Rapid Discovery of Antagonistic Human/Cyno CD200R1 Llama VHH Nanobodies Using Single B Cell Technology

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ABSTRACT

Nanobodies are a growing modality in antibody therapeutics, with FDA-approved treatments driving demand for VHH discovery. Their small size and typically longer CDR3 enables targeting challenging epitopes in solid tumors, overcoming limitations of larger antibodies. Here we used single B cell antibody discovery to rapidly identify a diverse set of potent, high affinity, neutralizing VHH from immunized llamas against CD200R1, an emerging therapeutic immune checkpoint target.

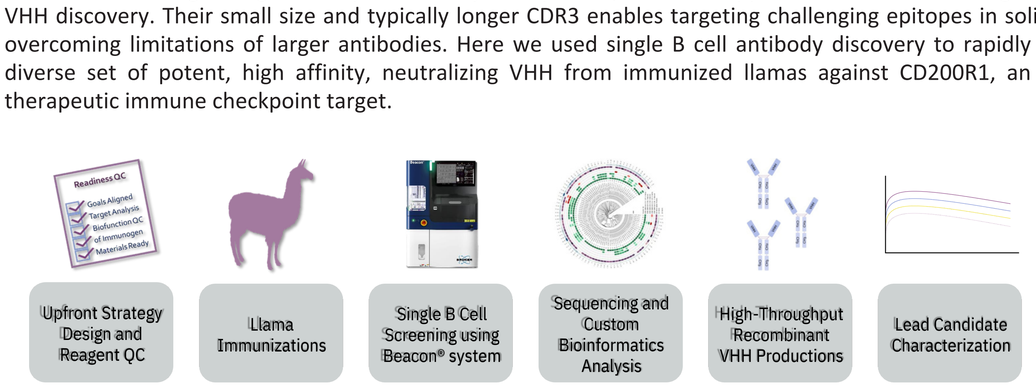

Figure 1. Curia's state-of-the-art single B cell screening workflow. An immunization and screening strategy is designed based on the desired features of the discovered VHH. Llamas are immunized and in-life plasma titer checks are used to monitor antibody generation and inform subsequent booster injections. Once sufficient titers are reached, the memory B cells are harvested from PBMC and activated into antibody secreted cells. These cells are then imported into the Beacon® system. Up to 80,000 individual B cells are clonally distributed into NanoPens® where they secrete IgG. Time-lapse fluorescent microscopy is used to screen the B cells. The top hits selected are advanced to cDNA synthesis for B cell receptor sequence recovery using Sanger sequencing. Lead candidates are selected for high-throughput recombinant productions using Curia's Tuna CHO™ platform. Additional characterization assays such as kinetics, functional neutralization, and epitope binning can be performed on purified VHH.

BACKGROUND

CD200R1 is a cell surface receptor expressed on $CD4^+$ and $CD8^+$ T cells and various populations of myeloidcells. The interaction between CD200R1 and its ligand, CD200, delivers an inhibitory signal leading to the suppression of immune responses. Targeting the CD200R1/CD200 pathway has demonstrated potential as an immunotherapeutic strategy as the ligand is expressed by various human cancers and blocking receptor-ligand interactions restores anti-tumor immune responses.

Curia's optimized high-throughput single B cell-based VHH discovery workflow, which leverages Bruker's Beacon® Optofluidic system and Curia's rapid gene-to-protein (GTP) productions, significantly accelerates identification of high quality, high affinity VHH leads (**Fig. 1**).

RESULTS

Immunogens and screening antigens were produced in-house using Curia's GTP services. Multiple production scales were utilized to obtain yields of 2.7 to 23.5 mg. Material QC was performed by ELISA to assess antigen binding to respective reference antibodies prior to campaign use.

Two llamas were immunized with human CD200R1-Fc and cyno CD200R1-Fc (human and cyno CD200R1 share 90% identity). Both llamas developed strong plasma titers after 57 days (**Fig 2**). Llama 11308 developed higher titer and was selected to be used for the discovery workflow. PBMC were harvested and memory B cells were isolated by positive selection. Isolated B cells were cultured and stimulated for 5 days to induce antibody secretion, proliferation, and plasma B cell development.

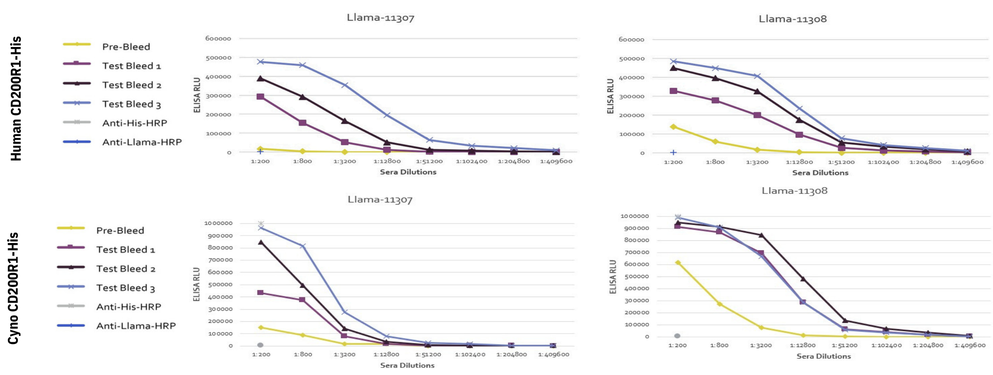

Figure 2. In-life plasma titer checks guide selection of llamas with highest huCD200R1 titer. Two Llamas were immunized with a mixture of human and cyno CD200R1-Fc. Blood plasma was collected at three time points (D16, D30, D57), diluted as indicated and tested for human and cyno CD200R1- HIS binding by ELISA. RLU: relative light unit.

The enriched memory B cells were loaded onto two OptoSelect® 20k chips on the Beacon® Optofluidic System. Each chip contains 20,000 NanoPen® chambers, each designed to isolate a single cell. Single cell penning efficiency was an average of 59%. Sequential on chip screening assays were performed to evaluate VHH secretion and binding to human and cyno CD200R1. 36% of penned single cells (7,854) were positive for Llama IgG 2/3. 4% (305) of the Llama IgG2/3 positive NanoPens® were positive for human CD200R1 binding and 55% (169) of those were cross-reactive with cyno CD200R1.

Automated and manual verification of hits that specifically bound to huCD200R1 and/or to cyCD200R1 and that are nanobody positive were marked for export and sequence recovery. cDNA synthesis and amplification for Sanger sequencing were then performed.

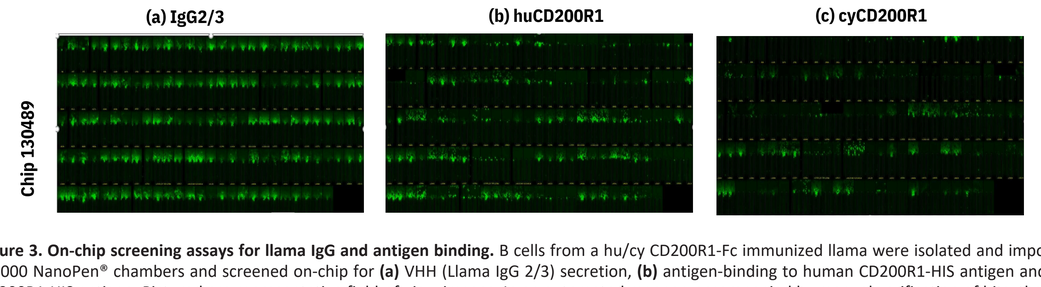

Figure 3. On-chip screening assays for llama IgG and antigen binding. B cells from a hu/cy CD200R1-Fc immunized llama were isolated and imported into 40,000 NanoPen® chambers and screened on-chip for **(a)** VHH (Llama IgG 2/3) secretion, **(b)** antigen-binding to human CD200R1-HIS antigen and **(c)** cyno CD200R1-HIS antigen. Pictured are representative field-of-view images. In an automated manner, accompanied by manual verification of hits, the Beacon® system identified hits by analyzing Alexa Fluor 488-positive blooms.

Using a custom bioinformatics pipeline, 31 unique variable region sequences were identified, aligned, and grouped into clonal families based on sequence identity (**Fig. 4**). A wide range of CDR3 lengths (from 5 to 23 residues) reflect diversity in the leads (**Fig. 5a**), and substantial sequence divergence from germline (>10% on average) indicates extensive affinity maturation *in vivo* in the immunized llama (**Fig. 5b**).

High-throughput (HTP) production of 31 VHH-huFc (hIGg4 isotype with S228P stabilizing and L235A effectorless function mutations) were performed utilizing 10 mL transient productions in TunaCHO™ cells (14- day process) followed by Protein A chromatography. Yields ranged from 2.6 to 12.3 mg (**Fig. 6a**). We next assessed VHH-huFc binding potency to huCD200R1 by ELISA. Potent VHH leads were discovered, with an average EC50 of 1.6 nM against huCD200R1 (**Fig. 6b,d**). Most (22 of 30) VHH-huFc were cynoCD200R1 cross-reactive (average EC50: 3.2 nM), which will facilitate preclinical development **(Fig. 6c,d)**. Binding kinetics were assessed by arrayed SPR (Carterra® LSA®). Approximately half of the leads were high affinity CD200R1 binders (KD < 10 nM) (**Fig. 7**). Importantly, most VHH neutralized CD200R1 and prevented CD200 binding, with a mean IC50 of 18.4 ± 5.7 nM (**Fig. 8**). Finally, we performed epitope binning by Carterra®LSA® and identified 6 distinctbins comprising 1 to 12 VHH (**Fig. 9**).

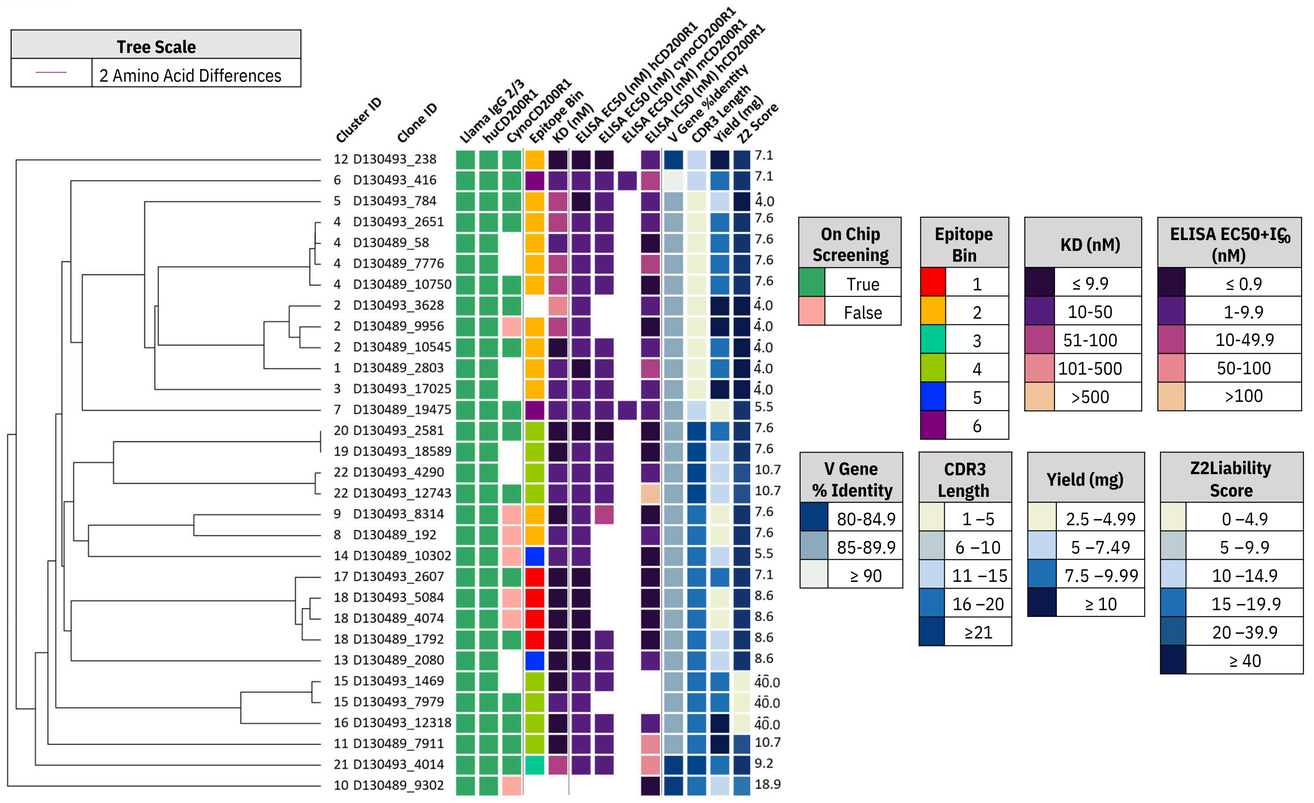

Figure 4. Phylogenetic tree of 31 VHH-huFc diverse and unique variable region sequences integrated with antibody characterization data. The figure displays the cluster ID (VHH with the same cluster ID share ≥ 94.5% amino acid sequence identity), on-chip screening results (e.g., green box indicating positivity for llama IgG 2/3, huCD200R1, or cyCD200R1), epitope bin classification (red, orange, and teal indicate competitive binding epitopes with CD200, see Fig 9b), KD values for huCD200R1, and ELISA potency (EC50) against hu/cy/moCD200R1 (the darker the shade indicates stronger affinity or potency, respectively). Additionally, ELISA IC50 values for CD200/CD200R1 neutralization are shown (darker shades indicates stronger neutralization), variable (V) gene percent identity to germline (the darker the shade reflects greater affinity maturation and divergence from germline), CDR3 length, recombinant production yield, and Z2 score (darker blue indicates a higher likelihood of predicted liabilities). Branch lengths are proportional to the number of amino acid changes between VHH variants.

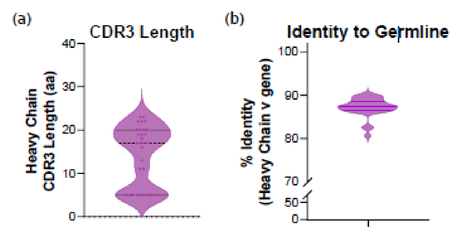

Figure 5. Analysis of CDR3 length and extent of affinity maturation *in vivo*. **(a)** CDR3 length ranged from 5 to 23 amino acids, 14.1 ± 1.2, mean ± SEM, n=31. **(b)** Percent identity to germline: 87.3 ± 0.4, mean ± SEM, n=31.

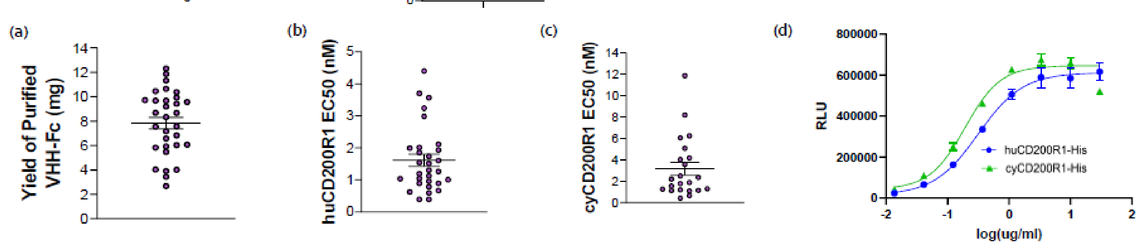

Figure 6. Recombinant production of VHH-huFc leads and assessment of binding potency against human and cyno CD200R1. (a) Recombinant antibody yields following small-scale (10 mL) high throughput production using Curia's TunaCHO℠ platform: 7.84 ± 2.63 mg, n=31. **(b)** huCD200R1 EC50 by ELISA: 1.6 ± 0.2 nM, n=30. **(c)** cyCD200R1 EC50 by ELISA, 3.2 ± 0.6 nM. **(a-c):** mean ± SEM **(d)** example EC50 curves for anti-CD200R1 VHH-Fc D130493_4290.

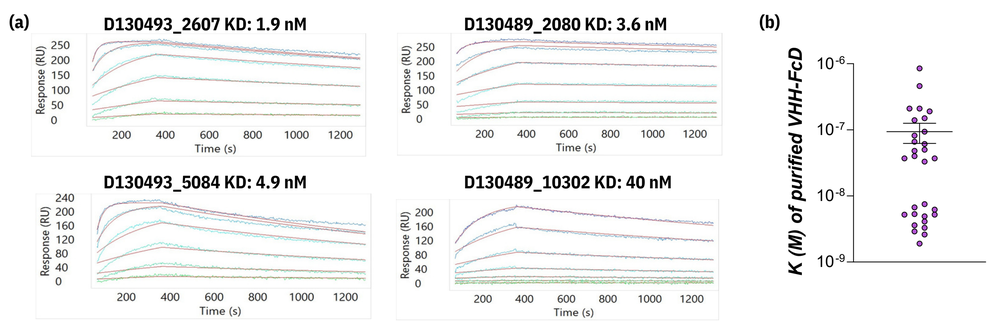

Figure 7. Assessment of VHH-huFc binding affinity to huCD200R1 by Carterra®LSA® SPR. Goat anti-human IgG Fc was coupled to an HC30M chip via Sulpho-NHS/EDC coupling chemistry and blocked with ethanolamine. 31 VHH-huFc were captured via anti-huFc lawn to create an array. HuCD200R1 was injected into the array and coupled kinetics were run. **(a)** Examples of coupled kinetics sensorgrams. CD200R1 association (Ka) was measured for 5 minutes followed by 15 minutes of dissociation (Kd) for binding kinetics assessment to four candidate VHH-huFc. **(b)** Affinities ranged from 1.9 to 850 nM.

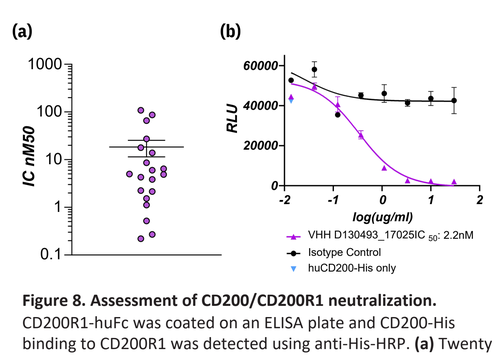

Figure 8. Assessment of CD200/CD200R1 neutralization. CD200R1-huFc was coated on an ELISA plate and CD200-His binding to CD200R1 was detected using anti-His-HRP. **(a)** Twenty VHH-huFc inhibited CD200-His binding to CD200R1 with IC50 ranging from 0.2 to 108 nM**. (b)** Example IC50 curve for anti-CD200R1 VHH-huFc D130493_17025 vs. Fc-control.

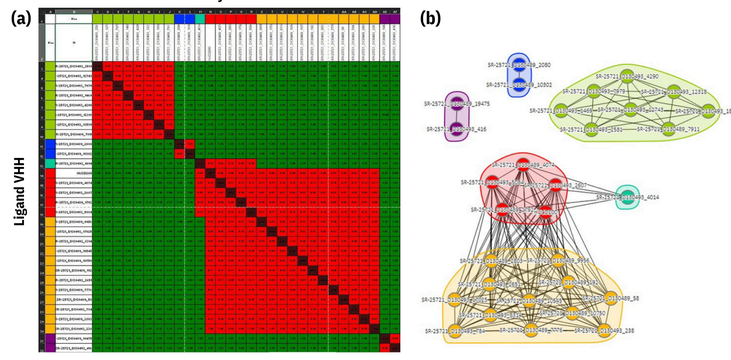

Figure 9. Epitope binning by Carterra®LSA® SPR. (a) Classical binning was performed and six distinct bins were established, all interactions were symmetrical. **(b)** Network plot. Teal, red, and orange bins are defined as a community. Solid lines indicate a symmetrical blocking relationship, where both in the pairing blocked as analytes. Any interactions between VHH that do not have a line connecting them are sandwiching interactions. Interestingly, 17 VHH bin with CD200 (teal, red, and orange bins, including example neutralizing VHH D130493_17025 from **Fig. 8b**).

CONCLUSIONS

- Curia's high-throughput single B cell workflow enables the rapid discovery of unique llama-derived VHH antibody sequences within one month of immunization, significantly accelerating discovery timelines. This platform efficiently identified and
- comprehensively characterized greatly diverse, high-affinity, functionally blocking anti- human and cyno CD200R1 VHH candidates, providing a robust platform for therapeutic development.
- The CD200R1-targeting VHH antibodies are potential checkpoint inhibitors and are available for licensing.

CONTACT US

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