statistically significant.

We next wanted to determine the relative efficacy of different antibodies targeting CD20, CD19, and CD52 on CDC and direct killing against primary ALL cells. No CDC was observed with any of the antibodies tested (data not shown). In a similar manner, CD20 targeted antibodies did not mediate cell death against ALL cells with (Figure 2.1B) or without (data not shown) an anti-Fc cross-linking antibody. This contrasts with alemtuzumab, the non-engineered XENP-5603 and engineered XmAb-5574 which promote modest direct killing in the presence of an anti-Fc cross-linking antibody. These data suggest that CD19 and CD52 directed antibodies mediate direct cytotoxicity against primary ALL cells, whereas CD20 directed antibodies do not.

2.3.2 XmAb-5574 mediates superior Antibody-dependent Cellular Cytotoxicity

Given the importance of NK cell mediated ADCC in other systems, we studied the ADCC potential of the anti-CD19 antibodies. XENP-5603 shows ADCC activity against ALL target
cells but the engineered XmAb-5574 shows significantly enhanced activity at all effector-to-target ratios tested (p <0.0001) (Figure 2.2A). Finally, we wanted to expand on the data in Figure 2.2 and determine if CD52, C20, and CD19 antibodies have differences in ADCC mediated toward ALL blast cells. Figure 2.2B demonstrates that the non-engineered XENP-5603 antibody directed at CD19 mediates greater ADCC as compared to rituximab, ofatumumab, and alemtuzumab. Furthermore, we again validated that ADCC was dramatically increased toward ALL blast cells with the Fc engineered antibody XmAb-5574. XmAb-5574 mediates significantly superior ADCC against ALL blast cells than alemtuzumab, rituximab, and ofatumumab at all effector-to-target ratios tested (p <0.0001 for all). These studies suggest that XmAb-5574 mediates significant and superior ADCC against ALL cells as compared to other CD20 and CD52 antibodies currently under clinical investigation for this disease.

Herein we have demonstrated that XmAb-5574, an Fc region engineered antibody, mediates potent ADCC toward human ALL cells at low effector to target cell numbers and modest direct killing of these cells with a cross-linking antibody. This effect is dramatic as compared to different CD20 antibodies that are FDA approved. Additionally, superiority is demonstrated for ADCC and direct killing when XmAb-5574 is compared to alemtuzumab, an antibody targeting CD52. Notably rituximab and also alemtuzumab have demonstrated some evidence of clinical activity in ALL. The finding that XmAb-5574 mediates significantly better effector cell mediated killing than these antibodies raises promise that this new antibody will have clinical activity in the disease.
2.4 Conclusions

Previous attempts to target CD19 with therapeutic antibodies or immune toxins have been unsuccessful. However, recent reports have suggested that a bispecific single chain antibody Blinatumomab has impressive activity in several different CD19 positive malignancies by redirecting T cells (via CD3) to CD19 bearing targets. In particular, blinatumomab was shown to be effective in eliminating minimal residual disease from 16 of 21 patients with CD19 positive ALL [17]. Other studies have shown blinatumomab has significant activity in different types of CD19+ B-cell malignancies including ALL [18, 19]. XmAb-5574 has been shown to mediate enhanced recruitment of FcγRIIIa, FcγRIIa and FcγRIIb bearing effector cells thereby enhancing ADCC mediated killing of CD19 bearing tumor cells[12, 13, 20]. While XmAb-5574 is predicted to efficiently recruit NK cells, monocytes, and macrophages to the ALL tumor cells, one might hypothesize that similar benefit observed with blinatumomab may be seen in the settings of both MRD and also active ALL. These data support phase I/II studies with XmAb-5574 in adult ALL.
2.5 References


Figure 2.1 Surface antigen density on ALL cells and direct cytotoxicity. (a) Alexa-488-labeled alemtuzumab, ofatumumab or XmAb-5574 used to quantify the surface levels of CD52, CD20 and CD19, respectively, on primary ALL patient samples by flow cytometry (n = 9). (b) ALL cells treated with 10 mg/ml of each antibody with 50 mg/ml of goat anti-human anti-Fc cross-linking antibody for 24 h and viability assessed by flow cytometry (n = 4).
Figure 2.2. XmAb-5574 mediates superior ADCC. (a) XmAb-5574 mediates enhanced ADCC compared with XENP-5603 against ALL patient cell targets with normal NK cell effectors (n=7 ALL patient cell targets with three NK cell effectors each) with 10 mg/ml of antibody. (b) XENP-5603 mediates superior ADCC compared with anti-CD20 and anti-CD52 therapeutic antibodies against ALL patient cell targets with normal NK cell effectors (n = 2 ALL patient cell targets with three NK cell effectors each) with 10 mg/ml of each antibody.
Chapter 3: Glycovariant Anti-CD37 Monospecific Protein Therapeutic exhibits enhanced

Effector cell mediated cytotoxicity against Chronic and Acute B-cell Malignancies

3.1 Introduction

CD37 is a tetraspanin transmembrane family protein that is expressed on the surface of mature, immunoglobulin-producing B cells [1] but not in CD10+, CD34+ and CD34- B cell precursors found in the bone marrow. Surface CD37 expression becomes strong in CD10- mature B-lymphocytes and its expression further increases as the B-lymphocytes continue to mature and move into the lymph nodes and peripheral blood. Finally, surface CD37 expression is lost in terminally differentiated plasma B cells [2, 3]. CD37 is also highly expressed on the surface of transformed mature B-cell leukemia and lymphoma cells but not on myeloma cells [3]. CD37 is dimly expressed on T cells, monocytes, and granulocytes and is not expressed on the surface of natural killer (NK) cells, platelet and erythrocytes [1] [2]. This limited expression makes it an ideal therapeutic target in B cell malignancies[2] such as Chronic Lymphocytic Leukemia (CLL) and Acute Lymphoblastic Leukemia (ALL).

CD37 was first examined as a potential therapeutic target in the late 1980s. Radio-labeled mouse monoclonal antibodies against CD37 were studied in B-cell lymphoma patients and were shown to produce anti-tumor responses [4] [5] [6]. However, due to the perceived targeting potential of CD20, CD37 as a therapeutic target was not further developed until recently with an
engineered monoclonal antibody mAb 37.1 that has been shown to be effective in preclinical models of B cell malignancies [7]. Furthermore, our laboratory has shown that a novel protein therapeutic directed against CD37, SMIP-016 induces more apoptosis in CLL B cells than rituximab [8] in vitro, when it is used alongside an anti-human Fc crosslinking antibody. Its mechanism of action is through a caspase independent pathway, which suggests it can be used in combination therapy with other caspase activation-dependent cytotoxic antibody therapies or chemotherapeutic agents, such as Fludarabine. The direct cytotoxic effect of SMIP-016 on CLL B cells is proportional to the amount of CD37 present on the cell surface, making it a highly selective therapy towards malignant B cells. Furthermore, SMIP-016 showed potent anti-lymphoma activity in a Raji/SCID xenograft mouse model. TRU-016, a humanized anti-CD37 SMIP molecule derived from SMIP-016, is currently in Phase II clinical trials and showing single agent activity in CLL [9].

In addition to direct killing, a major potential mechanism involved in TRU-016 tumor elimination is ADCC. SMIP-016 induced NK cells mediated antibody-dependent cellular cytotoxicity (ADCC) both in vitro and in vivo [8]. Monoclonal antibodies with bisected, complex, non-fucosylated oligosaccharides attached to the asparagine 297 residue in the CH2 region, bind with increased affinity to FcγRIIIa [10]. This glycoform engineering has been shown to enhance ADCC [11] through cells bearing FcγRIIIa, an important component in how monoclonal antibodies are clinically effective [12]. For example, afucosylated anti-CD20 antibodies show higher B cell depletion than their fucosylated counterpart by reaching saturated ADCC levels at lower concentrations and through improved FcγRIIIa binding [13]. In addition, it has been reported that antibodies lacking the core fucose in Fc oligosaccharides elicit high ADCC responses by two mechanisms [14]. On the effector cell side, afucosylated anti-CD20 antibodies were less inhibited by human plasma IgG. On the target cells, cells treated with non-fucosylated
anti-CD20 antibodies showed markedly stronger binding to NK cells than fucosylated anti-CD20 [14].

Due to the success of the parent compound SMIP-016, we sought to determine if modifying the Fc oligosaccharides of a SMIP protein would enhance its activity. Herein, we describe a second generation anti-CD37 SMIP molecule, SMIP-016\(^{GV}\), with an afucosylated Fc receptor binding region designed for enhanced effector function. Our data demonstrates SMIP-016\(^{GV}\) has enhanced effector function with NK cells and monocyte derived macrophages (MDM), making it an exciting novel CD37-targeted peptide therapeutic for B cell malignancies.

3.2 Materials and Methods

3.2.1. Production, purification and structural analysis of SMIP-016 and SMIP-016\(^{GV}\)

SMIP-016, clone 8g5, a DG44 CHO cell line transfected with SMIP-016 cDNA was expanded in shake flasks in Ex-Cell™ 302 CHO serum-free media (SAFC Biosciences, Lenexa, KS) supplemented with 1x non-essential amino acids (MediaTech), 1x sodium pyruvate (MediaTech), 4 mM L-glutamine (MediaTech), 500 nM methotrexate (MP Biomedicals, Solon, OH) and 1 mg/mL recombinant insulin (Recombulin – Invitrogen) in a 37°C, humidified incubator with 5% CO\(_2\) and the shaker set at 125 RPM.

For production of soluble SMIP-016 and SMIP-016\(^{GV}\), clone 8g5 was cultured in a fed-batch process in 3L Applikon bioreactors (Applikon Biotechnology, Foster, CA) in the media noted above without methotrexate. Cultures were fed CHO Feed Bioreactor Supplement (Sigma) at 1% of starting culture volume on days 2, 4, 6, 8 and 10 as well as glucose and glutamine as needed to maintain levels above 1 g/L and 1 mM, respectively. For SMIP-016\(^{GV}\) generation, a
freshly prepared and sterile filtered 400 mM stock solution of castanospermine (CS) (Phytex Australia Pty Ltd, New South Wales, Australia) in distilled/deionized water (MediaTech) was added at the initiation of culture to yield a final concentration of 400 uM. Cultures were harvested on day 12 and cleared of cell-related debris by passage through a SartoPure GF+ cartridge (5555305PO—SS) followed by sterilization through a Sartopore 2 filter (5445307H7—O—A) (Sartorius Stedim Biotech GmbH, Gottingen, Germany). SMIP-016 and SMIP-016<sup>GV</sup> were purified by identical means. Clarified supernatant was run over a MabSelect<sup>TM</sup> protein A resin (GE Healthcare) and protein eluted with a 25 mM acetate, 100 mM NaCl, pH 3.9 buffer. This protein containing solution was then neutralized to pH 5.5 with 550 mM MES buffer (JT Baker, Phillipsburg, NJ) after which it was run over a ceramic hydroxyapatite (CHT) column (Bio-Rad, Hercules, CA) and bound protein eluted with an 80 mM PO<sub>4</sub>, 140 mM NaCl, pH 7.0 buffer. As the latter buffer was higher in phosphate than that normally injected into animals, the CHT eluates were diluted 1:3 with 2 mM PO<sub>4</sub>, 140 mM NaCl, pH 7.0 for final formulation.

LC/MS analysis was used to confirm the peptide mass and to monitor changes in glycan distribution of the SMIP immunoglycoprotein generated in the absence (SMIP-016) and presence (SMIP-016<sup>GV</sup>) of CS. SMIP-016 and glycovariants thereof exist as a dimer under non-reducing conditions. Therefore, to simplify the analysis of the heterogeneous mixture of glycoforms, analysis was performed on the reduced, monomeric species. Prior to analysis, the molecule was reduced with 20 mM DTT in 4.8M guanidine. Twenty pmol of monomeric protein was then injected onto a POROS R1 10 µm column (Applied Biosystems, Carlsbad, CA) and eluted with acetonitrile into an ESI-TOF (Agilent Technologies, Santa Clara, CA) mass spectrometer detector. Deglycosylation prior to LC/MS analysis by treatment with peptide-N-glycosidase F (PNGaseF)(ProZyme) was used to confirm the parent peptide species. The resulting mass spectra were then deconvoluted and the glycan species identified by subtraction of the protein mass.
Resulting masses were analyzed using GlycoMod (SIB Swiss Institute of Bioinformatics) to correlate to known glycoforms. The relative abundance of individual glycopeptide species was then estimated by comparing intensities of the deconvoluted peaks of each species identified.

3.2.2. Binding affinity to target antigen CD37

Daudi cells (350,000/well) were plated in a round bottom 96-well plate in 1% paraformaldehyde (USB US19943). The cells were incubated for 30 minutes at 4°C, washed and resuspended in FACS Buffer. SMIP protein was diluted in FACS Buffer (2% FBS (Gibco) to Dulbecco’s PBS (Invitrogen) (v/v)), at concentrations ranging from saturation to background levels (72 µg/mL – 0.03 µg/mL), added to the appropriate wells, 50 µL/well, and the cells incubated for 30 minutes at 4°C. Any unbound reagents were removed and the cells were then incubated with a FITC tagged mouse antibody specific for the Fc portion of human IgG (Invitrogen, H10001C). The plate was incubated at 4°C in the dark for 45 minutes and unbound FITC anti-HuIgG was washed away. The cells were resuspended in 1% paraformaldehyde.

Each sample’s bound fluorescence was measured on a BD FACSCalibur flow cytometry system and analyzed with Cell Quest Pro software (Becton Dickinson, ver 5.2). The fluorescence intensity for each sample was plotted relative to the SMIP protein concentration. A dose response was generated and fit to a 4-parameter logistic (4-PL) curve using SoftMax Pro software (Molecular Devices, ver 5.3). The binding capacity of SMIP-016GV relative to SMIP-016 was determined by comparison of the titration curves.

3.2.3. Binding affinity to soluble CD16 low and high affinity receptor proteins

SMIP protein was diluted in FACS Buffer, at concentrations ranging from saturation to background levels (24 µg/mL – 0.011 µg/mL) and added to cells. Daudi cells were incubated for
30 minutes at 4°C. The custom soluble CD16:MuIgGFc fusion proteins were human CD16 (low or high affinity polymorphism) linked to a murine IgG Fc tail. The fusion protein was diluted in FACS Buffer to a saturating level (20 µg/mL) and added to the assay and incubated for an additional 30 minutes at 4°C to form a complex with the SMIP that had bound to the cell surface. Unbound reagents were removed from the well. The cells were then incubated with a PE-tagged F(ab’)2 antibody, specific to murine Fc (minimally reactive to human Fc) (Jackson 115-116-071). The plate was incubated at 4°C in the dark for 45 minutes. Any unbound PE conjugated antibody was removed. The cells were resusupended in 1% paraformaldehyde.

Each sample’s bound fluorescence was measured on a BD FACS Calibur flow cytometry system and analyzed with Cell Quest Pro software (Becton Dickinson, ver 5.2). The fluorescence intensity for each sample was plotted relative to the SMIP concentration. A dose response was generated and fit to a 4-parameter logistic (4-PL) curve using SoftMax Pro software (Molecular Devices, ver 5.3). The binding capacity to soluble low and high affinity CD16 receptor of cell-bound SMIP-016 GV relative to SMIP-016 was determined by comparison of the maximal “D”-parameter (maximal curve asymptote) values from the titration curves. An increase in the “D” value represents an increase in the binding activity for the corresponding sample.

3.2.4. Patient sample processing and cell culture

Blood was obtained from patients with informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the Institutional Review Board (IRB) of The Ohio State University (Columbus, OH) [15]. All patients had immunophenotypically defined CLL and had been without prior therapy for a minimum of 30 days at the time of collection. CLL cells were isolated from freshly donated blood with ficoll density gradient centrifugation (Ficoll-Plaque Plus, Amersham Biosciences, Piscataway, NJ). Enriched CLL B cells were prepared with
the use of the "Rosette-Sep" kit from StemCell Technologies (Vancouver, British Columbia, Canada) according to the manufacturer's instructions. Isolated cells were incubated in RPMI 1640 media (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 2mM L-glutamine (Life Technologies, Carlsbad, CA), and 56 U/mL penicillin/56 µg/mL streptomycin (Life Technologies) at 37°C in an atmosphere of 5% CO₂. Normal cells were obtained from either from Red Cross partial leukocyte preparations or anonymous donors as part of a second approved exemption protocol. Natural killer (NK) cells were negatively selected with Rosette-Sep kits (StemCell Technologies) according to the manufacturer’s instructions. Monocytes were positively selected using MACS system (Miltenyi Biotec, Cambridge, MA). The purity of enriched populations of normal cells was routinely checked with the use of PE labeled CD19, CD14, and CD56 staining by flow cytometry. The Daudi, Raji and 697 cell lines were obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

3.2.5. Assessment of apoptosis by flow cytometry

Cell viability was measured at various time points using Annexin V-FITC/PI staining followed by FACS analysis according to the manufacturer’s protocol (BD Pharmingen, San Diego, CA) as described previously [8]. Data were analyzed with CXP software package (Beckman-Coulter, Indianapolis, IN). At least 10,000 cells were collected for each sample and data were acquired in list mode. Results were expressed as the percentage of total Annexin V/PI negative cells over untreated control.
3.2.6. *In vitro treatment of cells with antibodies*

Primary CLL or ALL cells were suspended in complete media at a density of 1x10^7 cells/mL immediately after isolation. SMIP-016 and SMIP-016^GV were used a 5 µg/mL concentration unless otherwise noted. All antibodies (trastuzumab, rituximab, and alemtuzumab) were used at 10 µg/mL. The cross-linker, goat anti–human IgG (Fc specific) (Jackson Immunoresearch) was added to the cell suspension 5 minutes after adding the primary antibodies, at a concentration 5 times that of the therapeutic protein or antibody (i.e., 25 µg/mL for 5 µg/mL of SMIP-016). In addition, a group of samples with no treatment was collected as media control.

2.2.7. *Immunoblot Analysis*

Whole cell extracts were prepared as previously described by our group [16]. Equivalent amounts of protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Following antibody incubations, proteins were detected with chemiluminescent substrate (SuperSignal, Pierce). The following antibodies were used for detection: anti-Phospho tyrosine (4G10) and anti-GAPDH (both from Millipore, Billerica, MA).

3.2.8. *Antibody-dependent cellular cytotoxicity (ADCC) assay*

ADCC activity was determined by standard 4-hour ^51^Cr-release assay as previously described [13]. Briefly, ^51^Cr-labeled target cells (5x10^3 cells/ well) were incubated 30 minutes with 10 µg/ml of individual antibodies. Effector cells (NK cells from healthy donors or CLL patients) were then added at the indicated effector-to-target (E:T) ratios. After 4-hour incubation, supernatants were removed and counted on a Perkin Elmer (Waltham, MA) Wizard gamma counter. The percentage of specific cell lysis was determined by: % lysis = 100 x (ER – SR)/(MR
– SR), where ER, SR, and MR represent experimental, spontaneous, and maximum release, respectively.

### 3.2.9. Antibody-Dependent Cellular Phagocytosis (ADCP) Assay

Monocyte Derived Macrophages (MDMs) were derived from peripheral blood monocytes using Monocyte-Colony Stimulating Factor (R and D Systems, Minneapolis, MN) for seven days. They were fluorescently labeled with Min-Claret dye (Sigma). CLL cells were fluorescently labeled with PKH-67 (Sigma) and coated with antibody for one hour at 4°C. MDM and CLL cells were co-incubated for 30 minutes at a 1:5 E: T ratio, then colocalization of CLL with MDM was scored using flow cytometry and verified using microscopy.

### 3.2.10. Complement Dependent Cytotoxicity (CDC) Assay

CLL B cells were suspended at 10⁶/mL in RPMI 1640 media, media with 30% autologous plasma from the patient blood samples, or media with 30% heat-inactivated (56°C, 30 minutes) plasma. Cells were then treated with antibodies and incubated at 37°C for 1 hour, pelleted and resuspended with 100 μL 1% Formaldehyde with Live/Dead Stain (Sigma-Aldrich). The extent of CDC was measured by FACS analysis of percent of cells staining for dead.

### 3.2.11. Retroviral Transductions

A CD37 construct was designed with the human CD37 gene inserted onto a pBABE (Promega) backbone. The huCD37-pBABE vector, an empty pBABE vector, and a no vector 697 cell control were independently transfected into separate flasks of Phoenix Amphi™ cells (Orbigen) using a Calcium phosphate mediated transfection kit from Promega. The Phoenix Amphi™ cells
were designed by placing constructs for gag-pol and envelope protein for amphotropic viruses into 293T cells. The supernatant containing the retrovirus was collected from the Phoenix Ampho™ cells after two days and the 697 cells were resuspended in the viral supernatant along with polybrene (hexadimethrine bromide). The 697 cells were incubated with the virus for about 8 hours and then resuspended in fresh media. Puromycin was added as a selection agent after two days. The cells were grown under selection and then separated out in limited dilution cloning.

3.2.12. Assessment of antibody-binding and antigen surface density

Quantitative analysis of CD37 surface density was done using the Quantum Simply Cellular kit (Bangs Laboratories, Fishers, IN), according to the manufacturer's instructions.

3.2.13. NK cell and monocyte in vitro stimulation and cytokine release assays

For in vitro NK-cell stimulation experiments, SMIP protein or antibody were immobilized on a plate. Normal donor NK cells were plated at 2 x 10^5 NK cells/well. CD107a-FITC or IgG1-FITC control was added to the suspension at the start of the 4-hour incubation at 37°C. NK cells were harvested at the end of incubation period, stained with CD56-PE and analyzed by FACS for CD107a surface expression. For supernatant experiments, cell-free culture supernatants were harvested after 4-hours and analyzed for levels of IFN-γ by a Quantikine Human IFN-γ ELISA performed according to the manufacturer’s instructions (R&D Systems).

Primary Monocyes were incubated on immobilized antibodies or SMIP proteins for 24 hours and supernatant was harvested. TNF-α levels were measured using a Quantikine Human TNF-α ELISA, performed according to the manufacturer’s instructions (R&D Systems).
3.2.14. Live Cell Imaging

CLL cells were treated with 5 μg/mL of SMIP protein or a Type II antibody for 1-16 hours. Images were captured every hour using a Zeiss CCD camera (AxioCam Mrm, New York, NY) in ApoTome Microscope (Axio Observer.Z1; Carl Zeiss MicroImaging GmbH, Jena, Germany) and were analyzed with Zeiss Axiovision (Vs40) image acquisition software.

3.2.15. Statistical methods

Since each patient’s cells were under all conditions of each experiment, linear mixed effect models were used to estimate unrestricted covariance structures and produce robust hypothesis tests [18]. Holm’s method was used to adjust for multiplicity [19].

3.3 Results

3.3.1. Engineering and characterization of glycovariant SMIP protein

SMIP-016<sup>GV</sup> was generated by treating a DG44 CHO cell line transfected with SMIP-016 cDNA with castanospermine (CS). CS is a potent plant alkaloid inhibitor of α- and β-glucosidases, including those involved in N-linked processing of glycoproteins such as glucosidase I [20]. LC/MS glycoprofiling analysis of the products demonstrated that the observed mass species in both CS treated (SMIP-016<sup>GV</sup>) and untreated (SMIP-016) preparations were consistent with the expected amino acid sequence with a typical, heterogeneous mammalian glycosylation pattern. A slight difference in amino acid composition was noted between the two preps with a greater proportion of the SMIP-016<sup>GV</sup> monomers found to lack the C-terminal lysine compared to the
SMIP-016 monomers (89% vs 72%). With respect to the N-linked glycoforms, there was a near total reversal in the percentage of fucosylated and non-fucosylated glycan species between the two preparations (Figure 3.1A). For SMIP-016 the fucosylated complex glycoforms (94% of total) were composed of 80% G0F and 20% G1F species with the remaining 5% of glycans being represented by non-fucosylated high hexose forms of \((\text{Man})_3 + (\text{Man})_3(\text{GlcNAc})_2\) composition. In the case of SMIP-016\(^{GV}\), only 6% of the glycans were of fucosylated complex form (all G0F) while the remaining (95%) were of non-fucosylated high hexose form comprised mostly of \((\text{Hex})_7 + (\text{Man})_3(\text{GlcNAc})_2\) (93%) glycans with the remainder of \((\text{Man})_5 + (\text{Man})_3(\text{GlcNAc})_2\) composition (7%).

3.3.2. SMIP-016\(^{GV}\) and SMIP-016 have equal binding affinity for CD37

To test whether the glycovariant form of SMIP-016 had similar antigen binding affinity as the parent compound, both SMIP proteins were bound to Daudi cells, a human B-lymphoblastoid cell line derived from Burkitt’s lymphoma that expresses high levels of CD37. A FITC labeled anti-huIgG antibody was added to bind to the SMIP proteins and the bound fluorescence was measured by flow cytometry. As shown in the Figure 3.1B, SMIP-016 and its CS generated glycovariant SMIP-016\(^{GV}\) yielded virtually identical binding profiles on CD37\(^+\) Daudi cells, demonstrating that alteration of the N-linked glycosylation of SMIP-016\(^{GV}\) had no effect on target antigen binding of the protein.

3.3.3. The Fc portion of SMIP-016\(^{GV}\) has enhanced affinity for low and high affinity soluble Fc\(\gamma\)RIII

The single nucleotide polymorphisms (SNP) in Fc\(\gamma\)RIIIa resulting in either a valine (V) or phenylalanine (F) at position 158 results in low or high affinity receptors in humans and this has
been implicated to be indicative of response to rituximab immunotherapy [21] [22] [23]. However, this SNP has shown no correlation to response in CLL [24] [25]. In order to test the binding affinities to these receptors, SMIP-016 and SMIP-016GV were bound to CD37+ Daudi cells and their ability to bind to soluble versions of low and high affinity FcγRIIIa was tested by flow cytometry. SMIP-016GV demonstrated a 9.3-fold in binding over that observed with SMIP-016 for the FcγRIII low affinity receptor and a 2.1-fold increase in affinity for the high affinity FcγRIII receptor, respectively (Figure 3.1C).

3.3.4. SMIP-016 and SMIP-016GV show comparable levels of cytotoxicity through similar mechanisms

Direct cytotoxicity is one of the mechanisms of killing mediated by SMIP-016. To test whether the changes in the glycosylation pattern on this SMIP protein influenced its potential for direct cytotoxicity, we tested SMIP-016 and SMIP016GV on isolated primary CLL cells. SMIP-016GV mediates direct cytotoxicity in primary CLL B cells with goat anti-human Fc crosslinker in comparable levels to those seen with the parent (unmodified) protein, (Figure 3.2A). When cells are drugged for 48 hours along with anti-human Fc crosslinker, SMIP-016GV decreased cell viability by about 60 percent, which was significantly higher than rituximab (p <0.0001) but comparable to SMIP-016 (p = 0.708; Figure 3.2A). Therefore, both SMIP-016 and its glycovariant, SMIP-016GV show similar levels of direct cytotoxicity at the same concentration and time points, suggesting they have the similar potential for signaling of direct cytotoxicity in cells expressing CD37 on their surface. Furthermore, the SMIP proteins initiate apoptosis in a Type I antibody-like manner (Figure 3.2B) as compared to a Type II anti-CD20 antibody.

Zhao et al. has previously reported that SMIP-016 works in a novel caspase-independent pathway and that in vitro stimulation of CLL B cells with SMIP-016 along with an anti-Fc
crosslinker showed activation of protein tyrosine phosphorylation [8]. This was subsequently shown to involve SHP1 and a complex array of signaling via the ITIM motif of CD37 [26]. In order to test whether the SMIP-016\(^{GV}\) was signaling within the cells via the same molecular pathways, we stimulated CLL cells with SMIP-016 or SMIP-016\(^{GV}\) along with anti-human Fc crosslinker. Both SMIP-016 and SMIP-016\(^{GV}\) show similar patterns of increased tyrosine phosphorylation after 10 minutes of in vitro stimulation of CLL cells (Figure 3.2C). In particular, western blot analysis of cellular lysates with an anti-phospho tyrosine antibody revealed a predominant protein at ~65 kD in cells treated with SMIP-016 or SMIP-016\(^{GV}\), an observation previously described by our group [8] [26] and determined to be SHP-1 and Lyn phosphorylation. The identical binding affinity to the target antigen CD37 along with the equivalent amount of cytotoxicity against CLL cells via identical phosphorylation of downstream proteins collectively suggests that the parent compound SMIP-016 and the glycovariant SMIP-016\(^{GV}\) are working through similar molecular pathways in CLL cells to cause direct cytotoxicity, which have been previously described [26].

Next, we tested if SMIP-016\(^{GV}\) had any complement initiating capabilities. We have shown that the SMIP-016 is unable to mediate complement dependent cytotoxicity [8]. This is also seen with the SMIP-016\(^{GV}\) (Figure 3.2D). The alteration of the Fc glycosylation pattern does not affect the SMIP’s inability to initiate complement.

3.3.5. SMIP-016\(^{GV}\) shows enhanced Phagocytosis by Monocyte Derived Macrophages

We reported previously that NK cells, not monocytes were the major effector population mediating cytotoxicity with SMIP-016 [8]. However, the importance of the role of monocytes and macrophages in the function of therapeutic antibodies is emerging [27] [28] [29] [30],
especially in anti-CD20 therapy. Similar to what we had seen previously, we saw low ADCC function with SMIP-016 and SMIP-016\textsuperscript{GV} in monocytes against CLL B cells (data not shown).

In addition, plate bound SMIP proteins were not efficient at inducing cytokines in peripheral blood monocytes. TNF-\(\alpha\) production by monocytes stimulated with SMIP-016 and SMIP-016\textsuperscript{GV} for 24 hours was decreased compared to non-specific IgG or rituximab (Figure 3.3A). Furthermore, Monocyte Derived Macrophages (MDM) stimulated similarly for 24 hours also had lower TNF-\(\alpha\) production (data not shown).

To study if SMIP-016\textsuperscript{GV} could elicit a cytotoxic response from MDM, which express Fc\(\gamma\)RIIIA [27] [31], we tested it in Antibody Dependent Cellular Phagocytosis (ADCP) assays using MDM from normal donors. Primary CLL samples were coated with either SMIP-016 or SMIP-016\textsuperscript{GV} and subsequently co-incubated with MDM. The glycovariant SMIP protein mediates significantly more ADCP than the parent SMIP protein (p<0.05; Figure 3.3B).

3.3.6. SMIP-016\textsuperscript{GV} enhances NK cell activation and ADCC towards CD37 Expressing Targets

Previous studies have shown that changing the glycosylation pattern of the Fc portion of antibodies can influence their ability to elicit effector function by enhancing their binding affinity to Fc\(\gamma\)RIIIa on human immune cells [32] [14]. We saw similar enhanced affinity to Fc\(\gamma\)RIIIa with the SMIP-016\textsuperscript{GV} (Figure 3.1C). In addition, compared to SMIP-016, the SMIP-016\textsuperscript{GV} mediated enhanced activation of NK cells, as revealed by their increased expression of surface CD107a following stimulation with the SMIP proteins or control antibodies by 8-fold (p = 0.012) (Figure 3.3C) and by their 16-fold increase in IFN-\(\gamma\) production (p = 0.009, Figure 3.3D).

To determine whether the afucosylation of glycoproteins on the SMIP-016\textsuperscript{GV} engineered using TRU-ADhanCe\textsuperscript{TM} (glycovariant optimization technology) influence the molecule’s ability to elicit NK cell ADCC, we performed ADCC assays with NK cells from healthy donors against
CD37 positive (Raji) and negative (697) malignant B cell lines targets. Figure 3.3E demonstrates that SMIP-016\textsuperscript{GV} mediates significantly more (p = 0.0013 in all E:T ratios > 0) ADCC against Raji cells than SMIP-016. Contrasting with this, 697 is a human B cell precursor leukemia cell line that does not express CD37. SMIP-016 and SMIP-016\textsuperscript{GV} do not mediate any ADCC effects in 697 cells (Figure 3.3F), validating the antigen specificity of the two SMIP proteins.

3.3.7. NK cells from CLL patients show enhanced ADCC with SMIP-016\textsuperscript{GV}

Ziegler et al. showed in a landmark paper that the NK cells from CLL patients are deficient in activity. The NK cell activity was not detectable in patients with advanced disease and six times lower than control in patients with early disease \cite{33}. This finding has been challenged by others \cite{34}. To test whether CLL NK cells would be capable of mediating an effective response with SMIP-016\textsuperscript{GV}, we tested NK cells from patients with early stage disease as effectors in ADCC assays against Raji cells targets. Their response was compared to the response from NK cells isolated from healthy donors. The SMIP-016\textsuperscript{GV} showed increased levels of cytotoxicity than SMIP-016 with normal and CLL NK cells (p <0.0001 for both) (Figure 3.4A). The trend in response to the SMIP-016\textsuperscript{GV} from primary NK cells from CLL patients as in NK cells from healthy donors was comparable (p = 0.0154). Another statistical test showed that the difference between the healthy versus CLL NK cells and the SMIP-016\textsuperscript{GV} is not significant (p = 0.86), indicating that the superiority of SMIP-016\textsuperscript{GV} over SMIP-016 is consistent for CLL and normal NK cells. The enhanced cytotoxicity achieved by SMIP-016\textsuperscript{GV} with CLL NK cells validates the glycovariant SMIP-016 as being an excellent therapeutic in CLL patients.
3.3.8. **SMIP-016<sup>GV</sup> shows enhanced ADCC against primary CLL B cells**

Given the documented clinical activity of TRU-016, the clinical version of SMIP-016, in CLL, we next compared the ability of SMIP-016 with the SMIP-016<sup>GV</sup> to elicit effector function against primary CLL B cells. CLL B cells have wide range of CD37 on their surface [8]. SMIP-016<sup>GV</sup> was able to induce greater cytotoxicity in CLL cell targets as compared to SMIP-016 (p <0.0001; **Figure 3.4B**). SMIP-016<sup>GV</sup> mediates significantly greater ADCC than rituximab (p <0.0001) and is comparable to alemtuzumab (p = 0.691). However, SMIP-016<sup>GV</sup> is more specific to malignant B cells than the anti-CD52 antibody. The superiority of SMIP-016<sup>GV</sup> over SMIP-016 for ADCC was observed for all of the effector:target ratios tested (average across E:T ratios > 0 versus E:T = 0 for SMIP-016<sup>GV</sup> vs. SMIP-016, p <0.0001) (**Figure 3.4C**).

3.3.9. **SMIP-016<sup>GV</sup> at low concentrations is effective in enhancing NK cell ADCC**

Our initial ADCC experiments were done using 5 µg/mL of both SMIP proteins, the optimal dose found to be effective for direct cytotoxicity in CLL B cells [8]. Given the enhanced ADCC by SMIP-016<sup>GV</sup>, we hypothesized that this may maintain the enhanced ADCC at lower SMIP protein concentrations. It has been suggested that serum plasma and IgG can affect the concentrations of therapeutic monoclonal antibodies within the body [12] therefore efficacy at low concentrations is desirable for therapy. SMIP-016, SMIP-016<sup>GV</sup>, and rituximab were used at decreasing log concentrations in an ADCC assay using normal donor NK cells as effectors and CLL B cells as targets. The SMIP-016<sup>GV</sup> showed enhanced ADCC as compared to the SMIP-016 across a wide range of concentrations, even as low as 5 x 10<sup>-6</sup> µg/mL (p <0.0001 compared to both SMIP-016 and Rituximab, **Figure 3.4D**). The cytotoxicity with SMIP-016 reaches a plateau after the 0.005 µg/mL concentration while SMIP-016<sup>GV</sup> continues to increase. The trend in cytotoxicity for
SMIP-016\textsuperscript{GV} was significantly steeper than for SMIP-016 (p <0.0001) and for rituximab (p <0.0001).

3.3.10. SMIP-016\textsuperscript{GV} Enhances ADCC in cells expressing low levels of surface CD37

SMIP-016 mediated direct cytotoxicity has been shown to be dependent on antigen density \cite{8,26}. We wanted to test whether antigen density played a role in ADCC with the SMIP-016 and whether the glycovariant could overcome this. In order to test this, we created a model system by retrovirally transducing 697 cells (CD37 negative cell line) with a pBABE-CD37 vector construct and isolated clones with differing levels of surface CD37 by limiting dilution cloning. We validated that the clones were producing CD37 mRNA by RT-PCR (data not shown) and quantified the amount of surface CD37 protein by flow cytometry (Figure 3.5A). These clones expressed between 5000 and 80000 molecules of CD37 on their surface, substantially less than Raji cells which have hundreds of thousands of CD37 molecules. Low, medium and high CD37 antigen density clones were chosen to continue in our assays in order to test a range of CD37 antigen levels.

Our experimental system allowed us to determine if antigen density played a role in level of NK cell mediated ADCC by SMIP-016\textsuperscript{GV}. Using normal donor NK cells as effectors and the CD37-expressing 697 clones as targets, ADCC assays were performed to test varied amounts of antigen on the target cells against decreasing SMIP protein concentrations (Figure 3.5B). As described previously (Figure 3.3E), the parent 697 cells were not susceptible to ADCC by the CD37 specific SMIP protein. However, despite a relatively low level of CD37 expression in the 697-CD37 V1 clone (under 20,000 molecules/cell), SMIP-016\textsuperscript{GV} elicited excellent NK mediated ADCC in these cells, up to around 80% relative cytotoxicity (Figure 5B). In addition, the results showed that with SMIP-016\textsuperscript{GV}, cytotoxicity increased with increasing CD37 surface expression (p
Enhanced ADCC with increasing CD37 antigen was also seen in SMIP-016 treated cells (\( p = 0.0005 \)).

Interestingly, the expression of CD37 amplifies the response difference between the SMIP-016\(^{GV} \) and SMIP-016 in the low and medium CD37 expressing 697 cell clones, 697-CD37 V1 and V2. In 697-CD37 V3 the trend difference between the SMIP-016 and the glycovariant was not significant. This may be due to the high amount of cytotoxicity already obtained with SMIP-016. The SMIP-016\(^{GV} \) results in greater killing but reaches 100\%, thus reaching the maximal limit of the assay. Therefore, the SMIP-016\(^{GV} \) could be beneficial for B-cell malignancies with a low level of surface CD37.

3.3.11. **SMIP-016\(^{GV} \) shows enhanced ADCC function against Acute Lymphoblastic Leukemia cells**

Acute Lymphoblastic Leukemia (ALL) cells have been reported as having low surface expression levels of CD37 [3]. From our results in Figure 5, we hypothesized that the SMIP-016\(^{GV} \) would be capable of enhanced NK cell mediated ADCC against these leukemic cells. First, we quantified the levels of CD37 on the surface of primary ALL bone marrow cells, CLL cells and normal B cells (**Figure 3.6A**). With an average of about 12,000 molecules of surface CD37, the ALL cells have significantly lower surface levels of CD37. This is 3 fold lower than CLL cells (\( p = 0.0051 \)) which have an average of about 36,000 and 5 fold lower CD37 expression than normal peripheral B cells (\( p < 0.0001 \)) which have an average of about 63,000 on their surface. As shown previously [8], CLL cells have a wide range of CD37 expression.

Direct cytotoxicity was tested with SMIP-016, SMIP-016\(^{GV} \) and alemtuzumab in primary ALL samples. Viability analysis by flow cytometry at 24 hours revealed that both SMIP proteins
exhibited comparable cytotoxicity of 20 percent (Figure 3.6B) \( (p = 0.7425) \), while alemtuzumab mediated about 40 percent cytotoxicity in these cells.

Finally the primary ALL samples were used as targets in ADCC experiments with normal donor NK cell effectors. We saw a minimal response with SMIP-016 as compared to the control SMIP protein. The SMIP-016\textsuperscript{GV} was able to elicit an enhanced ADCC response against ALL samples, despite the low levels of surface CD37 (Figure 3.6C). This is significantly enhanced cytotoxicity than SMIP-016 \( (p < 0.0036 \) for all effector to target ratios tested). Collectively, this suggests SMIP-016\textsuperscript{GV} might serve as a potential therapeutic for ALL.

3.4 Discussion and Conclusions

Herein we report successful modification of the Fc binding region of a SMIP protein to effectively remove fucosylation in a manner that still allows efficient production of the protein for clinical use. SMIP-016 produced in CHO cultures containing CS results in an end product that is afucosylated. This modification of SMIP-016 does not affect its binding affinity to CD37 and subsequent direct cytotoxicity, nor does it alter SMIP-016\textsuperscript{GV} from signaling through mechanisms previously described by our group [8] [26]. From these data we hypothesize that SMIP-016\textsuperscript{GV}’s direct cytotoxicity is dependent on surface levels of CD37 on CLL cells and is irrespective of prognostic factors such as IgVH mutational status, Rai stage, and common cytogenetic abnormalities, as described with SMIP-016 [26]. However, SMIP-016\textsuperscript{GV} shows enhanced NK cell mediated ADCC against primary CLL B cells compared to its parent compound SMIP-016. We demonstrate that NK cell effectors from CLL patients are not as effective in SMIP-016-mediated ADCC as normal NK cell effectors. However, the level of ADCC obtained with SMIP-
016\textsuperscript{GV} and CLL NK cell effectors were similar to that achieved with the parent compound SMIP-016 and normal NK cell effectors, indicating that the SMIP-016\textsuperscript{GV} can overcome these deficiencies. Finally, we demonstrated that SMIP-016\textsuperscript{GV} mediates superior ADCC against both low surface level expressing 697 cell lines and also primary ALL cells, whereas SMIP-016 lacks activity against these. Collectively, these data provide a strong rational for clinical development of SMIP-016\textsuperscript{GV} in B-cell malignancies expressing high as well as low amounts of surface CD37.

With the success of antibody therapy, significant effort has gone into modifying the Fc domain to enhance effector cell recruitment, ability to fix complement, and also increased affinity for FcRn to enhance serum half life. These manipulations of function can occur through amino acid engineering via substitutions in the constant region of antibodies and have been extensively described \cite{35} \cite{36} \cite{37}. An alternative way to modify antibody effector function with enhanced recruitment of FcγRIIIa binding is to reduce Fc region fucosylation through genetic manipulation of the antibody producing cell line \cite{38} \cite{39}. This has been described for CD20 directed antibody therapeutics where studies have shown enhanced NK cell ADCC function at lower antibody concentrations \cite{13} \cite{40} \cite{41}. It provides the opportunity for using less therapeutic antibody thereby diminishing production and ultimately treatment cost. Modification of Fc glycosylation of clinically relevant alternative peptide therapies such as SMIP proteins has not been previously described. Afucosylation of the FcγR binding region had potential to affect stability or other properties important to viable process for commercial development. In this paper, we demonstrate that this process is feasible with SMIP-016 and provides a way to also enhance effector cell function of immunoglycoprotein therapeutics. Given SMIP-016 has already demonstrated single agent activity against CLL and low grade B-cell lymphoma\cite{9}, further pursuit of SMIP-016\textsuperscript{GV} that has enhanced NK cell effector cell function seems worthwhile.
The completed phase I study of TRU-016 in CLL has demonstrated that this agent has significant single agent activity in both symptomatic untreated CLL and also those patients having received 1-2 prior therapies [9]. Exploration of TRU-016 in ALL was not warranted based upon very modest expression of this antigen in less mature B cells [3]. Our in vitro data provide further justification for this decision as SMIP-016 lacks ADCC against ALL cells. However, studies done with low expressing CD37 transfected 697 cell lines suggested that SMIP-016GV was more effective at mediating ADCC at low copy number of CD37 antigen. Outside of kinase inhibitors targeting the Philidelphia chromosome positive ALL, little therapeutic progress has been made over the past two decades in the treatment of B cell ALL. A small proportion of patients are cured with intensive chemotherapy based approaches or allogeneic stem cell transplant, however many ultimately die from their disease. This is particularly true for elderly patients who do not tolerate current therapy well. Immunotherapy for adult ALL is being studied in clinical trials, with rituximab and alemtuzumab where very modest benefit has been observed [42] [43] [44] [45]. This in part relates to low antigen density of CD20 and populations of cells not expressing CD52 in ALL [45] [46] [47]. Herein, we demonstrate that SMIP-016GV is effective at NK cell mediated ADCC against primary ALL cells which have lower CD37 surface expression. This provides support for use of SMIP-016GV as a potential immune therapy of ALL.

In summary, our data suggest the potential use of the SMIP-016GV with enhanced ADCC function as a new alternative for therapy in B cell malignancies including CLL therapy. SMIP-016GV mediated cytotoxicity was mainly through FcγR mechanisms. The capacity of monoclonal antibodies to interact with FcγRs in humans has been shown to be vital for therapeutic efficacy [11]. This same concept seems to apply to SMIP proteins, with SMIP-016GV being highly effective against CLL and ALL cells due to interactions with FcγRs on effector cells.
3.5 References


17. Rafiq, S., et al., XmAb-5574 antibody demonstrates superior antibody dependent cellular cytotoxicity as compared to CD52 and CD20 targeted antibodies in adult acute lymphoblastic leukemia cells. Leukemia, 2012.
3.6 Figures

A. | Glycoform | Glycoform Composition | Glycoform Type | Relative Abundance (%) |
---|---|---|---|---|
1493 | (Man,Hex,HexNAc) + (B,Hex) + (GlcNAc) | Reconstituted Complex | 75 | 5 |
1607 | (Man,Hex,HexNAc) + (B,Hex) + (GlcNAc) | Reconstituted Complex | 12 | 0 |
1772 | (Man,Hex,HexNAc) | Native Repeat High | 1 | 0 |
1703 | (Man,Hex,HexNAc) | Native Repeat High | 1 | 7 |
2027 | (Man,Hex,HexNAc) | Native Repeat High | 1 | 10 |

B. Geo Mean Value vs Concentration (ng/ml) for SMIP-016 and SMIP-016<sub>GV</sub>

C. Geo Mean Value vs Concentration (ng/ml) for Low affinity and High affinity Receptors for SMIP-016 and SMIP-016<sub>GV</sub>

Figure 3.1. Characterization of SMIP-016<sub>GV</sub> molecule. (A) Data table from LC-MS glycoprofiling of SMIP-016 made with or without treatment with Castanospermine (CS) showing relative proportions of glycoform types. (B) Binding affinity of SMIP-016 and SMIP-016<sub>GV</sub> to target antigen CD37. (C) Enhanced binding of SMIP-016<sub>GV</sub> with both high and low affinity soluble FcγRIII.
Figure 3.2. SMIP-016 and SMIP-016$^{GV}$ show similar methods of direct cytotoxicity. (A) SMIP-016 and SMIP-016$^{GV}$ show comparable levels of direct cytotoxicity in primary CLL B cells (n = 11) (p = 0.0708). (B) SMIP-016 and SMIP-016$^{GV}$ kills cells in a Type I antibody-like manner. Microscopy images of CLL B cells (i) untreated (ii) Type II antibody (iii) SMIP-016 or (iv) SMIP-016$^{GV}$ for 16 hours (n = 3). (C) Both SMIP-016 and SMIP-016$^{GV}$ induced a similar pattern of tyrosine phosphorylation of cellular proteins at 65 kDa. CLL cells were treated with 5μg/mL of SMIP-016 or SMIP-016$^{GV}$ with anti-Fc cross-linking antibody in PBS for 10 minutes and phospho tyrosine proteins were detected by Western blot analysis using anti-phosphotyrosine antibody 4G10 (n = 6). (D) SMIP-016 and SMIP-016$^{GV}$ do not initiate complement dependent cytotoxicity in CLL B cells (n = 6).
Figure 3.3. SMIP-016\textsuperscript{GV} can mediate cytotoxicity through effector cells. (A) Intact antibodies, F(ab)’2, or SMIP bound to plates induced TNF-α from peripheral blood monocytes (n = 4). (B) SMIP-016\textsuperscript{GV} mediates enhanced Antibody Dependent Cellular Phagocytosis (ADCP) by Monocyte Derived Macrophages (MDM) of primary CLL cells compared to SMIP-016, as measured by flow cytometry (n = 3) (p < 0.05). (C) Induction of CD107a on the surface of CD56\textsuperscript{+} NK cells by SMIP-016\textsuperscript{GV}, as measured by flow cytometry (n = 6). (D) Enhanced induction of Interferon gamma from NK cells by SMIP-016\textsuperscript{GV} compared to SMIP-016 (p = 0.009) (n = 4). (E, F.) ADCC with normal donor NK cell effectors and Raji (E) or 697 (F) targets. SMIP-016\textsuperscript{GV} shows enhanced ADCC function in the CD37 expressing Raji cells as compared to SMIP-016 (p = 0.0013 of average of all E:T ratio > 0) but no activity in the 697 cells (n = 3).
Figure 3.3. Continued
Figure 3.4. SMIP-016\textsuperscript{GV} is effective against CLL B cells. (A) SMIP-016\textsuperscript{GV} shows comparable enhanced cytotoxicity trends in ADCC assays with primary normal donor or CLL NK cells used as effectors against Raji cell targets at an 25:1 E:T ratio (n = 12) (p = 0.01). The levels of cytotoxicity achieved with the CLL NK cells were significantly lower than what is seen with comparable treatments in the normal donor NK cells. (p = 0.0154 for SMIP-16\textsuperscript{GV} and p = 0.0104 for SMIP-016). (B) ADCC with normal donor NK cells against primary CLL B cell targets shows enhanced ADCC function with SMIP-016\textsuperscript{GV} compared to SMIP-016 (p <0.0001) . Each patient at each E:T ratio is represented by a dot (n = 15). (C) The enhanced ADCC function seen in (B) is sustained over all effector to target ratios tested (average SMIP-016\textsuperscript{GV} vs. SMIP-016 at E:T ratio > 0, p <0.0001). (D) Dose dependent ADCC function of SMIP-016\textsuperscript{GV} against CLL B cells. SMIP-016\textsuperscript{GV} shows significantly enhanced ADCC compared to SMIP-016 (p <0.0001) or rituximab (p <0.0001) even at the lowest concentration (n = 12). The trend showed significantly increased ADCC with SMIP-016\textsuperscript{GV} when compared with SMIP-016 (p <0.0001) or rituximab (p <0.0001). The non-specific Control SMIP showed less than 5% cytotoxicity at all concentrations tested (data not shown).
Figure 3.4. Continued
Figure 3.5. SMIP-016\(^{\text{GV}}\) mediates enhanced ADCC in cells expressing a range of surface CD37.

(A) Quantification of surface CD37 on parental 697 (697), 697-CD37 clones V1, V2 and V3 by flow cytometry. (B) ADCC assay with normal donor NK cell effectors against the different 697 cell clones as targets at a 25:1 E:T ratio. SMIP-016 and SMIP-016\(^{\text{GV}}\) were used in decreasing concentrations (n = 4 NK donors). Increasing surface CD37 levels enhanced ADCC with both the SMIP-016\(^{\text{GV}}\) (p = 0.002) and SMIP-016 (p = 0.0005).
Figure 3.6. SMIP-016\textsuperscript{GV} mediates effective NK cell ADCC against primary Acute Lymphoblastic Leukemia cell. (A) Quantification of surface CD37 by flow cytometry shows ALL cells ($n=9$) have significantly lower levels of surface CD37 than CLL B cells ($n=20$) ($p=0.0051$) and normal B cells ($n=5$) ($p<0.0001$). (B) SMIP-016 and SMIP-016\textsuperscript{GV} show comparable levels of modest direct cytotoxicity against primary ALL bone marrow samples ($n=4$) ($p=0.74$). (C) ADCC using ALL bone marrow samples as targets and normal donor NK cell effectors shows enhanced ADCC with SMIP-016\textsuperscript{GV} compared to SMIP-016. ($n=8$ ALL samples x 3 NK cell effectors each) ($p<0.0036$ for all E:T ratios tested).
Chapter 4: Comparative Assessment of Clinically Utilized CD20-directed Antibodies in Chronic Lymphocytic Leukemia (CLL) Cells Reveals Divergent NK cell, Monocyte and Macrophage Properties

4.1 Introduction

Expression of CD20 glycoprotein is tightly restricted to the surface of B cells, making it an ideal therapeutic target for antibody therapy. Over the past decade, it has become a well-validated target for therapy in B cell malignancies, mainly due to the approval of rituximab for Non-Hodgkins Lymphoma in 1997. Rituximab is chimeric monoclonal antibody that has revolutionized therapy in a variety of B cell malignancies, including chronic lymphocytic leukemia (CLL). In CLL, rituximab was shown to have modest single agent activity (reviewed in [1, 2]) but has shown greatest promise in combination with chemotherapy (chemoimmunotherapy), where retrospective phase II comparison studies [3] [4] and a recent prospective phase III study demonstrated prolongation of survival [5]. Despite its successes, not all patients respond to rituximab therapy and virtually all relapse. Improving the properties of rituximab to enhance its efficacy further is therefore highly desirable.

B cell depletion by rituximab and other anti-CD20 antibodies has been proposed to occur via several mechanisms. While many effector cells including Natural Killer (NK) cells, monocytes, macrophages, and granulocytes can mediate ADCC, several sentinel papers in mouse
models have suggested that B cell depletion with anti-CD20 or anti-CD19 antibodies are predominantly dependent on monocytes and their expression of FcγRIIa, FcγRIIIa, and FcγRIV[6] [7] [8]. Furthermore, others have suggested that Tumor Necrosis Factor-α (TNF-α) secreted by monocytes activates NK cells and this crosstalk mediates enhanced ADCC [9] [10]. In humans, NK cells have been suggested to be most important for rituximab tumor clearance based upon the FcγRIIIa single nucleotide polymorphisms (SNPs) expressed predominately in this cell type and result in a low or high affinity receptor that is highly predictive of antibody response [11] [12] and of normal B cell depletion[13]. In CLL, these same FcγRIIIa SNPs have no correlation with response[14] [15] or extended progression free survival[16]. The true importance of NK cells, monocytes, or other effector cells to CD20 antibody mediated killing in CLL remains controversial.

Other mechanisms of anti-CD20 mediated cytotoxicity including direct cell death and complement dependent cytotoxicity have also been documented. Direct cytotoxicity with Type I anti-CD20 antibodies such as rituximab generally require cross-linking with an anti-Fc directed antibody in vitro[17, 18], proposing to mimic in vivo binding to FcγR on effector cells. Evidence of in vivo apoptosis following rituximab treatment in CLL cells has supported this as a mechanism of action [19]. However, a recent study has challenged this by using a novel mouse model with a FcγR lacking the active immune tyrosine activating motif (ITAM) that demonstrated little in vivo activity with CD20 antibodies [20]. Type II anti-CD20 antibodies lack the need for cross linking and offer a potential advantage clinically by promoting homotypic adhesion and actin-dependent, lysosome-mediated cell death [21]. Complement Dependent Cytotoxicity (CDC) with rituximab occurs but the antigen density on CLL cells limits killing by this mechanism [22] [23]. Additionally, up-regulation of complement protection antigens CD55 and CD59 may occur after rituximab based therapy [24] [25].
Based on the success of rituximab in NHL and CLL, the next generation of anti-CD20 therapeutic antibodies is emerging, intelligently engineered to enhance efficacy of anti-CD20 therapy via different mechanisms of action (Table 4.1). Ofatumumab (Arzerra) is a human, Type I antibody that uniquely binds to the small and large extracellular loop of CD20 [26]. It has been shown to induce potent CDC in vitro compared to rituximab at low concentrations and low antigen density [27] [26]. Clinically, ofatumumab produced clinical responses in more than 50% of fludarabine and alemtuzumab refractory CLL patients with modest toxicity [28] [29] and is active in patients irrespective of prior treatment with rituximab [30]. It is currently approved for this indication. GA101 (Obinutuzumab) is a Type II humanized anti-CD20 antibody that promotes direct killing without in vitro cross-linking and has an afucosylated Fc domain engineered for enhanced FcγRIIIa binding [31-36]. Direct cell death and ADCC by NK cells is superior with GA101 as compared to rituximab against malignant B cells [31]. A phase I study with GA101 in CLL showed favorable efficacy in relapsed/refractory patients with modest toxicity [37]. The follow up phase II study of 20 relapsed and refractory CLL patients demonstrated promising blood clearance of tumor cells but only a 20% response by IWCLL 2008 response criteria [38]. This response criterion has a 50% reduction of nodal mass by CT assessment for partial response which was the most frequent reason for not attaining this. Prior studies with other therapeutic antibodies have not mandated CT for assessment of response but rather relied on physical exam measurements alone. Response in nodal disease may also have been influenced by dose of GA101 and larger studies will be required to ascertain the true efficacy of this antibody.

Whereas ofatumumab and GA101 have each been compared to rituximab in CLL, no study has compared these two antibodies or examined each ones interaction with monocytes or macrophages. Herein we report such a detailed analysis demonstrating ofatumumab and GA101...
are both superior to rituximab but each has varied ability to activate different effector cell types that might bear relevance to combination based strategies in CLL.

4.2. Materials & Methods

4.2.1. Patient sample processing and cell culture

Blood was obtained from patients with informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the Institutional Review Board of The Ohio State University (OSU). All patients examined in this series had immunophenotypically defined CLL and had been without prior therapy for a minimum of 30 days at the time of collection. CLL PBMC were isolated from freshly donated blood with Ficoll density gradient centrifugation (Ficoll-Plaque Plus, Amersham Biosciences, Piscataway, NJ). Enriched CLL fractions were prepared with the use of the "Rosette-Sep" kit from StemCell Technologies (Vancouver, British Columbia, Canada) according to the manufacturer's instructions. Isolated cells were incubated in RPMI 1640 (Life Technologies, Grand Island, NY) media supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 2mM L-glutamine (Life Technologies, Carlsbad, CA), and 56 U/mL penicillin, 56 µg/mL streptomycin (Life Technologies) at 37°C in an atmosphere of 5% CO₂. Normal cells were obtained from Red Cross partial leukocyte preparations, and natural killer (NK) cells were negatively selected with Rosette-Sep kits (StemCell Technologies) according to the manufacturer’s instructions. Monocytes were positively selected using MACS system (Miltenyi, Cambridge, MA). The purity of enriched populations of normal cells was routinely checked with the use of CD19, CD56, and CD14-PE staining by flow cytometry. Normal samples were from anonymous donors as part of a
second exemption protocol approved by the institutional review board at OSU. The Raji and THP-1 cell lines were obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. The NK92 CD16+ cells have been previously described [39].

4.2.2. In vitro treatment of cells with antibodies

Cells were suspended in complete media at a density of 1 x 10^7 cells/mL immediately after isolation. All therapeutic antibodies were used at 10 μg/mL unless otherwise noted. Where indicated, the Fc specific goat anti–human IgG crosslinker (Jackson Immunoresearch, West Grove, PA) was added to the cell suspension 5 minutes after adding the primary antibodies, at a concentration 5 times that of the primary antibodies (i.e., 50 μg/mL for 10 μg/mL). In addition, a group of samples with no treatment was collected as media control.

4.2.3. Assessment of apoptosis by flow cytometry

The apoptosis of cells was measured using annexin V-FITC/PI staining followed by FACS analysis according to the manufacturer’s protocol (BD Pharmingen, San Diego, CA) as described previously [40]. Results are presented as percentage cytotoxicity, which is defined as (% annexin V^+ and/or PI^+ cells of treatment group) / (% annexin V^+ and/or PI^+ cells of media control) x 100. FACS analysis was performed using a Beckman Coulter FC500 cytometer (Beckman Coulter, Indianapolis, IN). Ten thousand events were collected for each sample and data were acquired in list mode.
4.2.4. *Complement Dependent Cytotoxicity (CDC)*

CLL B cells were suspended at $10^6$/mL in RPMI 1640 media, media with 30% plasma from the patient blood samples, or media with 30% heat-inactivated (56°C, 30 minutes) plasma. Cells were then treated with antibodies and incubated at 37°C for 1 hour, pelleted and resuspended in 1% Formaldehyde with Live/Dead Stain (Sigma-Aldrich). The extent of CDC was measured by FACS analysis of percent staining for dead.

4.2.5. *Antigen Quantification*

Quantitative analysis of CD20 surface density was done using the Quantum Simply Cellular kit (Bangs Laboratories, Fishers, IN), according to the manufacturer's instructions.

4.2.6. *In vitro stimulation and cytokine assays*

For in vitro NK cell stimulation experiments, wells of a 96-well flat-bottom plate were coated with 10 or 20 μg/mL of respective antibody in PBS. Freshly isolated NK cells were plated at 2x10^5 cells/well. CD107a-FITC or isotype control (BD Pharmingen) was added to the suspension at the start of the 4-hour incubation at 37°C. NK cells were harvested at the end of the 4-hour incubation period and stained with CD56-PE and analyzed by FACS for CD107a surface expression.

For NK cell cytokine experiments, cell-free culture supernatants were harvested after 4-hours of stimulation with immobilized antibody and analyzed for levels of IFN-γ by A Quantikine Human IFN-γ ELISA, performed according to the manufacturer’s instructions (R&D Systems). For monocytes and MDM experiments, cell-free culture supernatants were harvested after 24-hours of stimulation with immobilized antibody and analyzed for levels of TNF-α by A
Quantikine Human TNF-α ELISA, performed according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). For monocytes and MDM assays using R-848 (Enzo, Farmingdale, NY), the cells were pretreated overnight with 1 μM R-848 before stimulated with immobilized antibody, as described above.

4.2.7. Real-time reverse transcription–polymerase chain reaction

Monocytes were stimulated 18 hours with immobilized antibodies. Cells were collected and total RNA was extracted using TRizol (Life Technologies). Real-Time PCR for TNF-α was performed using pre-designed TaqMan® Gene Expression Assays and ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA).

4.2.8. Antibody-dependent cellular cytotoxicity (ADCC) assay

ADCC activity was determined by standard 4-hour 5¹Cr-release assay. 5¹Cr-labeled target cells (5x10³ cells/ well of B CLL or Raji cells) were incubated 30 minutes with various concentrations of antibodies. Unbound antibodies were washed off and cells were placed in to 96-well plates. Effector cells (NK cells from healthy donors or CLL patients) were then added to the plates at the indicated effector-to-target (E:T) ratios. After 4 hour incubation, supernatants were removed and counted on a Perkin Elmer (Waltham, MA) Wizard gamma counter. The percentage of specific cell lysis was determined by: % lysis = 100 x (ER – SR)/(MR – SR), where ER, SR, and MR represent experimental, spontaneous, and maximum release, respectively.

4.2.9. Antibody-dependent Cellular Phagocytosis (ADCP) Assay

Monocyte Derived Macrophages (MDMs) were derived from peripheral blood monocytes using Macrophage Colony-Stimulating Factor (M-CSF) (R&D Systems) at 20 μg/mL for 5 to 7 days.
The MDM were fluorescently labeled with Min-Claret dye (Sigma-Aldrich). CLL cells were fluorescently labeled with PKH-67 (Sigma-Aldrich) and coated with antibody for one hour at 4°C. MDM and CLL cells were coincubated for 30 minutes at a 1:5 Effector: Target ratio, then colocalization of CLL with MDM was scored using flow cytometry and verified using microscopy.

4.2.10. Lipid Rafts and Immunoblot Analysis
Lipid raft fractions were isolated using a sucrose gradient, as previously described by our group [41]. Purity of lipid raft fractions was determined by using cholera-toxin. Whole cell extracts were prepared as previously described by our group [42] with the addition of phosphatase inhibitor cocktail 1 and 2, protease inhibitor cocktail P8340 and 1 mM phenylmethylsulfonyl fluoride (all from Sigma-Aldrich) to the lysis buffer. Equivalent amounts of protein were separated on polyacrylamide gels and transferred onto nitrocellulose membranes. Following antibody incubations, proteins were detected with chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL). The following antibodies were used for detection: anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-ERK1/2 (Cell Signaling Technologies, Danvers, MA); Anti-pan phosphor-tyrosine antibody 4G10 and anti-GAPDH (Millipore, Billerica, MA), Cholera-toxin (Sigma-Aldrich), and Actin (Santa Cruz, Santa Cruz, CA). The FcγRIIa and FcγRIIb antibodies have been previously described [43].
4.3 Results

4.3.1. GA101 mediates superior direct cytotoxicity with or without anti-Fc crosslinking antibody

Direct signaling to apoptosis has been shown to play a role in anti-CD20 therapy [19] [44]. GA101, as a Type II antibody, promotes about 25% cell death of CLL B cells, with a range of 0 to 61%, in the absence of an Fc crosslinker. This is significantly more than the type I antibodies ofatumumab or rituximab in the absence of a crosslinking antibody (Figure 4.1A) (p = 0.0012 or 0.013, respectively). This direct cytotoxicity study was further extended to compare the three anti-CD20 antibodies along with the Fc cross-linking antibody in a larger sample of patients (n = 19) (Figure 1B). No enhanced cytotoxicity was observed with GA101 treatment when treated along with an Fc cross-linking antibody. GA101 mediated significantly greater cytotoxicity with an Fc cross-linking antibody than rituximab (p = 0.003) or ofatumumab (p = 0.03) (Figure 4.1B). Collectively, these studies suggest the Type II antibody GA101 mediates superior direct cytotoxicity without requirement for an Fc cross-linking as compared to the Type I antibodies. However, ofatumumab mediated greater direct cytotoxicity with the Fc cross-linking antibody than rituximab (p = 0.0687).

4.3.2. Ofatumumab mediates superior complement-dependent cytotoxicity

Initiation of complement can be a potent method of cytotoxicity for therapeutic antibodies [45] [46]. In order to test the ability of the three CD20-directed antibodies to initiate complement, we examined CLL cells for sensitivity to complement-mediated killing. As shown in Figure 4.1C, ofatumumab demonstrates ~30% CDC in CLL cells, significantly greater than either rituximab or GA101 (p = 0.0001). This enhanced CDC with ofatumumab was maintained regardless whether fresh or frozen primary CLL samples were used as targets (data not shown). Quantification of
CD20 antigen on CLL patient B cells showed a wide range of CD20 surface expression (from 17,000 to 600,000) (Figure 4.1D) and a weak correlation ($r^2 = 0.3$, $p = 0.026$) between the level of CDC induced by ofatumumab and CD20 surface expression, as depicted in Figure 4.1E. There was no correlation between surface CD20 and CDC seen with rituximab ($p = 0.96$) or with GA101 ($p = 0.836$). Collectively, these data demonstrate that ofatumumab is superior to GA101 or rituximab in mediating CDC against CLL cells.

4.3.3. GA101 stimulates enhanced NK cell activation compared to Ofatumumab or rituximab

NK cells are the major effector cell population implicated in mediating ADCC. ADCC is initiated through FcγRIIIa (CD16) engagement [47] and engineered antibodies that are either mutated or lack a core fucosylation in the Fc region generally have enhanced affinity for this receptor. As an afucosylated antibody, GA101 has been reported to mediate enhanced NK cell ADCC [32, 34-36]. In order to compare GA101 to the other anti-CD20 antibodies in terms of NK cell function, we first examined NK cell activation by looking at the activation marker CD107a. Normal donor NK cells were stimulated with immobilized antibody and CD107a expression was measured by flow cytometry. As shown in Figure 4.2A, GA101 significantly induces more CD107a expression on NK cells than ofatumumab ($p = 0.02$) or rituximab ($p = 0.005$). Furthermore, GA101 promoted a significant amount of IFN-γ release from stimulated normal donor NK cells as compared to ofatumumab and rituximab ($p = 0.0008$) (Figure 4.2B). There was no significant difference observed in IFN-γ production between ofatumumab and rituximab. Collectively, these data suggest GA101 is a better activator of NK cells as compared to ofatumumab or rituximab.

Binding of FcγRs on immune cells and recruitment of these receptors into lipid rafts is necessary for signaling within effector cells. Therefore, we next evaluated the recruitment of
FcγRIII to lipid rafts on NK cells post-stimulation with immobilized anti-CD20 antibody. In order to obtain sufficient cells to isolate lipid rafts, we utilized the NK92 cell line that has been stably transfected to express FcγRIII [39]. These cells were briefly stimulated with immobilized antibodies and lipid raft fractions were isolated with subsequent assessment by immunoblot to study if FcγRIII was differentially recruited into the rafts. As seen in the representative blot in Figure 4.2C, FcγRIII was recruited equally into the raft fraction with GA101 and with ofatumumab. Rituximab showed recruitment to a lesser degree. Analysis of the lipid raft with cholera-toxin revealed the presence of GM1 ganglioside binding activity in the raft but not in the non-raft fractions demonstrating purity of the lipid raft preparations used in these studies.

4.3.4. GA101 mediates enhanced NK cell ADCC as compared to ofatumumab and rituximab

Activation of NK cells, as measured by CD107a and IFN-γ production, and recruitment of FcγRIII to lipid rafts often correlates with ADCC potential [48]. To confirm superiority of GA101 in NK cell function, normal donor NK cells were used as effectors against CLL B cell targets in standard 51Cr release assays. As expected with an afucosylated Fc region engineered antibody, GA101 mediated significantly more ADCC than ofatumumab (p <0.0001) or rituximab (p<0.0001) at 5 μg/ml of antibody (Figure 4.2D). However, at decreasing concentrations of antibody, the enhanced ADCC seen with GA101 is no longer apparent. At concentrations of 0.05 μg/ml and less, GA101 is no longer significantly more effective than ofatumumab (p = 0.9761). In addition at those lower antibody concentrations ofatumumab is superior in ADCC function compared to rituximab (p <0.0001).

NK cells from CLL patients have been reported to have decreased effector function [49] and are most relevant to the studies pursued herein. To test these engineered antibodies with NK cells from CLL patients, ADCC assays were performed using NK cells from twelve patients with
early stage disease as effectors against Raji cell targets (Figure 4.2E). At 10 μg/ml concentrations GA101, ofatumumab, and rituximab demonstrate insignificant difference in ADCC between NK cells from CLL patients and normal donor NK cells (p = 0.141, 0.464, 0.085, respectively), although the CLL cells have an overall trend toward decreased function. Collectively, these data support the findings of others that CLL patients NK cells may have a very modest defect in NK cell ADCC [50, 51] against CD20-targetted cells, however GA101 remains superior to ofatumumab or rituximab at NK cell-mediated ADCC.

4.3.5. The Anti-CD20 antibodies differentially stimulate monocytes

Monocytes have been implicated as being the most important effector cell in how CD20-directed antibodies work in vivo in murine models [6] [8]. Similar to the antibody effector function of NK cells, monocyte and monocyte derived macrophage activation and phagocytosis is governed by the interplay between FcγRI, FcγRIIa, FcγRIIb and FcγRIIIa which are all expressed in these cells [52] [53]. In order to compare GA101 and ofatumumab to rituximab in these cells, we tested production of TNF-α cytokine by monocytes from normal donors. Monocytes were stimulated with immobilized GA101, ofatumumab or rituximab and cells or supernatants were collected for mRNA and TNF-α analysis respectively. As seen in Figure 4.3A, monocytes have decreased mRNA levels of TNF-α following stimulation with GA101 as compared to rituximab or ofatumumab (p = 0.0018, <0.0001, respectively) and produce less TNF-α (p = 0.35, 0.018, respectively) (Figure 4.3B).

4.3.6. GA101 demonstrates inferior ADCP as compared to ofatumumab or rituximab

MDMs have also emerged as especially important in anti-CD20 antibody clearance of B cells, particularly in murine models [6]. These macrophages have been demonstrated with other
antibodies to phagocytose antibody-coated cells predominantly through FcγRIIa, with some contribution from FcγRI and FcγRIIIa [54] [47]. We first examined the ability of immobilized GA101, ofatumumab, or rituximab to activate MDM, generated from normal peripheral blood monocytes (PBM) as measured by TNF-α production. MDM cells exposed to plate-bound Ofatumumab or rituximab produced higher levels of TNF-α, as compared to GA101 (p = 0.7, 0.34, respectively) (Figure 4.3C). These data support monocytes and MDM cells demonstrate inferior activation with GA101 as compared to ofatumumab or rituximab.

We next examined the ability of GA101, ofatumumab, and rituximab to mediate Antibody-dependent Cellular Phagocytosis (ADCP) by MDMs. Phagocytosis was tested by co-incubating MDM with antibody coated CLL cells. Both cells were dyed with fluorescent membrane dyes and the cells were analyzed by flow cytometry. Percent Phagocytosis was determined by double positivity for both dyes, indicating MDM that had ingested CLL cells and the results were validated by microscopy. Contrary to the TNF-α data, all three of the anti-CD20 antibodies show ADCP capability against CLL B cells (Figure 4.3D), with ofatumumab exhibiting the greatest ADCP (60% ± 8.8) as compared to rituximab (48% ± 17.8) and GA101 (41% ± 16.7). Ofatumumab is able to mediate significantly more ADCP as compared to GA101 (p = 0.0036) and rituximab (p = 0.03). Collectively, these data demonstrate that non-FcγR engineered ofatumumab and rituximab mediate superior MDM activation and phagocytosis as compared to GA101.

4.3.7. The anti-CD20 antibodies differentially signal in monocytes

We next sought to investigate the mechanism of differential TNF-α release and phagocytosis by the CD20-directed antibodies. Initial studies examined if there was differential global-tyrosine protein phosphorylation as measured by 4G10 immunoblots from monocytes
stimulated with immobilized GA101, ofatumumab, or rituximab for 5 to 7 minutes. Immunoblot analysis shown in Figure 4.4A demonstrates that GA101 has reduced pan tyrosine phosphorylation compared to the non Fc engineered ofatumumab or rituximab in the monocytic THP-1 cell line and normal donor monocytes (data not shown). As the Fc binding region is responsible for binding to FcγR and ultimately recruitment to lipid rafts where activation signaling is mediated, we examined for differential phosphorylation and recruitment of FcγRIIa to lipid rafts by immobilized GA101, ofatumumab, and rituximab. Phosphorylation pattern of both the activating FcγRIIa (Figure 4.4B) and inhibitory FcγRIIb (Figure 4.4C) in monocytes exposed to the different immobilized CD20 antibodies does not explain the differential TNF-α release.

Since phosphorylation of the receptor did not explain the differential activation of monocytes, we hypothesized potential differences in recruitment of the Fcγ Receptors into signaling lipid rafts. In Figure 4.4D we demonstrate that GA101 does not induce recruitment of the activating FcγRIIa to lipid rafts in the THP-1 cell line. This is in contrast to what is observed with the positive control (IgG), ofatumumab, and rituximab where robust recruitment of this ITAM containing FcγR occurs. Analysis of the lipid raft with cholera-toxin revealed the presence of GM1-ganglioside binding activity only in the raft, demonstrating purity of the lipid raft preparations (Figure 4.4D, left panel).

Next, to see if other receptors such as FcγRIIIa and FcγRI were differentially recruited we investigated the effect of CD20-directed antibodies on γ-chain recruitment to lipid rafts (Figure 4.4E). Whereas FcγRIIa contains its own cytosolic ITAM motif, the other activating Fcγ Receptors, namely FcγRIIIa and FcγRI associate with an ITAM containing common γ-chain to initiate activating signals. There were no differences in recruitment of the common γ-chain
between the three anti-CD20 antibodies tested, indicating no differences in FcγRIIIa and FcγRI recruitment. Therefore, GA101 lacks recruitment of only FcγRIIa into lipid rafts in monocytes.

To further explore if downstream signaling is differentially diminished in GA101 stimulated monocytes, we examined alteration of phospho-ERK following exposure to immobilized antibodies. Downstream phosphorylation of ERK following FcγR crosslinking has been demonstrated by others to be critical in the induction transcription factors such as NFκB and c-fos and subsequent expression of cytokines [55] [56]. In Figure 4.4F we demonstrate that GA101 results in decreased phosphorylation of ERK (T202/Y204) as compared to ofatumumab or rituximab in THP-1 cells. This was further verified in primary monocytes (data not shown). These results collectively suggests that GA101 demonstrates diminished recruitment of FcγRIIa to lipid rafts and decreased signaling in monocytes as compared to ofatumumab and rituximab.

4.3.8. Combination of anti-CD20 antibodies with TLR-agonist R-848 enhances MDM cytokine production

Toll-like receptor (TLR) 7/8 agonists work predominantly through cytokine production [57]. R-848, a TLR 7/8 agonist, has also been shown to increase expression of the activating Fc receptors FcγRI and FcγRIIa, as well as the common γ–chain. Furthermore, monocytes treated with R-848 decreased expression of the inhibitory receptor, FcγRIIb [58]. Given this positive link between the TLR7/8 and FcγR pathways, we set out to determine if the cytokine deficiency seen with the anti-CD20 antibodies could be enhanced with R-848. Monocytes or MDM were pretreated overnight with R-848 and subsequently stimulated for 24 hours with immobilized antibodies. TNF-α levels was measured in the supernatant by ELISA. Monocytes treated with R-848 followed by stimulation with GA101 showed no increase in TNF-α production, similar to R-848 alone stimulated cells (p = 0.92) (Figure 4.5A). In contrast, MDM pre-treated with R-848
showed significantly increased TNF-α produced by GA101 stimulated MDM over R-848 alone stimulation (p = 0.0071) (Figure 4.5B) to levels that were similar to the other anti-CD20 antibodies. This suggests that combination therapy of GA101 with R-848 may rescue the decreased cytokine release phenotype seen from MDM.

4.4 Discussion

Herein, we have shown the divergent effector properties of three clinically relevant anti-CD20 antibodies. Both GA101 and ofatumumab are superior to rituximab in separate ways. GA101 displays superior direct cytotoxicity without an in vitro crosslinking antibody. Its engineered Fc region elicits enhanced NK cell stimulation and IFN-γ release, and subsequently superior NK cell-mediated ADCC. Conversely, ofatumumab exhibits superior complement activation against primary CLL cells, independent of CD20 antigen density. It mediates the greatest MDM mediated ADCP. In addition, rituximab and ofatumumab elicited TNF-α release from both monocytes and MDM, while GA101 exhibited decreased response. Although high levels of TNF-α production post therapeutic antibody infusion may not be clinically desirable [59] [60] [61], TNF-α release from mononuclear cells is needed for death signal to target cells and for crosstalk to NK cells [9] [10]. In addition, although GA101 was not able to elicit cytokine response from MDM, it still had moderate ADCP function. This may suggest separate pathways are involved. Lastly, monocytes showed an overall decrease in tyrosine-phosphorylated proteins post stimulation with GA101 and decreased recruitment of FcγRIIa into lipid rafts.

The impact of Fc region afucosylation of antibodies on monocytes function has only been implied by studies looking at the binding affinity of these antibodies to FcγRII. Low-fucosylated
antibodies have moderate to no enhanced binding to FcγRIIa, FcγRIIb, or FcγRIa compared to their high-fucosylated counterparts [62] [63]. This would imply that monocytes, which predominantly express FcγRIIa and FcγRIIb, should not be differentially affected by glycoengineered antibodies. However, these systems only look at Fc region binding affinities to receptors and not how the receptors interact with the antibodies, i.e. recruitment to rafts and signaling. This process has not been described with Fc-engineered antibodies. Our results suggest that perhaps there may be differences in interaction with alternative FcγRs due to glycoengineering.

Lack of recruitment of FcγRIIa into lipid rafts has numerous implications for signaling in monocytes. FcγRIIa has a cytosolic ITAM motif, a sequence of conserved amino acids found on many immune receptors that contains a tyrosine that can be phosphorylated by activating kinases. Unlike GA101, the non-Fc engineered anti-CD20 antibodies are able to recruit FcγRIIa to the rafts, indicating that perhaps Fc engineering for enhanced binding to FcγRIII may affect IgG interactions with alternative FcγRs. This lack of recruitment to the rafts post GA101 treatment leads to decreased activation as reflected by a decrease in pan tyrosine phosphorylated proteins. This is further confirmed by diminished phosphorylation of ERK, a downstream target of FcγRIIa signaling, and functionally by decreased cytokine production.

Decreased activation of monocytes and MDM by GA101 may have clinical implication for therapy. Given the role of these cell types in anti-CD20 mediated B cell depletion in mouse models, this may explain the potential diminished efficacy seen with GA101 clinically in CLL and NHL [64] [65, 66]. GA101 showed lymphocyte depletion until week 25 but disease progression in patients with high tumor burden [67]. Furthermore, our studies show that NK cell-mediated ADCC is not maintained at superior level with low antibody concentrations, making dosing schedules quite relevant. The deficiencies in monocyte and MDM cytokine production by
GA101 can be overcome with combination therapy TLR 7/8 agonists such as R-848. TLR 7 or
7/8 agonist have been successfully used in the clinic (reviewed in [68]) and have been shown to
have antitumor responses in murine models [69].

The interaction of FcγRs on effector cells with engineered Fc portions is vital in antibody
therapeutics. While engineered antibodies are designed to be effective activators of CLL NK
cells in vitro [70], the in vivo role of these antibodies in activation of NK cells, monocytes, and
MDM is controversial. Differential effects of the novel CD20 directed antibodies reported here
underline the importance in choosing the ideal CD20 antibody as a single agent or in combination
in the context of the functional competency of the relevant effector cell populations in CLL and
other B cell malignancies.
4.5. References


### 4.6. Tables

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**Table 4.1.** Anti-CD20 antibodies in development. CLL = Chronic Lymphocytic Leukemia, NHL = Non-Hogkins Lymphoma, RA = Rheumatoid arthritis
Figure 4.1. Anti-CD20 antibodies differentially mediate direct cytotoxicity and complement dependent cytotoxicity. (A.) GA101 mediates significantly greater direct cytotoxicity in CLL cells treated without a crosslinking antibody as compared to ofatumumab or rituximab (p = 0.0012 or 0.013, respectively) at 48 hours (n = 8). (B.) GA101 mediate significantly greater direct cytotoxicity on CLL cells with an Fc crosslinking antibody than rituximab (p = 0.003) or ofatumumab (p = 0.03). Flow cytometry results after treatment with 10 μg/ml of each antibody with 50 μg/ml of goat anti-human anti Fc crosslinking antibody for 48 hours (n = 19). (C.) Ofatumumab mediates enhanced Complement dependent Cytotoxicity (CDC) with human serum on previously frozen CLL B cells (p = 0.0001, n = 19). All antibodies used at 10 μg/ml. (D.) Quantification of CD20 on the surface of CLL cells by flow cytometry (n = 19). (E.) Ofatumumab shows a weak correlation between surface CD20 density and CDC (n = 19) as compared to GA101 and rituximab ($r^2 = 0.3$, p = 0.026).
Figure 4.1. Continued

C. % Dead

D. Number of CD20 molecules/cell

E. % Cytotoxicity (normalized to crosslinker)

Number of CD20 molecules/cell

- Rituximab
- Ofatumumab
- GA101
Figure 4.2. NK cell function with anti-CD20 antibodies. (A.) GA101 stimulates significantly enhanced CD107a expression on primary NK cells than ofatumumab or rituximab (p = 0.02 or 0.005, respectively), as analyzed by flow cytometry (n = 8). (B.) Immobilized GA101 induces significantly more IFN-γ release from primary NK cells than ofatumumab or rituximab (p = 0.0008), as measured by ELISA (n = 8). (C.) GA101 and ofatumumab show recruitment of FcγRIII to lipid rafts. Immunoblot analysis for FcγRIII of lipid raft fraction made from stimulating NK92 CD16+ cells with immobilized antibodies for 5-7 minutes (results shown are representative of three independent experiments). Cholera-toxin blot on the right of panel 2C indicates purity of raft versus nonraft fractions. R = Raft, NR = NonRaft (D.) ADCC with normal NK cell effectors against CLL B cell targets in decreasing antibody concentrations (n = 4 CLL B cell targets with 3 NK cell effectors each). GA101 shows enhanced ADCC at higher concentrations as compared to ofatumumab or rituximab (p<0.0001). (E.) ADCC with CLL or normal NK cell effectors against Raji cell targets (n = 12) shows insignificant difference in ADCC between NK cells from CLL patients and normal donor NK cells with anti-CD20 antibodies (p = 0.141, 0.464, 0.085, for GA101, ofatumumab, and rituximab respectively), although the CLL cells have an overall trend toward decreased function.
Figure 4.2. Continued

C.

D.

E.

E:T 25:1

% Relative Cytotoxicity

Antibody Concentrations (µg/ml)

Rituximab

Ofatumumab

GA101

E:T 25:1

% Relative Cytotoxicity

No Antibody

Rituximab

Ofatumumab

GA101

Normal NK cells

CLLNK cells

105
Figure 4.3. Monocyte and Monocyte Derived Macrophages function with CD20-directed antibodies (A.) GA101 stimulates less TNF-α messages levels in primary monocytes compared to ofatumumab or rituximab (p = 0.0018, <0.0001, respectively), as measured by RT-PCR. (n = 8). (B.) Immobilized GA101 induces significantly decreased TNF-α release from normal donor monocytes as compared to rituximab (n = 6) (p = 0.018). (C.) Immobilized GA101 induces decreased TNF-α release from normal donor Monocyte Derived Macrophages (MDM) than ofatumumab or rituximab (n = 5). (D.) Ofatumumab stimulates significantly more Antibody Dependent Phagocytosis by normal donor MDM of CLL B cells targets than GA101 or rituximab (p = 0.0036 or 0.03, respectively), as measured by flow cytometry (n = 6).
Figure 4.4. Anti-CD20 antibodies signal differentially in Monocytes. (A.) Immobilized GA101 induces decreased pan tyrosine-phosphorylated proteins response in THP-1 cells as compared to Ofatumumab or rituximab (n = 4). (B.) There is no differential phosphorylation of FcγRIIb after stimulation with GA101 and ofatumumab. Primary monocytes stimulated 5-7 minutes on antibody coated plates and immunoblots tested for FcγRIIb (n = 3). (C.) There is no differential phosphorylation of FcγRIIa after stimulation with GA101 and ofatumumab. Pan phospho-tyrosine protein immunoprecipitation done on primary monocytes stimulated with plated antibodies for 5-7 minutes and immunoblots tested for FcγRIIa (n = 3). (D.) FcγRIIa is not recruited to lipid rafts after stimulating THP-1 cells with immobilized GA101 (representative of three independent experiments). FcγRIIa recruitment is seen with ofatumumab and rituximab. Cholera-toxin blot on the right of panel 4D indicates purity of raft versus nonraft fractions. R = Raft, NR = NonRaft (E.) The common γ chain is not differentially recruited to lipid rafts in the same samples as 4D, indicating no differential recruitment of FcγRI or FcγRIII (representative of three independent experiments) in THP-1 cells with the anti-CD20 antibodies. (F.) Immunoblot analysis shows decreased phosphorylation of ERK in THP-1 cells after stimulation with immobilized GA101, as compared to ofatumumab and rituximab (results shown is a representative of three independent experiments).
Figure 4.4, Continued
Figure 4.4. Continued
Figure 4.5. Combination therapy of anti-CD20 antibodies and R-848. (A.) Pretreatment of primary monocytes with R-848 enhances overall production of TNF-α by rituximab, ofatumumab, or GA101 (n = 6), as measured by ELISA that is not significant over unstimulated monocytes (p = 0.49, 0.59, 0.92, respectively). (B.) Pretreatment of primary MDM with R-848 leads to increase in TNF-α production after stimulation with rituximab, ofatumumab, or GA101, which is significantly enhanced over unstimulated monocytes (p = 0.0005, 0.0005, 0.0071, respectively) (n = 6).
Chapter 5: Conclusions and Perspectives

5.1. Conclusions

Herein we have studied various engineered antibodies and protein therapeutics against different therapeutic antigens in adult acute lymphoblastic leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL). These represent promising new therapeutics for patients.

Anti-CD19 therapy has been previously reported in CLL [1]. However, adult ALL is associated with poor clinical outcome and has lacked progress with respect to introduction of new effective therapies. XmAb-5574 is an Fc region engineered antibody designed to have increased affinity for FcγRIIa/IIIa thereby demonstrating ability to mediate enhanced NK cell activation and antibody dependent cellular cytotoxicity (ADCC) against CD19^+ cells. Herein we compared XmAb-5574 ADCC, direct killing and complement dependent cytotoxicity (CDC) to several antibodies (rituximab, ofatumumab, and alemtuzumab) currently approved for other indications and which have demonstrated some evidence of clinical activity in ALL. Despite similar target antigen expression of CD19, CD20, and CD52 on ALL tumor cells, XmAb-5574 mediates significant ADCC at lower target:effector ratios as compared to alemtuzumab, rituximab, and ofatumumab. Modest direct killing with Fc antibody cross-linking was observed with XmAb-
These results suggest ALL cells are uniquely sensitive to CD19 targeted FcγR engineered antibodies and provide justification for clinical trials of XmAb-5574 in adult ALL. Future experiments with these antibodies could study the significance of targeting CD19 antigen in ALL cells. Given the biological role of CD19 in B cell signaling, knowing the importance of targeting this antigen may shed light on how therapeutic antibodies are effective in ALL.

CD37 is a tetraspanin family protein that represents a novel therapeutic target in B cell malignancies. TRU-016 is a SMIP™ (monospecific protein therapeutic) molecule against CD37 that is currently in Phase II trials in CLL and Non-Hodgkin’s Lymphoma (NHL). In an attempt to enhance the ADCC function of SMIP-016, the chimeric version of TRU-016, SMIP-016\textsuperscript{GV} was engineered with a modification in a glycosylation site in the Fc domain. The wild-type and glycovariant SMIP proteins mediate comparable Type I antibody-like direct cytotoxicity in the presence of anti-human Fc crosslinker and show a similar tyrosine phosphorylation pattern post-treatment. However, NK cells stimulated with the SMIP-016\textsuperscript{GV} exhibit enhanced activation and release 3-fold more IFN-γ compared to SMIP-016. SMIP-016\textsuperscript{GV} shows enhanced ADCC function against cells expressing CD37 with NK cell effectors derived from both normal and CLL-affected individuals. Enhanced ADCC is observed against CLL cells and is sustained at concentrations of SMIP-016\textsuperscript{GV} as low as 5E-6 µg/mL on cells expressing minimal CD37 antigen. In support of the biological relevance of this, SMIP-016\textsuperscript{GV} mediates effective ADCC against primary ALL cells with low surface expression of CD37. Collectively, these data suggest potential use of the novel therapeutic agent SMIP-016\textsuperscript{GV} with enhanced effector function for B-cell malignancies, including CLL and ALL therapy.

Future experiments with SMIP-016\textsuperscript{GV} can be done to study if the enhanced cytotoxicity at low concentrations and low cell densities by SMIP-016\textsuperscript{GV} is maintained in vivo. This can be
done in xenograft SCID models using the 697-CD37 clones that express varying amounts of surface CD37. However, since the glyco-engineering on the SMIP-016\textsuperscript{GV} is for enhanced binding to human FcγRIII, these studies may not provide clear superiority of the modified protein therapeutic. Transgenic mice with human FcγRIII [2] may provide more clear results, but may present a challenge for engraftment. In addition, given that the signaling pathway is elucidated for SMIP-016 cytotoxicity via CD37 [3], combination therapies with specific kinase inhibitors that target the PI-3K pathway and SMIP-016\textsuperscript{GV} can be tested to enhance Fc-mediated effects and direct cytotoxicity on CLL B cells.

Finally, CD20 is a widely validated, B cell specific target for therapy in B cell malignancies. Rituximab is an anti-CD20 antibody that when combined with chemotherapy prolongs survival of CLL patients. Ofatumumab and GA101 are CD20 directed antibodies developed as alternative agents to rituximab in CLL based upon different properties of enhanced direct killing, NK cell-mediated ADCC, or CDC. Despite wide spread study, ofatumumab and GA101 had not been directly compared to one another, nor studied for interaction with monocytes and macrophages that are critical to CD20 mediated antibody efficacy in murine models. In CLL cells, we showed that direct killing is greatest with GA101 and CDC with ofatumumab. GA101 promotes enhanced NK cell activation and ADCC at high antibody concentrations. Ofatumumab has superior antibody dependent cellular phagocytosis (ADCP) with monocyte derived macrophages (MDM). GA101 demonstrated reduced activation of monocytes with diminished pERK, TNF-α release, and FcγRIIa recruitment to lipid rafts. These data demonstrate GA101 and ofatumumab are superior to rituximab against CLL cells in different mechanisms of potential tumor elimination. These findings bear relevance to potential combination strategies with each of these anti-CD20 antibodies in the treatment of CLL.
Our results with GA101 as compared to the non-Fc engineered antibodies suggest that afucosylated antibodies may differentially interact with FcγRIIa and differ in recruitment potential to lipid rafts. Future work may aim to determine if afucosylation indeed decreases recruitment of FcγRIIa to lipid rafts and decreased monocyte/macrophage function. Furthermore, it would be interesting to observe if amino acid engineered antibodies also differentially recruit FcγRs and have decreased activation of these cells. Ideally, these experiments would be done by taking a parent antibody and either afucosylating the Fc portion or introducing amino acid residue changes that are known to enhance FcγRIII binding. Detailed comparisons of these variants would help elucidate the full properties of antibody modifications and bear relevance to the future antibody engineering.

In addition, the most important mechanism of action of therapeutic antibodies in CLL is yet to be determined. This is a complicated task outside of human studies. However, one can imagine doing preclinical studies using novel and complex mouse models. One method would involve crossing the transgenic human FcγR expressing mice [4] with ones that are known to develop CLL-like B cell leukemia, such as the Tcl-1 transgenic mouse model [5]. This model could be used to systematically deplete various cell types or complement and study efficacy of treatment. It would require the use of mouse antibodies against mouse antigens, a problem could be circumvented by using a Tcl-1 mouse model that expressed a human target antigen, but this model has not yet been described. These experiments would be complex but would lend insight into the in vivo mechanisms of clinically relevant antibodies.
5.2. Future Perspectives

Natural antibodies have a wide array of subclasses and isotypes that help to tailor the body’s immune response to be specific to invading pathogen or cellular debris. Monoclonal antibodies have utilized the natural mechanisms of action of immunoglobulin and hence introduced a new class of therapy for cancer that is more specific than traditional chemotherapy. New antibody engineering techniques have resulted in antibody and protein therapeutics that are enhanced for specific mechanisms.

Although there has been a wide array of in vitro and mouse-modeling work done, the most important mechanism of action of rituximab, as well as other therapeutic antibodies, in humans is yet to be determined. The diverse modification and engineering strategies for new and emerging antibody-based therapeutics that are present in the field today represent the disconnect between preclinical findings and clinical relevance. The relative importance of each arm of antibody function and its relevance in vivo is unknown. Even within FcγR-mediated antibody function, it is unclear which FcγR is most important for therapy. Given the differential expression pattern of FcγR on effector cells, this also raises the question of which cell population is vital for efficacy.

Realistically, one mechanism probably does not account for the entire in vivo response and success of therapeutic antibodies. Response to antibody treatment may be a result of the combination of the specific antigen being targeted and priming of the immune system through FcγRs and complement. Furthermore, the ideal type of engineering on a therapeutic antibody may be dependent on the relevance of the mechanism or cell type being activated and the
particular cancer being treated. Factors to consider would include the location of the malignant
cells, the antigen density on these cells and tumor burden. For example, macrophage activation
may be more desirable in a cancer that has tissue infiltration. Or complement activation may not
be desirable in patients with high tumor burden, since this may result in tumor lysis syndrome [6].
Hence, there is most likely not one ideal type of antibody engineering that will be successful
against all malignancies.

One area of improvement in the designing of superior antibody therapeutics is
determining better target antigens. Ideally, these antigens should be tumor specific. Most
biological therapies for leukemia are directed against cell populations and do distinguish between
normal and malignant cells in this population. Furthermore, the ideal therapeutic antigen would
be a signaling molecule that is a part of the necessary signaling pathway for the cancer cells’
survival. A successful example of this is trastuzumab, an anti-HER2 humanized antibody for
breast cancer. HER2/neu is a tyrosine kinase receptor found on the membrane of cells and is
involved in proliferation and cell survival. It is overexpressed in 20-30% of invasive breast
cancers. Trastuzumab binds to the HER2 receptor and prevents the activation of its intracellular
tyrosine kinase and therefore prevents downstream survival signaling [7]. Designing antibodies
against signaling molecules like this requires intensive research but provides more intelligently
chosen targets.

Furthermore, understanding the signaling pathways of the target antigen can also lead to
hypothesis based combination therapies, such as antibody/protein therapeutics with specific
kinase inhibitors. A prime example of this is the elucidation of the dual signaling properties of
CD37 using a monospecific protein therapeutic, SMIP-016 [3]. CD37 contains both inhibitory
and survival signaling domains. Knowing the kinases involved in the pro-survival arm of CD37, like PI-3K, can lead to combination strategies that are hypothesis driven.

The future of antibody-based therapeutics is promising as superior functioning molecules are emerging due to intensive research into the biology of immunoglobulin molecules and therapeutic targets. Novel therapeutics being tested include dual specific and bispecific antibodies and protein therapeutics that bring together signaling molecules [8] [9] and possibly different cell types. In addition, although antibody conjugates have been studied for many decades, new variants are showing promise in leukemic and solid cancers [10]. These include therapeutic antibodies labeled with radiation, drugs, or protein toxins. Finally, T cell chimeric antigen receptors (CAR) therapy is another area of growing interest. CARs are monoclonal antibody derived single chain Fv (scFv) fragments that are expressed on the surface of T cells and have intracellular signaling domains [11]. They help hone T effectors cells to malignant cells and have shown promising efficacy in CLL and ALL patients [11] [12]. Overall, antibody-based therapeutics represent an area of great promise for cancer patients.
5.3. References


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