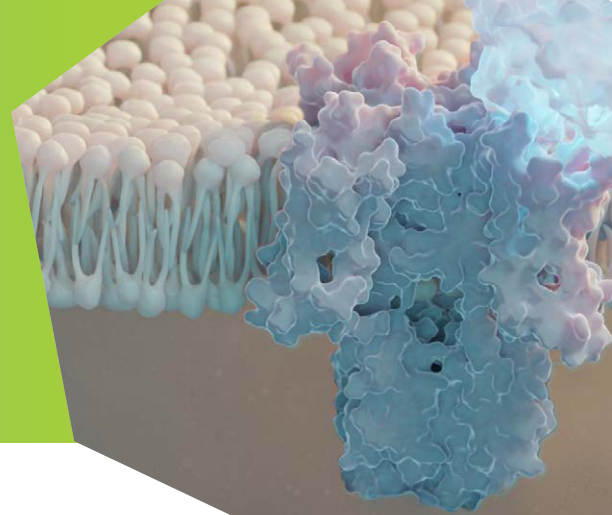# Discovery of diverse GPCR-targeted antibodies

The following data was presented as a poster at the **US Antibody Engineering & Therapeutics Conference 2023**.

INTRODUCTION

Generating diverse GPCR-targeted antibodies

There are more than 270 potential G protein-coupled receptor (GPCR) antibody drug targets spanning a wide range of indications, including oncology, autoimmunity, and metabolic disease.[1] Despite being high-value therapeutic targets, GPCRs have been largely intractable to conventional antibody discovery technologies.[2] Due to the deep complexity in GPCR structure and function, there are challenges at each stage of discovery.

Robust, target-specific immune responses are limited by high cross-species homology and a small number of available epitopes.[2] In addition, target-binding hits are extremely rare, reducing the chances of finding functional and developable leads.[3]

In this case study, we leveraged our discovery and development engine to find diverse antibodies against an established GPCR target, chemokine receptor 8 (CCR8). CCR8 is highly and selectively expressed on tumor-associated regulatory T cells in multiple cancer types.[4] Antibodies against CCR8 represent a promising therapy to treat solid tumors that have progressed on standard immune checkpoint inhibitors.[5,6,7]

To be effective, antibodies against CCR8 should be human/humanized and show favorable developability properties, highly specific binding, and potent antibody-dependent cellular cytotoxicity (ADCC) reporter activity.

Amplifying diversity, enriching for function, and optimizing developability

A combination of protein and DNA immunization campaigns was used to generate robust immune responses. We captured hundreds of diverse antibodies using deep single-cell screening. High-throughput function-based screening was used to down-select antibodies based on potency. Selected antibodies were optimized for developability using structure-guided protein engineering technologies paired with high-throughput antibody assessment.

OUTCOME

A diverse panel of anti-CCR8 antibodies optimized for developability and function

Selected molecules displayed desired properties, including:

- **Diversity:** derived from two animal species, including two humanized mouse strains
- **Potency:** strong ADCC reporter activity that is similar to a clinical benchmark
- **Developability:** favorable biophysical properties that have been de-risked by pre-emptive liability remediation

Using high-throughput immunization, protein engineering, and antibody assessment technologies, we demonstrate a strategy to identify optimized antibodies against this high-value and previously intractable class of antibody targets.
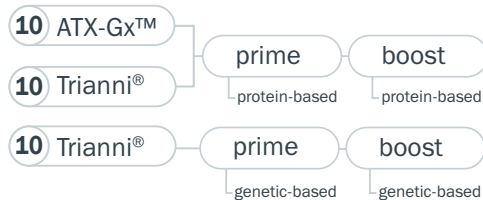
272 diverse CCR8-binding hits

To amplify antibody diversity we initiated multiple immunization campaigns in parallel, including two strains of humanized mice, two strains of rats, and diverse antigen formats (Figure 1A). We screened 2.6 million cells using a microfluidic-based single-cell screening assay and identified 633 CCR8-binding hits (Figure 1B). We progressed 272 unique sequences into high-throughput expression and characterization. This diverse set of antibodies was derived from two species and included 91 clonal families (Figure 1C).

FIGURE 1. 272 diverse CCR8-specific antibodies were identified from immunization and deep single-cell screening

(A) Two strains of humanized mice and two strains of wild-type rats were immunized using recombinant protein- or genetic-based formulations. Antibody titers were measured by flow cytometry using cells expressing human CCR8. **(B)** Single-cell screening assays were used to screen 2.6 million single cells and identify CCR8-specific antibodies. Representative output images of a CCR8-specific hit in a single chamber from our single-cell screen are shown. Antibodies that bind to CCR8-expressing cells were detected using fluorescence micro-scopy and images were analyzed using machine vision. **(C)** Antibody sequence diversity was visualized using Celium™, and 272 target-binding antibodies were progressed to high-throughput expression and characterization. Clusters represent clonal families based on V genes, J genes, and CDR3 lengths.
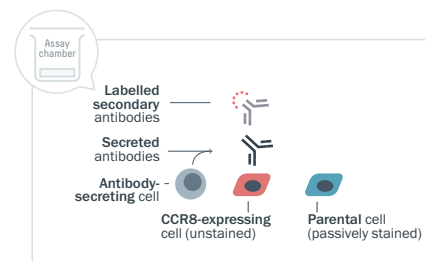

Ⓐ Immunization strategy

Humanized mice

10 ATX-Gx™
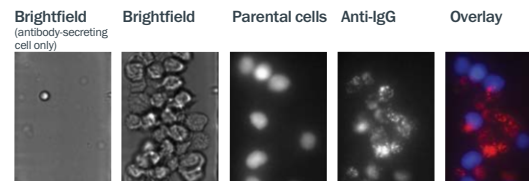10 Trianni® — prime (protein-based) — boost (protein-based)

10 Trianni® — prime (genetic-based) — boost (genetic-based)

Rats

6 Sprague-Dawley
6 Wistar — prime (genetic-based) — boost (genetic-based)

32 animals screened

Ⓑ Single-cell screening

Single-cell screening assay *Side view*

- Labelled secondary antibodies
- Secreted antibodies
- Antibody-secreting cell
- CCR8-expressing cell (unstained)
- Parental cell (passively stained)

Cell screening images *Top view*

Brightfield (antibody-secreting cell only) | Brightfield | Parental cells | Anti-IgG | Overlay

Legend
- ● Antibodies bound to CCR8-expressing cells
- ● Parental cells

2.6M single cells screened

633 hits recovered

Ⓒ Antibody sequence diversity

Humanized mice

ATX-Gx™	64 unique sequences	41 clonal families
Trianni®	96 unique sequences	18 clonal families

Wild-type rats

Sprague-Dawley	34 unique sequences	16 clonal families
Wistar	78 unique sequences	16 clonal families

Animal ID
- 01
- 02
- 03
- 04
- 05
- 06
- 07
- 08
- 09
- 10
- 11
- 12

Clonal families

Heavy chain clonal family* | Light chain clonal family*

*Same V gene, J gene, CDR3 length
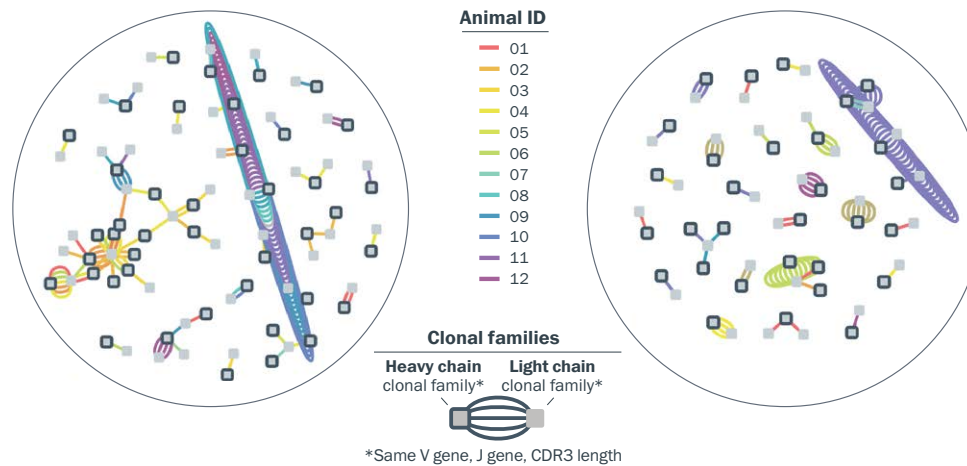
272 unique sequences

194 functional anti-CCR8 antibodies

We enriched the panel for antibodies with the most critical phenotype — potent ADCC reporter activity. Over 70% of expressed antibodies met the desired functional activity and we selected 24 highly potent molecules for optimization and characterization (Figure 2A).

To de-risk the panel early in development and optimize antibodies for clinical success, we identified sequence liabilities *in silico* and used this data to guide pre-emptive liability remediation (Figure 2B). The 24 selected antibodies showed a range of liabilities known to impact antibody quality, such as N-glycosylation sites, non-canonical cysteines, and deamidation motifs. In Figures 3 and 4 we describe the remediation approach of two example antibodies: (1) remediation of two sequence liabilities in a humanized mouse antibody and (2) humanization of a rat-derived antibody.

FIGURE 2. From 194 functional antibodies, 24 highly potent molecules were selected for high-throughput characterization and liabilities were mapped to guide pre-emptive remediation

(A) 194 of 272 expressed antibodies met the desired functional activity, measured in an ADCC reporter assay with activity quantified using luminescence. **(B)** Potential sequence liabilities of the 24 selected antibodies were identified *in silico* and mapped using proprietary bioinformatics pipelines. Antibody clonal diversity and liabilities were visualized using Celium™.
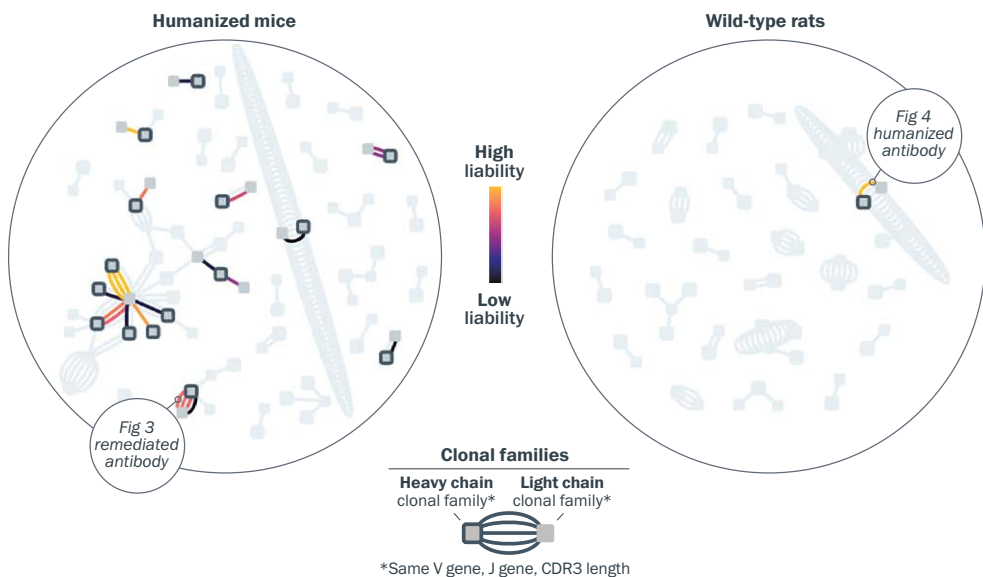
RLU: Relative luminescence units; FOI: Fold of induction.

(A) High-throughput screening of ADCC reporter activity
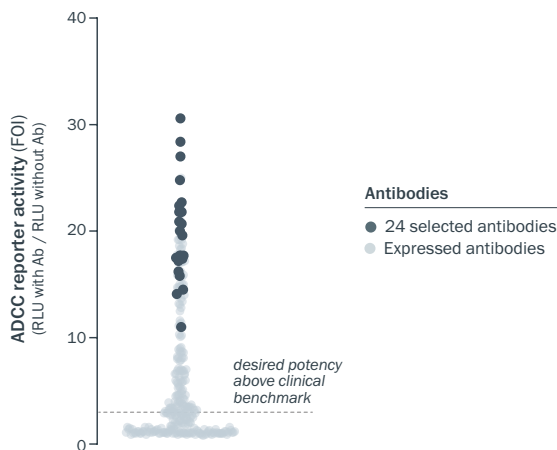
(B) *In silico* liability score overlaid with antibody sequence diversity

24 unique sequences | 17 clonal families

24 highly potent antibodies optimized for developability

Remediation of sequence liabilities in a humanized mouse-derived antibody

In silico developability was assessed in depth to reveal potential sequence and structural liabilities, as well as mitigation strategies for the 24 selected functional antibodies. As an example, two high-risk sequence liabilities – an N-glycosylation motif and a free cysteine – were identified in an antibody derived from a humanized mouse. To neutralize these liabilities we leveraged the clonal family sequence diversity identified during discovery and reverted sequences to germline motifs (Figure 3A). Homology modeling was used to support risk assessment of liabilities and potential substitutions.
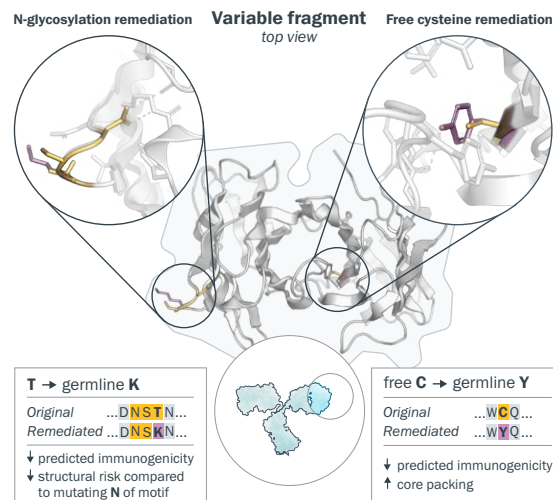
Remediated variants matched or exceeded parental function (Figure 3B) and binding (Figure 3C), as shown in ADCC reporter and flow cytometry assays, respectively. Consistent with the successful remediation of the two sequence liabilities, we observed reduced apparent molecular weight in CE-SDS and improved yield in several of the remediated variants (Figure 3C).

FIGURE 3. Remediated variants show enhanced function and yield compared to the parental antibody

(A) Homology modeling of a humanized mouse-derived antibody guided the remediation strategy. (B) Functional activity was measured in an ADCC reporter assay in three-fold serial dilutions starting at 10 nM and data were normalized to an isotype control; activity was quantified using luminescence. (C) Variants with a remediated N-glycosylation site (indicated by reduced apparent molecular weight in CE-SDS) displayed improved yield and cell binding compared to the parental antibody. Extended developability profiles of remediated variants were favorable, including desired stability, high monodispersity, and low self-association.
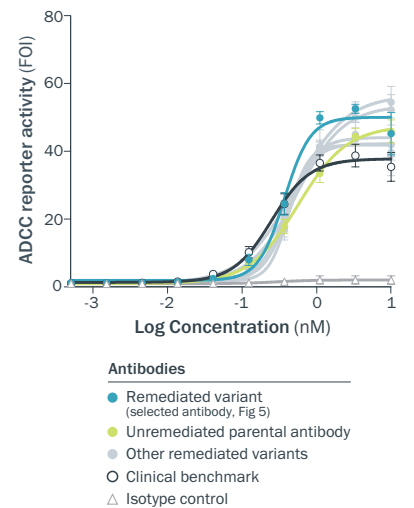

Ⓐ **Homology modeling**
Remediation strategy for a selected antibody with two high-risk liabilities

N-glycosylation remediation **Variable fragment** *top view* **Free cysteine remediation**

T → germline K
Original ...DNS**T**N...
Remediated ...DNS**K**N...
↓ predicted immunogenicity
↓ structural risk compared to mutating **N** of motif

free C → germline Y
Original ...WC**Q**...
Remediated ...WY**Q**...
↓ predicted immunogenicity
↑ core packing

Ⓑ **Functional assessment**
Liability remediation enhanced ADCC reporter activity

Antibodies
● Remediated variant (selected antibody, Fig 5)
● Unremediated parental antibody
● Other remediated variants
○ Clinical benchmark
△ Isotype control

Ⓒ **Analytical and biophysical characterization**
Liability remediation generated variants with favorable developability profiles
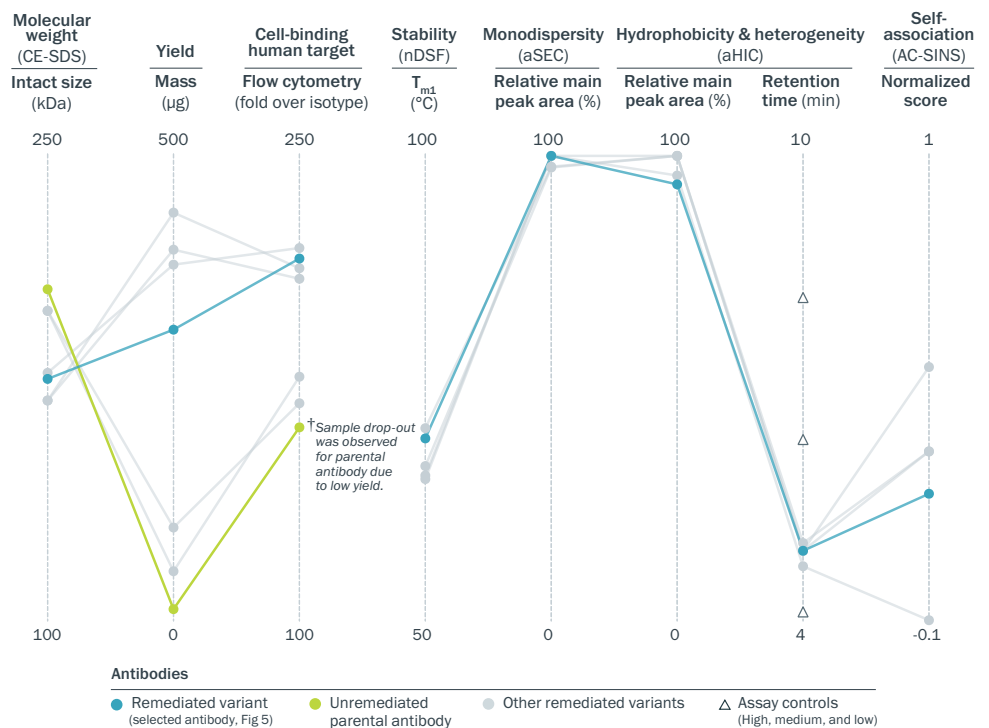
†*Sample drop-out was observed for parental antibody due to low yield.*

Antibodies
● Remediated variant (selected antibody, Fig 5)
● Unremediated parental antibody
● Other remediated variants
△ Assay controls (High, medium, and low)

Structure-guided humanization of a highly potent rat-derived antibody

A rational approach was used to reduce predicted immunogenicity of a highly potent rat-derived antibody. We grafted complement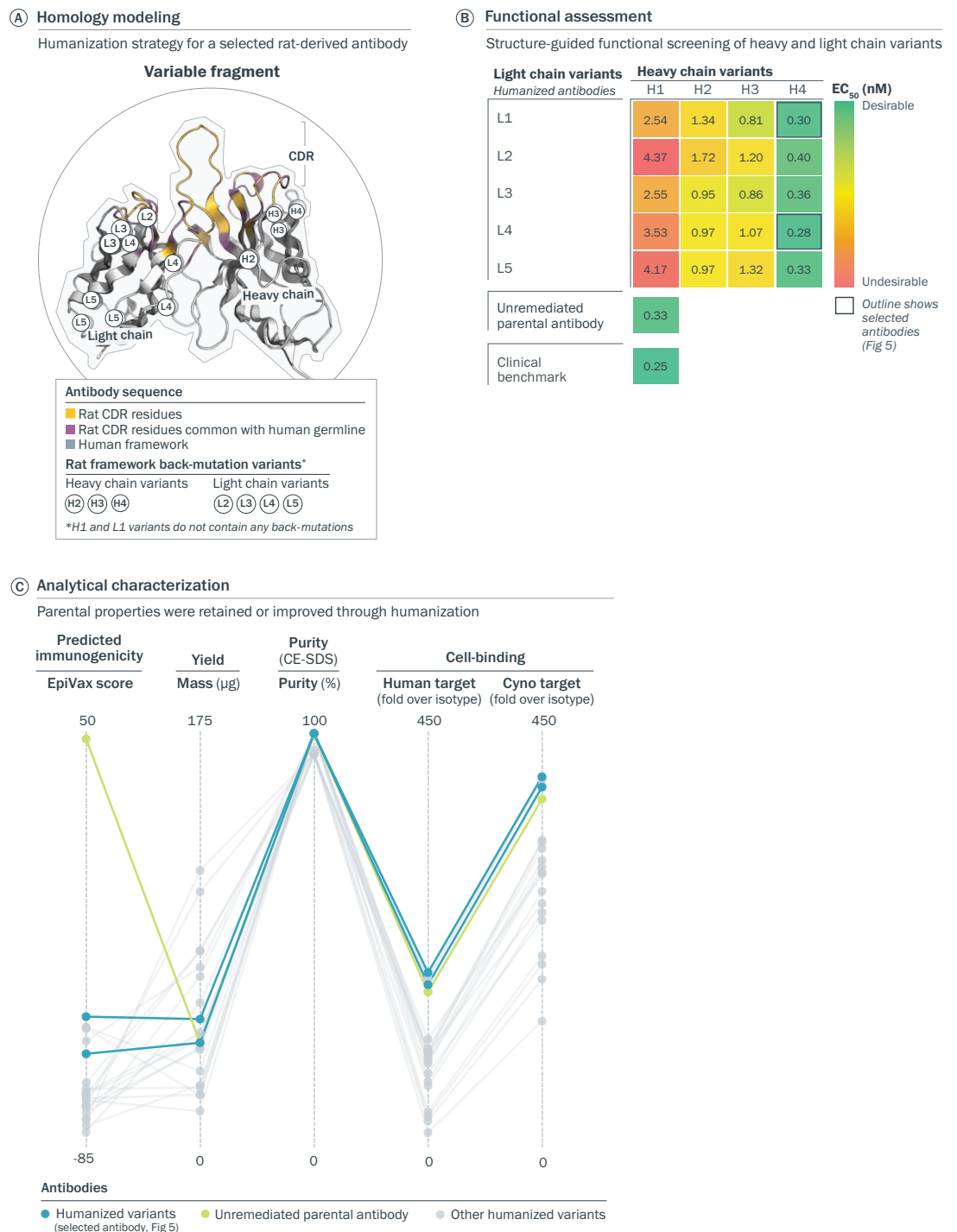arity-determining regions (CDRs) from the parental antibody onto a human germline framework, designed rat back-mutations in the heavy and light chains at key positions, and assessed likely impacts on structure with homology modeling (Figure 4A).

Humanized antibodies were produced and characterized in high-throughput assays to identify the optimal combination of heavy/light chain back-mutations that would reduce immunogenicity while retaining potent ADCC reporter activity. Multiple humanized variants showed reduced predicted immunogenicity, high potency, and favorable binding profiles (Figure 4B, C).

FIGURE 4. Structure-guided humanization was used to identify candidates with optimal profiles for predicted immunogenicity and function

(A) Homology modeling guided the assessment of residues targeted for humanized back-mutations on the light (L1-5) and heavy chains (H1-4). (B) The impact of heavy/light chain back-mutations on function was assessed in a rational, combinatorial approach and measured in an ADCC reporter assay as described in Figure 3B. (C) Humanization generated variants with reduced predicted immunogenicity and maintained or improved target-binding, yield, and purity compared to the parental antibody.

(A) Homology modeling

Humanization strategy for a selected rat-derived antibody

Variable fragment

Antibody sequence
- Rat CDR residues
- Rat CDR residues common with human germline
- Human framework

Rat framework back-mutation variants*

Heavy chain variants	Light chain variants
H2 H3 H4	L2 L3 L4 L5

*H1 and L1 variants do not contain any back-mutations

(B) Functional assessment

Structure-guided functional screening of heavy and light chain variants

Light chain variants *Humanized antibodies*	Heavy chain variants			
	H1	H2	H3	H4
L1	2.54	1.34	0.81	0.30
L2	4.37	1.72	1.20	0.40
L3	2.55	0.95	0.86	0.36
L4	3.53	0.97	1.07	0.28
L5	4.17	0.97	1.32	0.33

Unremediated parental antibody	0.33

Clinical benchmark	0.25

EC_{50} (nM)
Desirable
Undesirable

Outline shows selected antibodies (Fig 5)

(C) Analytical characterization

Parental properties were retained or improved through humanization

Predicted immunogenicity	Yield	Purity (CE-SDS)	Cell-binding	
EpiVax score	Mass (μg)	Purity (%)	Human target (fold over isotype)	Cyno target (fold over isotype)
50	175	100	450	450
-85	0	0	0	0

Antibodies
- Humanized variants (selected antibody, Fig 5)
- Unremediated parental antibody
- Other humanized variants

Anti-CCR8 antibodies with favorable developability and functional profiles that are similar to a clinical benchmark

Remediated and wild-type antibodies were expressed and characterized in high-throughput to down-select optimal antibodies. The highly diverse panel included both wild-type and remediated antibodies with favorable developability properties (Figure 5A) and potencies (Figure 5B) similar to a clinical benchmark molecule.

FIGURE 5. Strategic selection of diverse, developable antibodies with a range of potencies

(A) Selected antibodies have favorable developability profiles, including desired purity, monodispersity, relative surface hydrophobicity and heterogeneity, stability, hydrodynamics, and self-association with scores normalized to high and low controls. (B) Selected antibodies were similar to a clinical benchmark molecule in preliminary ADCC assessment. Functional activity was measured with an ADCC reporter assay as described in Figure 3B. (C) ADCC assay EC_{50} values and 95% confidence intervals are shown.
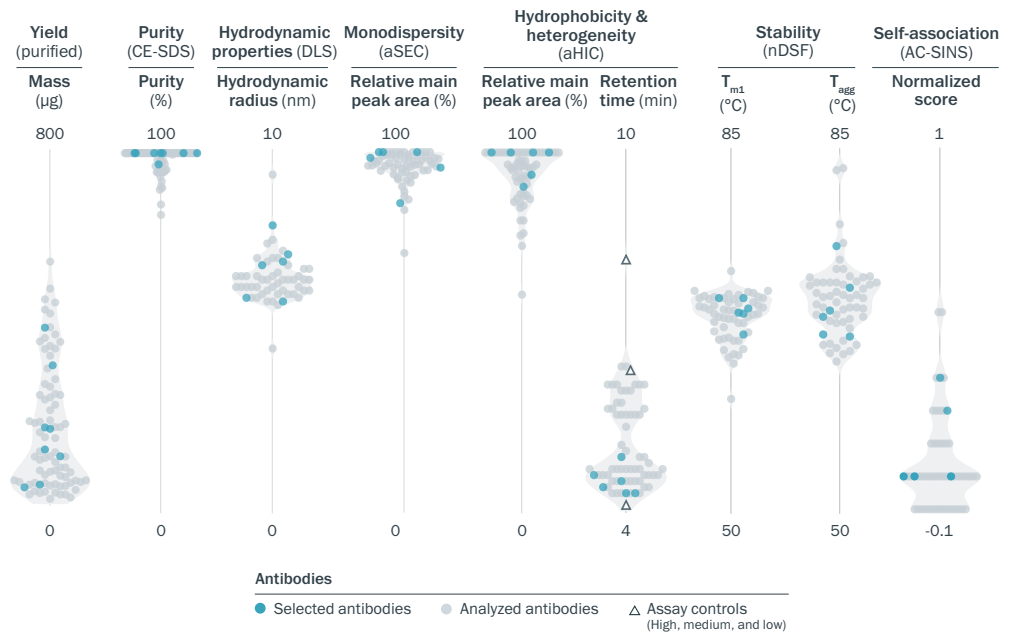
ADCC reporter activity
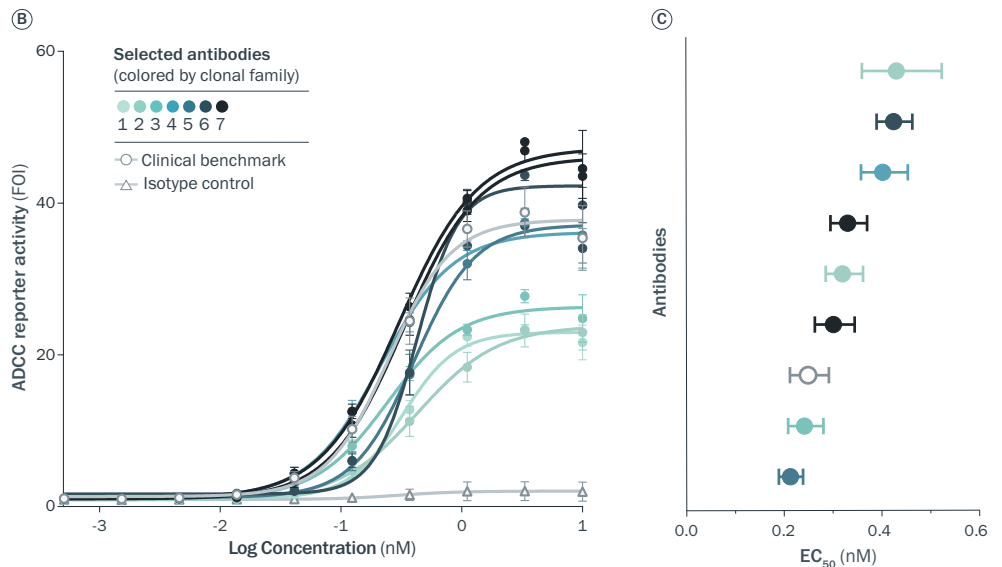
CONCLUSION

We used our antibody discovery and development engine to generate a diverse panel of anti-CCR8 antibodies that are highly potent and extensively de-risked. Our technologies and team address the unique challenges that hinder the discovery and development of antibody therapies against difficult transmembrane protein targets. Pairing a comprehensive immunization strategy with deep single B cell screening is key to identifying a large number of diverse hits against difficult GPCR targets. High-throughput developability and functional assessment, combined with structure-guided protein engineering, results in functional antibodies that are optimized for key characteristics critical to clinical success. We are leveraging these technologies against multiple GPCR and ion channel targets to unlock access to this high-value class of antibody therapeutics and treat disease areas of high unmet medical need.

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