Comprehensive Profiling of Approved Anti-CD20 mAbs Using Fc Effector Function Platform

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INTRODUCTION

CD20, a transmembrane calcium channel crucial for B cell activation, proliferation, and differentiation, has emerged as a pivotal therapeutic target in treating B-cell malignancies and autoimmune diseases. Its selective expression on B-cells from the late pre-B cell stage through to memory cells, but not on early pre-B cells or plasma cells, makes it an ideal target for monoclonal antibodies (mAbs). This selectivity allows for effective B-cell depletion while preserving long-term immune memory and enabling B-cell reconstitution post-treatment.

Initially developed for B cell proliferative disorders like non-Hodgkin's lymphoma and chronic lymphocytic leukemia, anti-CD20 mAbs have since expanded to treat autoimmune conditions such as rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis. The clinical benefits of these therapies extend beyond simple B cell depletion, potentially stemming from the loss of dysregulated B cell functions, inflammatory cytokine production, and T cell activation. Rituximab, the first approved anti-CD20 mAb introduced in 1997, paved the way for subsequent generations of antibodies designed to enhance efficacy and reduce immunogenicity.

Despite the evolution of anti-CD20 mAbs, there remains a need for a comprehensive understanding of their mechanisms of action and clinical implications. This study employs the advanced SeromYx Fc effector function platform to profile the Fc effector functions of approved anti-CD20 mAbs. By utilizing a well-characterized recombinant human full-length CD20 VLP from ACROBiosystems as an antigen, the study aims to compare the biophysical binding and immune cellular effector functions of the first three approved anti-CD20 mAbs. Additionally, it seeks to identify novel Fc effector functions that could enhance therapeutic efficacy while also potentially impacting safety risks. Through this detailed analysis, the study strives to contribute valuable insights for the optimization of existing anti-CD20 therapies and better guide the development of next generation mAbs with improved clinical outcomes.

Results

Characterization of anti-CD20 mAb binding to CD20-VLP

The study compared Rituximab (RTX), a Type I first-generation mouse/human chimeric mAb, with Ofatumumab (OFA), a Type I second-generation fully human mAb, and Obinutuzumab (OBZ), a Type II third-generation fully humanized mAb with non-fucosylated modification. To ensure a fair comparison, all antibodies were obtained from a single vendor source i.e., they underwent identical standard operating procedures for production, release and handling. For the antigen, high-quality full-length human CD20 virus-like particles (VLPs) were utilized (Figure 1). These VLPs, developed using ACROBiosystems' platform based on the HEK293 expression system, offer a versatile solution for expressing and studying transmembrane proteins in a near-native environment. The CD20 VLPs were validated for bioactivity and dynamic light scattering (DLS) data, ensuring the antigen's structural integrity, functionality, and homogeneity. This VLP format provides several advantages, including proper protein orientation, density, and preservation of post-translational modifications, crucial for maintaining conformational epitopes and mimicking CD20's natural presentation on B-cells. The use of VLP-based CD20 enables various assays, including antibody screening, bioactivity assessment, and flow cytometry, bridging the gap between in vitro studies and cellular systems for more physiologically relevant comparisons of the anti-CD20 antibodies.

Initially, an on-bead ELISA was employed to evaluate the binding affinity between the anti-CD20 antibodies and

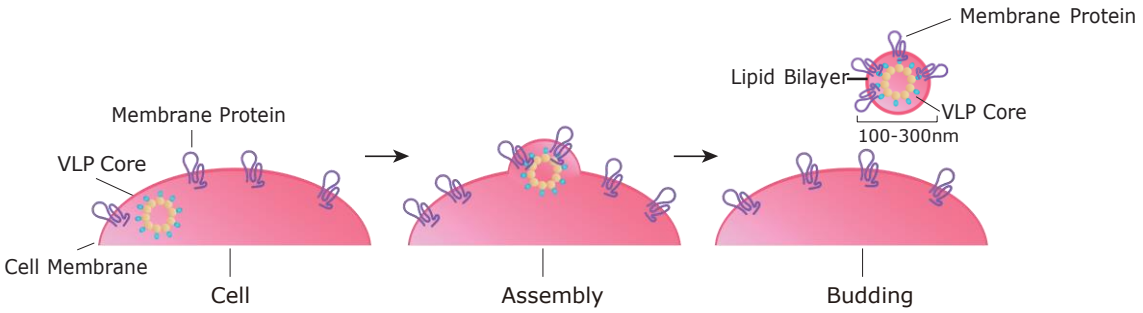


Figure 1. *Virus-like particle (VLP) technology platform expresses transmembrane proteins on the host cell surface.*

CD20-VLP. The results revealed that Type I anti-CD20 mAbs, RTX and OFA, exhibited close to twice the amount of binding to CD20-VLP compared to the Type II mAb, OBZ (Figure 2). This observation is consistent with the known behavior of Type I mAbs, which exhibit enhanced binding due to their ability to translocate CD20 into lipid rafts, as compared to Type II mAbs.

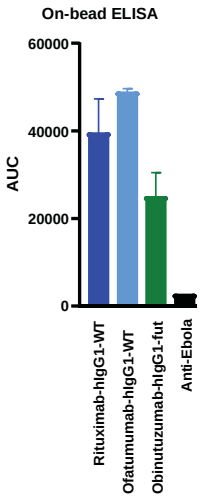


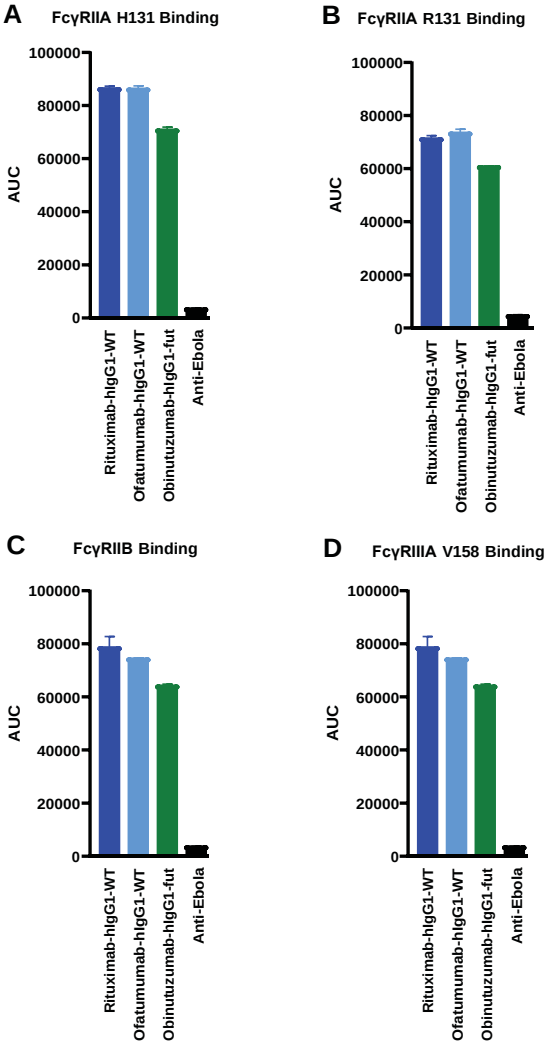
Figure 2. *On-bead CD20-VLP ELISA.*

Fc receptor binding profiles of anti-CD20 mAbs in the presence of CD20

Monoclonal antibody Fc characterization often focuses on the bipartite complex of Fc receptor and antibody. However, physiologically, immune complexes involve the simultaneous engagement of antigen, antibody, and Fc receptor, and this higher-order complex elicits Fc-mediated immune functions that impact product safety and efficacy. Therefore, bipartite binding studies involving antibody and Fc receptors without antigen is not sufficient to assess Fc mediated biological responses. We conducted an array of tripartite Fc receptor and C1q binding assays to predict the engagement of different types of immune cells. These experiments measured the binding interactions among CD20-VLP, mAbs, and specific Fc receptors or C1q in the presence of antigen, providing a baseline understanding of the immune complex interactions.

All three mAbs demonstrated strong binding to both activating FcγRIIA H131 and R131 variants, indicating potential engagement with various immune cells, including monocytes, neutrophils, dendritic cells, eosinophils, and basophils (Figure 3A & 3B). Additionally, the mAbs exhibited binding to inhibitory FcγRIIB, which may facilitate counterbalancing engagement with the same immune cell types (Figure 3C).

Consistently strong binding to both FcγRIIIA V158 and F158 variants was observed across all three mAbs predicting robust antibody-dependent cellular cytotoxicity (ADCC) activity through NK cell engagement (Figure 3E & 3F). Binding to FcγRIIIB was also evident for all mAbs, predicting engagement with neutrophils (Figure 3D). Overall, Type I mAbs, RTX and OFA, exhibited consistently higher binding than the Type II mAb, OBZ, across these Fcγ receptor tripartite binding assays.



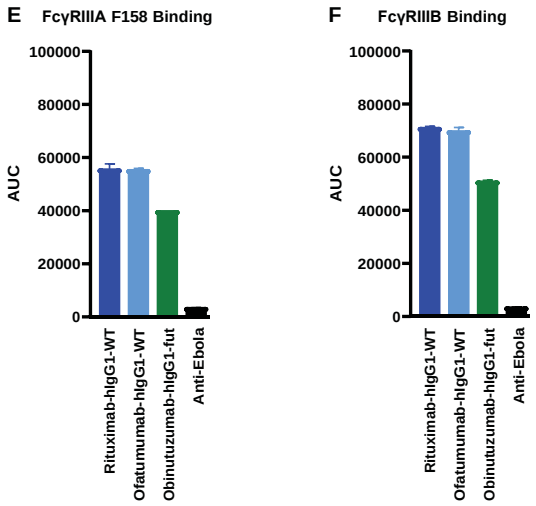


Figure 3. *Tripartite Fc receptor binding predicts engagement of immune effectors. CD20-VLP conjugated fluorescent microspheres were used to assess the interaction of anti-CD20 mAbs with Fc receptors: A) FcγRIIA H131; B) FcγRIIA R131; C) FcγRIIB; D) FcγRIIIA V158 E) FcγRIIIA F158; F) FcγRIIIB.*

RTX has demonstrated robust complement-dependent cytotoxicity (CDC), and OFA was developed to further enhance this activity whereas OBZ was engineered to elicit much lower complement activity via afucosylation. This rank order was faithfully maintained in our biophysical study with OFA showing the strongest C1q binding, followed by RTX and OBZ (Figure 4).

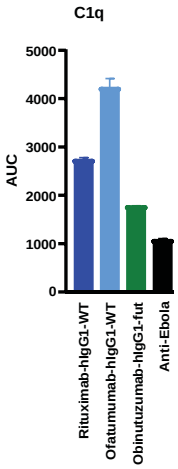


Figure 4. *Tripartite C1q binding of anti-CD20 mAbs. CD20-VLP conjugated fluorescent microspheres were used to assess the interaction of anti-CD20 mAbs with C1q.*

Comparing anti-CD20 mAbs in canonical Fc effector functional cell-based assays

To assess the canonical Fc effector functions of anti-CD20 mAbs, we conducted four assays: antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), CDD and antibody-dependent complement deposition (ADCD).

All three anti-CD20 mAbs, RTX, OFA and OBZ, elicited robust ADCP activity in undifferentiated monocytes (Figure 5A). In contrast, the ADCC assay using CD20-expressing Raji cells revealed enhanced activity for the next generation mAbs, OFA and OBZ, compared to RTX (Figure 5B). The strong ADCC activity observed for OFA and OBZ indicates their superior ability to recruit and activate natural killer (NK) cells, leading to more effective lysis and killing of target cells. In the CDC assay, we observed the predicted hierarchy of complement dependent cytotoxicity among the mAbs against CD20 found on the surface of Raji cells. OFA exhibited the highest CDC activity, followed closely by RTX, while OBZ elicited significantly lower CDC activity (Figure 5C). These findings correlate strongly with the tripartite C1q binding and ADCD activities against CD20-VLP displayed by these mAbs (Figure 5D).

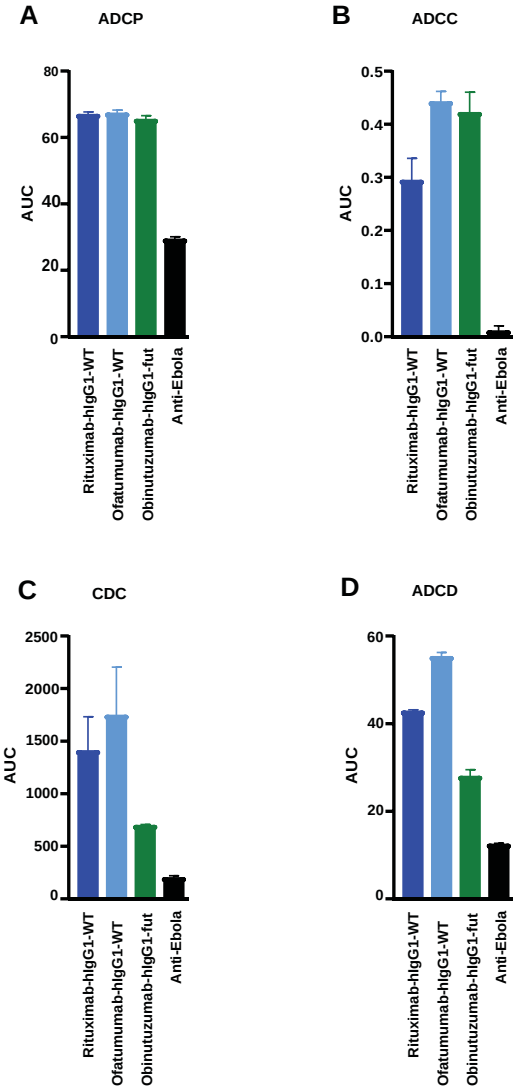


Figure 5. *Confirmation of known Fc effector functions of anti-CD20 mAbs. The ability of anti-CD20 mAbs to A) induce phagocytosis of antigen-functionalized fluorescent beads by undifferentiated monocytes was demonstrated in the ADCP assay, B) elicit NK-cell mediated specific lysis of Raji cells was evaluated in the ADCC assay, C) effect complement dependent cell death of Raji cells was shown in the CDC assay, and D) engage the complement system via C3b was shown in the ADCD assay.*

Uncovering novel Fc effector functions of anti-CD20 mAbs

Additional cell-based functional assays available on the SeromYx platform were utilized to detect potential novel anti-CD20 mAb Fc effector functions. We report our novel findings on antibody-dependent neutrophil phagocytosis (ADNP) and antibody-dependent eosinophil phagocytosis (ADEP) activities.

In the ADNP assay, all three anti-CD20 mAbs demonstrated robust neutrophil-mediated phagocytosis activity (Figure 6A). This finding suggests that the tested antibodies effectively engage and activate neutrophils to phagocytose target cells.

Similarly, in the ADEP assay, the three anti-CD20 mAbs exhibited strong eosinophil-mediated phagocytosis activity (Figure 6B). This result indicates that the antibodies can also effectively activate eosinophils and trigger eosinophil-mediated clearance of target cells.

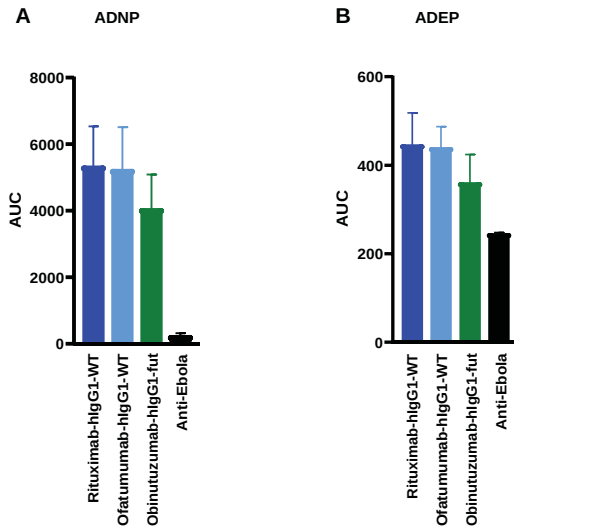


Figure 6. *Novel Fc effector functions of anti-CD20 mAbs. The ability of anti-CD20 mAbs to A) induce phagocytosis of antigen-functionalized fluorescent beads by neutrophils isolated from healthy human volunteers was assessed in the ADNP assay and B) induce primary human eosinophil-mediated phagocytosis of antigen-functionalized fluorescent beads was assessed in the ADEP assay.*

Summary

This study offers significant insights into the profiling of approved anti-CD20 monoclonal antibodies (mAbs) using the SeromYx Fc effector function platform. By employing high-quality, full-length human CD20 virus-like particles (VLPs) from ACROBiosystems, we achieved a physiologically relevant assessment of antibody binding and effector functions. This enabled a detailed comparison between Type I (Rituximab and Ofatumumab) and Type II (Obinutuzumab) anti-CD20 mAbs, revealing distinct binding profiles and effector function capabilities. Our findings indicated that Type I mAbs demonstrated stronger binding to CD20-VLPs and to Fc receptors in the presence of antigen compared to the Type II mAb, highlighting how structural differences could influence their mechanisms of action.

We observed an overall correlation between biophysical tripartite binding assays and effector cell function assays, validating the predictive utility of tripartite binding assays for mAb effector functions. Importantly, the discovery of robust antibody-dependent neutrophil phagocytosis (ADNP) and eosinophil phagocytosis (ADEP) activities for anti-CD20 mAbs significantly broadens our understanding of their potential in vivo mechanisms. These findings suggest that the involvement of neutrophils and eosinophils could impact the efficacy and safety of these mAbs in diverse disease states and tissue environments. Additionally, the differentiation between Type I and Type II mAbs across multiple assays underscores the importance of these distinctions in therapeutic applications and next generation antibody design.

In conclusion, broadly profiling Fc effector function using the SeromYx Fc effector function platform not only recapitulated the known Fc effector functions of anti-CD20 mAbs but also uncovered novel potential mechanisms of action. These insights have substantial implications for optimizing current anti-CD20 therapies and developing new, more effective mAbs. Furthermore, the CD20-VLP system presents an opportunity to design and characterize mAbs with tailored effector function profiles for specific therapeutic applications, potentially leading to more personalized and effective treatments for a variety of diseases.

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About ACROBiosystems

ACROBiosystems is a cornerstone enterprise of the pharmaceutical and biotechnology industries. Our mission is to help overcome challenges with innovative tools and solutions from discovery to the clinic. We supply life science tools designed to be used in discovery research and scalable to the clinical phase and beyond. By consistently adapting to new regulatory challenges and guidelines, we deliver solutions, whether it comes through recombinant proteins, antibodies, assay kits, GMP-grade reagents, or custom services. We empower scientists and engineers dedicated towards innovation to simplify and accelerate the development of new, better, and more affordable medicine.

About Seromyx

SeromYx Systems leverages high-throughput cell and bead-based assays, coupled with machine learning computational analysis, to advance the design and development of therapeutic monoclonal antibodies. By profiling the functional interactions between antibodies and innate immune cells, we enable biotechnology and pharmaceutical companies to develop targeted therapies with precision. Our platform provides the most comprehensive Fc effector function profiling to help identify antibody functions for targeting pathogens, infected cells, or tumors.

We offer an extensive suite of biophysical and functional assays to support the entire lifecycle of monoclonal antibody development, from candidate selection to IND and CMC filing. With a GCLP-certified platform and robust data interpretation, SeromYx is a trusted partner in the development of monoclonal therapies that address critical health challenges and unmet medical needs.

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