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# Selection and characterization of single domain antibodies against human CD20

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ABSTRACT

CD20 is a membrane protein with four integral membrane regions and a large extracellular loop between residues 142 and 187, which serves as a target binding region for rituximab (RTX) and most other anti-CD20 monoclonal antibodies. It is highly expressed in B-lymphoma cells and B lymphocytes and often serves as a target for immunotherapy. In this study, we developed single domain antibodies (sdAbs) for the sensitive detection of CD20. To achieve this, an immune sdAb library was prepared from llamas immunized with a fusion between the large loop from CD20 and Hoc, a highly antigenic protein from the T4 bacteriophage, (CD20-Hoc). By subtracting binders to recombinant Hoc during the biopanning, potential anti-CD20 sdAbs were selected, sequenced, and characterized for their binding affinity to CD20-Hoc fusion versus Hoc. Twenty five clones grouped into three different families based on CDR3 sequence were identified as potential CD20 binders. The binding kinetics of representative sdAbs from each class and RTX were evaluated by surface plasmon resonance (SPR). Most of the sdAbs that were evaluated show binding affinities to CD20-Hoc in the nM range, and class A sdAbs, exhibited ≥40-fold increase in affinity for CD20-Hoc versus Hoc. When the binding of the sdAbs to CD20 on SU-DHL-4 cells was evaluated by flow cytometry, only class A sdAbs displayed strong binding to CD20 and recognized DHL cells in a concentration dependent manner.



## 1. Introduction

The Human CD20 molecule consists of 297 amino acids and is a non-glycosylated 33–35 kD integral membrane protein expressed at high levels in B lymphocytes and most B-cell lymphomas (Tedder and Engel, 1994). It is part of the MS4A family of proteins, which are predicted to have four intermembrane regions and contains their C and N-termini within the cell. It is an ideal target for antibodies (Abs) as it does not shed from B-cells and is not internalized after antibody treatment (Cragg et al., 2005). More evidence shows that Ab treatment triggers complement-dependent cytotoxicity (CDC) and binding to CD20 triggers cell signaling that affects cell viability (Teeling et al., 2006; Deans et al., 2002). In fact, Rituximab (RTX), a human/mouse chimeric anti-CD20 antibody, was the first therapeutic monoclonal Ab approved by Food and Drug administration (FDA) for the treatment of B-cell Non-Hodgkin lymphomas (NHLs). Although RTX has been widely used in the treatment of NHL, only 48% of patients respond and complete responses are less than 10% (Cheson and Leonard, 2008). Thus, there is still a need to develop alternative CD20 specific Abs that may provide better therapeutic results or be useful for diagnostic and imaging applications.

A large extracellular CD20-loop formed by residues 142–187 between the third and fourth intermembrane region is the target binding domain for most of the anti-CD20 monoclonal Abs, including RTX, which specifically binds the epitope constituted by residues 163–187 (Teeling et al., 2006; Du et al., 2007). Therefore, the CD20-loop is often chosen as an alternative antigen to raise anti-CD20 Abs, especially as the full length of recombinant CD20 tends to aggregate in the expression host bacteria or cells (Anbouhi et al., 2012). To ensure the loop displays with proper secondary conformation and to increase its antigenicity, the CD20-loop is often expressed as a fusion with a carrier protein for use in immunizing the animals.

Single domain antibodies (sdAbs, also called nanobodies) are small antigen binding domains (~15 kD) that consist of the heavy chain variable region (VHH) derived from heavy chain antibodies (HcAb) exclusive to camelids. Although sdAbs have been evalu-

*Abbreviations:* RTX, rituximab; SdAb, single domain antibody; CDR, complementarity determining region; FR, framework region; Bt, biotinylated; NA-PE, NeutrAvidin–phycoerythrin conjugate; Ab, antibody; CDC, complement-dependent cytotoxicity.

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**A**. Human CD20 peptide sequence:

**$^{142}$KI SHF LKM ESL NFI RAH TPY INI YNC EPA NPS EKN SPS TQY CYS IQ$^{187}$.**

**B**. Human CD20 and T4 Hoc protein fusion.


**C**. Protein sequence for CD20-Hoc fusion.

**MGKISHFLKMESLNFIRAHTPYINIYNCEPANPSEKNSPSTQYCYSIQ**LEMTFTVDITPKTPTGVIDETKQFTAT
PSGQTGGGTITYAWSVDNVPQDGAEATFSYVLKGPAGQKTIKVVATNTLSEGGPETAEATTTITVKNKTQTTTLAV
TPASPAAGVIGTPVQFTAALASQPDGASATYQWYVDDSQVGGETNSTFSYTPTTSGVKRIKCVAQVTATDYDALS
VTSNEVSLTVNKKTMNPQVTLTPPSINVQQDASATFTANVTGAPEEAQITYSWKKDSSPVEGSTNVYTVDTSSVG
SQTIEVTATVTAADYNPVTVTKTGNVTVTAKVAPEPEGELPYVHPLPHRSSAYIWCGWWVMDEIQKMTEEGKD
WKTDDPDSKYYLHRYTLQKMMKDYPEVDVQESRNGYIIHKTALETGIIYTYPAAALEHHHHHH

**Fig. 1.** Sequences for CD20 peptide, fusion of CD20 and T4 Hoc. (A) The large loop region in Human CD20 includes the residues 142–187 from CD20 protein. Residues, A170 and P172, in red font are critical to form an epitope for Ab binding. (B) CD20 peptide was cloned into N-terminus of T4 Hoc (highly antigenic T4 outer capsid protein) through NcoI- XhoI site. (C) Amino acid sequence for CD20-Hoc fusion. CD20 peptide is in red font and Hoc is in blue font. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ated for therapeutic and imaging applications (Unciti-Broceta et al., 2013; Muyldermans, 2013), sdAbs specific to human CD20 have not previously been described. Most current CD20 immuno-reagents are mAbs and their scFv derivatives (Press, 2000; Foon, 2000; Schultz et al., 2000). Due to their smaller size, sdAbs have improved tumor penetration compared to full length conventional antibodies. An additional consequence of this property is their rapid normal tissue clearance, making them compatible for use with short half-life radionuclides such as $^{68}$Ga and $^{18}$F for PET imaging and $^{211}$At for targeted radiotherapy (Vaneycken et al., 2011; Pruszynski et al., 2013). SdAbs are able to direct treatment to the tumors with minimal off target impact, which should greatly enhance the prospects for patients. The availability of anti-CD20 sdAbs for both imaging and therapeutic applications would be a significant advancement, as they can be prepared with high homogeneity and their small size will allow for the facile development of fusion constructs in the future (Liu et al., 2016). Fusion constructs, such as with the biotin binding protein rhizavidin/streptavidin have the potential to yield a new generation of imaging and treatment options that could augment or supplant current immunoreagents. In addition, sdAbs have superior properties such as solubility and thermostability, thus they have the potential to provide reagents with improved shelf life and less degradation or aggregation prior to patient use (Revets et al., 2005; Siontorou, 2013).

In this study, we immunized llamas with a fusion of the CD20 large loop region with a highly antigenic carrier protein, T4 Hoc (highly antigenic outer capsid protein) and developed an immunized llama phage display library capturing the VHH repertoire of the animals for further selection of anti-CD20 sdAbs. The sdAbs selected from this library were characterized by binding to a genetic fusion of the CD20 peptide with Hoc, a highly antigenic protein from the T4 bacteriophage, by surface plasmon resonance (SPR) and CD20 displayed on B-lymphoma cells by flow cytometry.

## 2. Material and method

### 2.1. Preparation of recombinant Soc, Hoc, and CD20-Hoc fusion

Synthesized oligo-nucleotides encoding the CD20 peptide (Fig. 1A) with flanking NcoI and XhoI sites were inserted into a T7 expression plasmid, pet22b, containing a pelB leader sequence to direct expressed proteins to the periplasmic space to produce the plasmid CD20 p22b. The T4 Hoc gene (NCBI gene ID: 1258642) (Fig. 1B), 1128 bp in length and amplified by PCR, was cloned to CD20 p22b plasmid through the C-terminal XhoI site, creating the CD20-Hoc p22b plasmid. The sequence for the recombinant CD20-Hoc p22b plasmid was confirmed by Sanger sequencing (Fig. 1C). Similarly, fusions of CD20 with Soc (NCBI gene ID 1258783), T4 small outer capsid protein, were also constructed (CD20-Soc p22b). For expression of unfused Soc and Hoc, the Soc or Hoc gene were cloned to pet22b through NcoI and XhoI sites. T4 Hoc or Soc gene was amplified using T4 genomic DNA as a PCR template. T4 genomic DNA is a gift from Dr. Lindsay Black at University of Maryland at Baltimore. The recombinant Soc, Hoc, and CD20-Hoc plasmids were each then co-transformed into Tuner (DE3) with the pHelp plasmid (Hayhurst and Harris, 1999). Transformed bacteria were then grown at 30 °C for 3 h before the temperature was decreased to 25 °C. Chaperone production was induced by the addition of 80 μg/mL of Arabinose, followed 30 min later by 0.25 mM IPTG induction of the Soc, Hoc or CD20-Hoc production. After 2.5 h of IPTG induction, bacterial cells were centrifuged down and the pellets were subjected to osmotic shock and IMAC extraction according to the previously published protocol (Hayhurst et al., 2003; Turner et al., 2015). Following IMAC extraction, recombinant proteins were further purified from other protein contaminates or aggregates through Superdex 200 10/300 GL column (GE Healthcare) on a BioLogic DuoFlow chromatography system (Bio-Rad).

### 2.2. Immunization of llamas and construction of CD20 immunized phage display library

Purified CD20-Hoc was used to immunize two llamas (Triple J Farms, Bellingham, WA). Following immunization, peripheral blood lymphocytes were obtained from the buffy coat and the total RNA was isolated using QIAamp RNA Blood Mini kit (Qiagen Inc., Valencia, CA) according to manufacturer's protocol. Purified RNA was converted to cDNA using superscript RTIII (Life Technologies, Grand Island, NY) and oligo-dT according to manufacturer's protocol. The heavy chain variable domain fragments were amplified from PCR reaction using degenerated primers (Ghahroudi et al., 1997) and our previously published modified protocol (Goldman et al., 2006). The immune library derived from llamas immunized with CD20-Hoc was obtained by cloning the variable domain fragments into the phage display vector, pECAN21 (Goldman et al., 2006; Liu et al., 2007). Gel purified variable domain PCR fragments and pECAN21 DNA cut with SfiI (New England Biolab Inc, Ipswich, MA) were ligated overnight at 15 °C with a 3:1 ratio of insert to vector. The ligation mixture was then transformed to XL1 Blue cells (Agilent Technologies Inc, Clara, CA) using electroporation. Phage displaying sdAbs were prepared from the library according to the previous protocol (Liu et al., 2013). Representative clones were sequenced to assess the library quality and diversity.